Cloning the construct HVA1 into a binary vector by Gateway multisite System and genetic transformation of faba bean (*Vicia faba* L.) with HVA1 gene for improving drought tolerance.

Abdelwahd R.[1,*], Gan Q.[2], Udupa S. M.[3], Diria G.[1], Mentag R.[1], Ibriz M.[4] and Iraqi D.[1]

1) Biotechnology Research Unit, National Institute of Agricultural Research (INRA), B.P. 415, Avenue de la Victoire, Rabat, Morocco
2) Department of Agronomy, Iowa State University, Ames, USA
3) ICARDA-INRA Cooperative Research Project, International Center for Agricultural Research in the Dry Areas (ICARDA), B.P. 6299, Rabat, Morocco.
4) Université Ibn Tofail, Faculté des sciences, Département de Biologie B.P. 133 Kénitra, Morocco

* Corresponding author: Abdelwahd Rabha
E-mail: rabhaab@yahoo.fr,
Phone number, + (212) 666 313 941

Abstract

Agrobacterium tumefaciens is the preferred method for transformation of legume. Commonly, the genes to be transferred by this technique are cloned between the left and right T-DNA borders of so-called binary T-DNA vectors that can replicate both in E. coli and Agrobacterium. In this study, the integrated protocol for cloning genes of interest from PCR to Agrobacterium transformants via the Gateway multisite System was used to insert a novel construct containing HVA1 sequence, double Cauliflower mosaic virus (CaMV) 2x35S promoter and Nos terminator into an Agrobacterium binary vector pTF101.gw3. The binary vector generated pTFGat101.11 containing HVA1 gene construct was then introduced into A. tumefaciens EHA101 strain. Using the transformation protocol based on direct organogenesis from cotyledonary node explants, we succeeded to obtain 2% of regenerated transgene shoots of faba bean that integrate HVA1 construct. The bar gene (marker gene) integration was confirmed by PCR.

Key words: Faba bean, Cloning, HVA1 gene, *Gateway multisite System.*

Clonage de la construction HVA1 dans le vecteur binaire par le système multisite Gateway et la transformation génétique de la fève (*Vicia faba* L.) avec le gène HVA1 gène pour l'acquisition de la tolérance à la sécheresse.

Resumé

Transformation par Agrobacterium tumefaciens est la méthode préférée pour la transformation des légumineuses. Communément, les gènes transférés par cette technique sont clonés entre les frontières gauche et droite d'ADN-T (Transferred-DNA) des vecteurs binaires qui se répliquent à la fois dans E. coli et Agrobacterium. Dans cette étude, le nouveau protocole de clonage des gènes d'intérêts "Gateway multisite System" a été utilisé pour insérer une nouvelle construction contenant la séquence HVA1, le promoteur double virus de la mosaïque du chou-fleur (CaMV) 2x35S et le terminateur Nos dans un vecteur binaire pTF101.GW3. Le vecteur binaire généré pTFGat101.11 contenant la construction de gène HVA1 a été ensuite introduit dans la souche A. tumefaciens EHA101. En utilisant le protocole de transformation basé sur l'organogenèse directe à partir des nœuds cotylédonaires, nous avons réussi à obtenir 2% des pousses transgéniques de fève qui intègrent la construction de HVA1. Cette intégration a été confirmée par PCR de gène marqueur bar.

Mots clés : *Vicia Faba* L., clonage, gène HVA1, *Systéme multisite Gateway.*

استنساخ جين HVA1 بطريقة متعدد الابواب وادخال الجين « multisite gateway » في الفول من اجل مقاومة الجفاف

عبدالواحد ربحة، كنكلي كان، أدوب سريبد، ديرية غزلان، منتاك رشيد، ابريز محمد، عراقي ادريس

ملخص

ان الهندسة الوراثية بالبكتيريا الأجرعية المورمة «Agrobactérium tuméfaciens» هو الأسلوب الفعال عند التعديل الجيني للقطاني . عادة الجينات المرغوب ادماجها بهده الطريقة يجب ان تكون بين يمين و يسار الحمض النووي المحمول «T –DNA» من اجل اكثاره داخل القولونية «E. Coli» والاجرعية «Agrobactérium tuméfaciens». في هده الدراسة تم استخدام بروتوكول جديد «Gateway multisite System» من أجل ادماج صيغة جينية جديدة تحتوي على مورثة HVA1 . الناقل التنائي المستنبط pTFGat101.11 المحتوي على المورثة HVA 1 تم ادماجه في السلالة الجرعية EHA 101. وقد تم استعمال هده الأخيرة على العقد البرعومية للفول لزراعة النسيج و الهندسة الوراثية للفول. حيث حققنا نسبة نجاح تصل الى 2 في المائة من النباتات التي أدمجت الجين المقاوم للجفاف. هدا الأدماج تم تأكيده بواسطة الواسمات الجزئية عن طريق تقنية PCR .

الكلمات المفتاح : الفول، الاستنساخ، جينHVA1 ، كتواي ملتيسايت.

Introduction

Faba *bean* (*Vicia faba* L.) is an important cool season legume mainly grown in West and Central Asia, North Africa, Southern Europe, China and Ethiopia. Faba bean is used as human food in developing countries and as animal feed, mainly in industrialized countries. In Morocco, drought is the most important environmental stress affecting crop production including faba bean. The most effective way of managing this stress is through use of drought tolerant faba bean cultivars. Traditional breeding approaches aimed at developing drought tolerant lines are hampered by lack of availability of good sources of tolerance in the cultivated germplasm and complexity of inheritance of these traits as they are controlled by many genes. Recent developments in functional genomics had allowed the identification and isolation of a large number of stress-inducible genes involved in drought tolerance in various plant species such as Arabidopsis, rice, barley (Jewell *et al*. 2010). Significantly, the introduction of many stress-inducible genes via genetic transformation resulted in improved plant stress tolerance in various plant species (Zhang *et al*., 2004; Umezawa *et al*., 2006). Therefore, this technique offers suitable alternative and rapid approach for improvement of stress tolerance.

In plants, transgenic approach has been successfully used to introduce and over express the barley *HVA1* gene encoding for late embryogenesis abundant (LEA) protein in wheat, oats, rice, and strawberries (Bahieldin *et al*. 2005; Maqbool *et al*. 2002; Wang *et al*. 2004).

Agrobacterium tumefaciens is the preferred method for transformation of a wide range of plant species. Commonly, the genes to be transferred by this technique are cloned between the left and right T-DNA borders of so-called binary T-DNA vectors that can replicate both in *E. coli* and *Agrobacterium*. However, HVA1 gene was cloned in pBluestript vector which is not a binary vector. So, to use this gene in Agrobacterium mediated transformation of *faba bean*, we must clone it in a binary vector. In recent years, Gateway® cloning technology (Invitrogen Co.) has developed a fast and reliable alternative cloning methodology based on bacteriophage λ site-specific recombination to clone the gene of interest into binary vectors of a larger size (5 to 12 kb) in order to obtain transgenic plants via Agrobacterium-mediated T-DNA transformation (Karimi *et al*., 2002 and Ruqiang and Qingshun Quinn, 2008). Thus, in terms of its efficiency when compared to traditional DNA cloning, Gateway® cloning technology has proved to be extremely useful for gene cloning into a larger size of vectors (Curtis et Grossniklaus, 2003). Many Gateway® compatible binary vectors have been made available (Karimi *et al*., 2002; Curtis et Grossniklaus, 2003). So, the first step of our work is to clone the *HVA1* gene construct (currently cloned into pBluescript plasmid) into Agrobacterium binary vectors such as pTF101.gw3 by using gateway multisite technology. The second step is to introduce the resulting binary vector containing HVA1 gene into *Agrobacterium strain*. The third ste p is to transform cotyledonary nodes of faba bean using the *HVA1* transformed Agrobacterium, to increase its tolerance to drought.

Materials and methods

1. Cloning of *HVA1* gene in a binary vector pTF101.gw3

This part of the work was carried out in collaboration with Prof. Kan Wang, from Iowa State University, Ames, USA.

The *HVA1* gene construct pBY520 (*HVA1* cloned in pBluestript vector) was kindly provided by Prof R. Wu, Cornell University, Ithaca, USA. The construct pBY250 contains cassette consisting of the rice *Act1* promoter, the HVA1 sequence, and *Pin3* terminator (Fig.1). In order to insert a novel construct containing HVA1 sequence, double *Cauliflower mosaic virus* (CaMV) 2x35S promoter and Nos terminator into a binary vector pTF101.gw3, we used gateway multisite technology. This technology allows assembling multiple DNA segments in a single highly efficient and specific *in vitro* LR clonase reaction (Ruqiang and Qingshun Quinn, 2008). The Multisite Gateway Three-Fragment Vector Construction Kit is used to generate the Entry clone and the expression clone: The key steps are as follows:

1.1. Creating the entry clone containing HVA1 *gene*

The MultiSite Gateway® donor vector pDONR™221(Invitrogen) was used to clone attB1 and attB2-flanked PCR products. The key steps were as follows:

1. Designed PCR primers by using Vector NT software for HVA1gene amplification, with an addition of 4-nucleotide GGGG at the 5' end of the forward primer followed by 25 bp attB1 site and 20 bp of HVA 1 gene sequences. For reverse primer, the addition of four guanine followed by 25 bp attB2 and 25 bp of HVA gene.

HVA1,5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCAGTTCGTGTTTGTTTG

HVA2,5'-GGGGACCACTTTGTACAAGAAAGCTGGGTATTTTTTTTTTTTTTTTTCGATCAC

2. These primers are used to amplify DNA sequence of HVA1 gene by using pBY250 plasmid containing the HVA1 gene as a template The PCR reaction conditions were as follows:

PCR reactions contained 1ul of 0.5 pmol/µl of each primer, 2ul of 50 mM Mg2+, 2ul of 2.5 mM each dNTP, 1 ul of 50 ng template DNA, 5 ul buffer and 0.5 ul of Pfx DNA Polymerase and water to reach 50ul of mix.

PCRs were incubated at 94°C for 30 sec, followed by 35 cycles of 94°C for 30 sec, 58°C for 1 min, and 68°C for 5 min , with a final incubation at 68°C for 10 min and 4°C Hold.

3. The obtained PCR products flanked by *att*B site were submitted to Agarose gel electrophoresis to check PCR products, after which the desired band was cut and purified using the QIAquick Gel Purification Kit. (According to the manufacturer instructions).

4. BP Recombination Reaction

We used the PCR products in BP recombination reactions with the donor vectors pDONR™221 to generate the entry clones containing DNA sequences of *HVA1* gene. 7ul of PCR products (150ng/ul) were mixed with 1ul pDONR™221 vector (150 ng/ul) in the reaction buffer. To each sample, add 2 µl of BP Clonase™ II enzyme mix (Invitogen) and incubated overnight at 25°C. To stop the reaction 2 µl of proteinase K solution was added and the mixture was incubated for 10 min at 37°C.

5. 1 µl of the cloning PB reaction mixture was used to transform 50 µl of the competent *E. coli* cells (DH5α) using electroporation method. After electroporation, the cells were spread on LB medium plate containing 50µg/mL kanamycin, and incubated overnight at 37°C.

6. Some colonies, selected randomly, were inoculated in liquid medium and then plasmid DNA was extracted using the miniprep kit according to manufacturer instruction (Invitrogen). The integration of the insert is checked by electrophoresis after restriction enzyme digestion using PvII enzyme.

7. LR Recombination reaction:

Three entry clones (AttL4- **2x35S**-attR1 + AttL1-**HVA1**-AttL2 +AttR2-**Nos**-AttL3) were used in a single Multisite Gateway LR recombination reaction with a specially designed destination vector pTF101.gw3 to create the expression clone of interest.

Note: AttL4-2X35S- attR1 and AttR2-Nos-AttL3) entry vectors and destination vector pTF101.gw3 were donated by Iowa State University.

in a 10 µl MultiSite Gateway® LR reaction, 145 ng of destination vector pTF101.gw3, 100ng of tree entry vectors 4.5 ul TE and 2 ul of LR Clonase™ Plus enzyme mix were added and the reaction was incubated overnight at 25°C. The reaction was stopped by 1ug of proteinase K solution and incubation at 37°C for 10 min.

8. We took 3–5 µl of the LR reaction mixture to transform competent *E. coli* cells (DH5α) by electroporation, spread the transformation on LB plate containing 50 µg/ ml spectinomycin and incubated overnight at 37°C. We harvested all colonies from the plate and isolated plasmid DNA. The mixture contains the non-reacted plasmids of the Entry clone, as well as the recombination plasmid between the Entry clone and the Destination vector. But E. *coli* cells containing non-reacted plasmids of the Destination vector and by-product plasmids of the LR recombination reaction failed to grow because these plasmids contain the ccdB gene which is toxic to the cell growth of *E. coli* stain.

9. We confirmed the construct integration in a binary vector by electrophoresis analysis after digestion with PvuII and Hind III enzymes.

1.2. Agrobacterium transformation to select the recombinant expression clone

We used electroporation method to transform competent *Agrobacterium* cells *EHA101*. We added 150 µL YEP liquid media and incubated at 28°C for 4 hr with gentle shaking; then transferred and spread all cells on YEP plate containing 50µg/mL spectinomycin and incubated 2 to 3 days at 28°C. Since the unreacted entry clones were removed by the selection due to lack of *Agrobacterium* replication origin, only the recombinant destination plasmid can be selected in *Agrobacterium*.

2. Transformation of faba bean with HVA1 gene using Agrobacterium

2.1. Bacterial strains and gene constructs

The binary vector generated pTFGat101.11 containing the HVA1 gene and bar gene construct which confers resistance to the glufosinate herbicide (basta) was transformed into *Agrobacterium strains* EHA 101 (kindly provided by Dr Kan Wang, Iowa State University, USA) by electroporation. Then it was used for genetic transformation of faba bean.

2.2. Explants preparation

Transformation procedure developed in our laboratory based on direct organogenesis from cotyledonary node explants was used. Healthy and uniform seeds of 'Defès' cultivar were surface-sterilized by 70% ethanol for 1 min followed by soaking in 30% sodium hypochlorite (bleach) with a few drops of Tween 20 for 20 min. Before soaking in sterile distilled water overnight, the seeds were subsequently rinsed 3 times with sterile distilled water. The peeled seeds were soaked in 1g/l PVP solution for 1 hour to reduce the production of phenols and cultured on MS/2 medium (Half MS salts) (Murashige and Skoog, 1962). The cotyledonary node explants (3–4 mm) were excised from 6 days old in vitro raised seedlings by removing both the cotyledons, and excising both epicotyls and hypocotyls approximately 1 and 2 mm respectively from above and below the nodal region with a very sharp razor blade wetted by the EHA101 Agrobacterium strain. Then the explants were incubated in the Agrobacterium suspension for 30 min.

2.3. Preparation of the bacterial suspension

For preparation of the bacterial suspension culture, 1 ml of *A. tumefaciens* strain *EHA101* harboring pTFGat101.11 plasmid was allowed out from a -80°C glycerol stock culture and precultivated on Yep solid medium containing 50 µg/ ml spectinomycin at 28°C during 1 to 2 days. Single colonies of the bacteria (EHA101) were grown overnight (16 hr) on a shaker at 28 °C and 250 rpm in YEP liquid medium containing appropriate antibiotics. Once the A. tumefaciens cell culture (A-culture) reaches density of 0.8 at OD600nm, the culture is ready for inoculation. The Agrobacterium culture was diluted with MS liquid medium amended with 2 mg/l TDZ and 2 mg/l BA (MSTB).

2.4. Inoculated Plant selection on selection media

After inoculation, each explant was placed on appropriate cocultivation medium lined with filter paper. Cocultivation was continued for 3-4 days at 24°C. After cocultivation, the explants were washed in sterile water containing 400 mg/l of cefotaxime. The explants were subsequently cultured on appropriate medium MSTB containing 3 mg/l glufosinate (glufosinate-ammonium) for *bar* selection.

Results and Discussion

In order to clone the HVA1 gene in a binary vector, we chose the gateway multisite technology, because we do not have the plasmid map of pBY250 to choose the best restriction enzymes for plasmid and vector to clone the gene of interest by using the strategy based on digestion and ligation. The second objective is to change the rice Act1 promoter by double Cauliflower mosaic virus (CaMV) 2x35S promoter. The Gateway MultiSite recombinational cloning framework is ideally suited for constructing versatile collections of genetic elements to be assembled in predetermined order, orientation, and open reading frame (ORF) registry (Karimi *et al*., 2005, 2007) and to be linked at a single time by an LR reaction (Karimi *et al*., 2007). In collaboration with Iowa State University, the construct containing 2x35S promoter, *HVA1* gene, and *Nos* terminator was subcloned in a gateway binary vector pTF101.gw3 by using the cloning technique "multisite gateway". This technology has provided a fast and reliable alternative to the cloning of sequences into large acceptor plasmids (Liu *et al*., 1998; Hartely *et al*., 2000). Gateway binary vectors have proved also to be useful for high-throughput plant transformation (Karimi and al.2002; Dubin and al., 2008). Because the site-specific recombinant mechanism is precise, gateway multisite technology allows to insert and to clone multiple DNA segment in one vector in a predefined order and orientation (Liu *et al*., 1998; Hartely *et al*., 2000). In the new construct, we changed rice Act1 promoter by double *Cauliflower mosaic virus* (CaMV) 2x35S promoter to transform faba bean. In fact, the 35S promoter has been widely and successfully used for expression of transgenic in plants (Kamo *et al*, 2000). The level expression of uidA gene derived by 35S promoter is higher than the expression of the same gene derived by Act 1 promoter in dicotyledonous species (Kamo *et al*, 2000).

We created an entry clone by first generating a HVA1 PCR product containing *att*B sites (Fig.2), which required two overlapping PCR reactions due to the addition of a 25 bp *att*B site on both ends of the HVA1 PCR product. This *att*B-PCR product was then used in BP reaction with a donor vector pDONR™221 to generate the entry clones containing DNA sequences of *HVA1* (Fig.3). Then we used this clone and two others entry clones containing respectively 2x35S promoter and Nos terminator in LR reaction (Fig.4) with a gateway binary vector pTF101.gw3 (Fig.2) to generate the expression clone of interest genes (HVA1, 2x35S and Nos). The binary vector generated is pTFGat101.11 (Fig 5) containing *HVA1* gene. This vector contains the appropriate border sequence to aid the transfer of T-DNA in plant genome and *bar* gene resistance to allow selection of putatively transgenic plants on basta medium.

The construct integration in pTF101.gw3 binary vector was confirmed by electrophoresis after double digestion with Hind III and PvuII enzymes (Fig.6). The number and the size of bands obtained confirm this integration.

pTFGat101.11 plasmid containing HVA1 gene construct were subsequently introduced into EHA101 strain of *A. tumefaciens*. The resistant colonies to spectonomycin were used for faba bean transformation (as described below). Using the transformation protocol (Abdelwahd *et al*., 2009), we succeeded in obtaining a number of regenerated transgene shoots from cotyledonary node. After a number of subcultures on fresh medium added with glufosinate phosphinothricin (basta), some of these shoots survived under selection pressure (Fig.7).

To confirm the integration of phosphinothricin acetyltransferase (bar) gene, which confers resistance to herbicide basta, through PCR analysis, genomic DNA PCR of selected bar expressing plants and one non-transformed plant was carried out using bar gene-specific primers. The bar gene was detected as 400 bp fragment, in the transgenic plants. The amplified fragments were of the same size as was expected (Fig.8). The data indicated that transgenic plants expressing bar gene have been successfully produced via Agrobacterium mediated transformation of faba bean with 2 % for transformation efficiency designed by (bar integrated shoots number/inoculated explants number). Thus, we can deduce the possible integration of HVA1 gene to be confirmed by molecular techniques and physiological tests.

pBY520 map

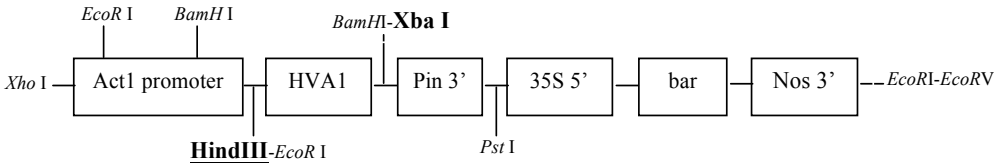

Fig.1. Map of HVA1 construct gene in pBY520 plasmid.

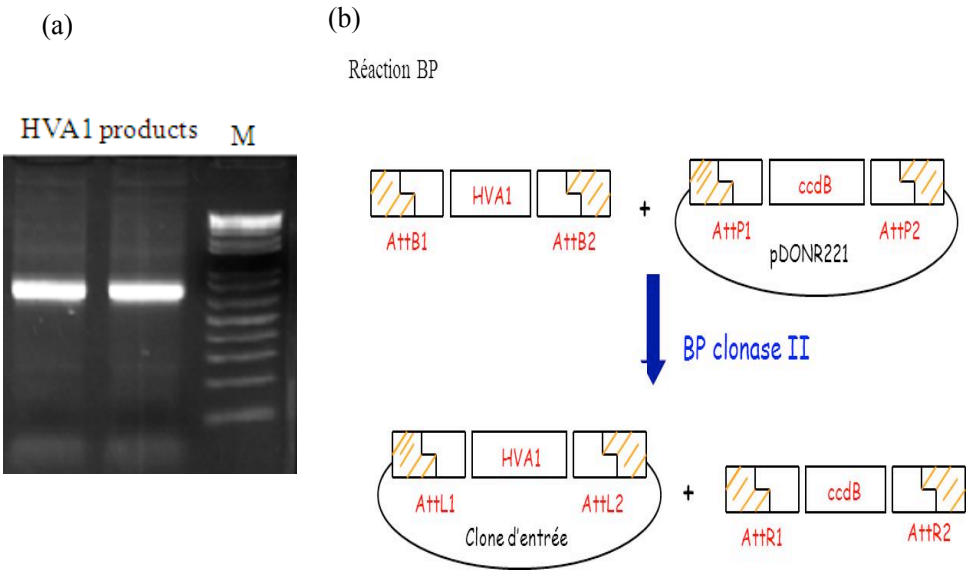

Fig.2. Gateway BP: (a) PCR product "HVA1 fragment" flanked by attB sites amplified (L1, L2) by PCR reaction with the recommended attB primers, M: Marker. (b) Schematic drawing of BP recombination of a PCR product "HVA1 fragment" flanked by attB sites with a Gateway donor vector pDONR™221 to generate entry vector pDONR221/HVA1sequence.

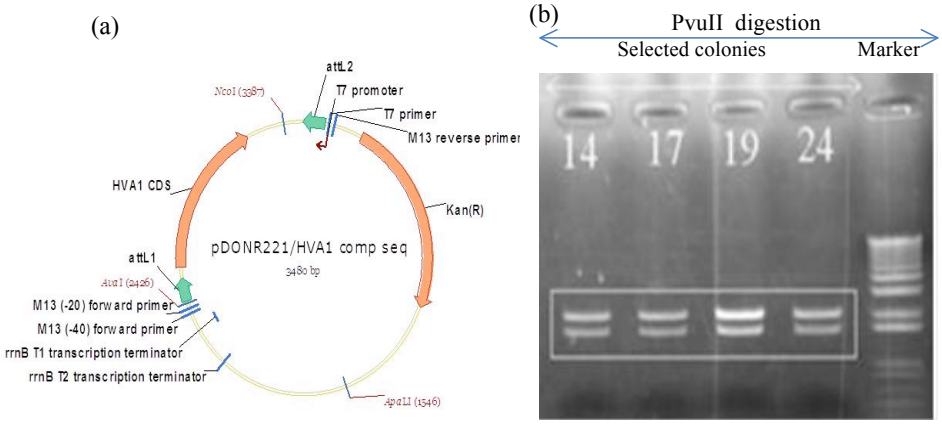

Fig.3. Gateway BP: (a) Map of entry vector pDONR221/HVA1sequence generated after gateway BP reaction. (b) the integration of the insert (HVA1 flanked by attB sites) in pDONR221 is confirmed by electrophoresis analysis of the PCR product using PvuII enzyme.

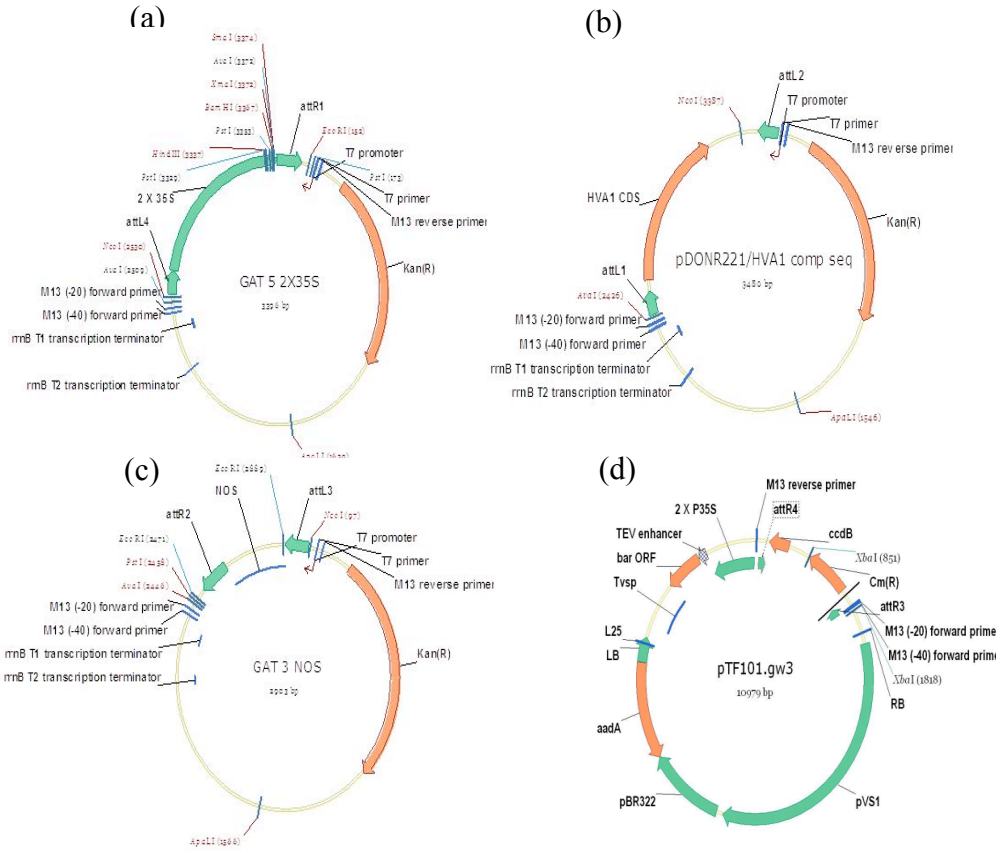

Fig.4. Multisite Gateway LR: Maps of the three entry clones bearing the DNA fragments (a) "2x35S", (b) "HVA1" and (c) "Nos" with the (d) MultiSite Gateway destination vector pTF 101.gw3 (Iowa state university) used in LR recombination reaction to generate pTFGat101.11 containing HVA1 new construct.

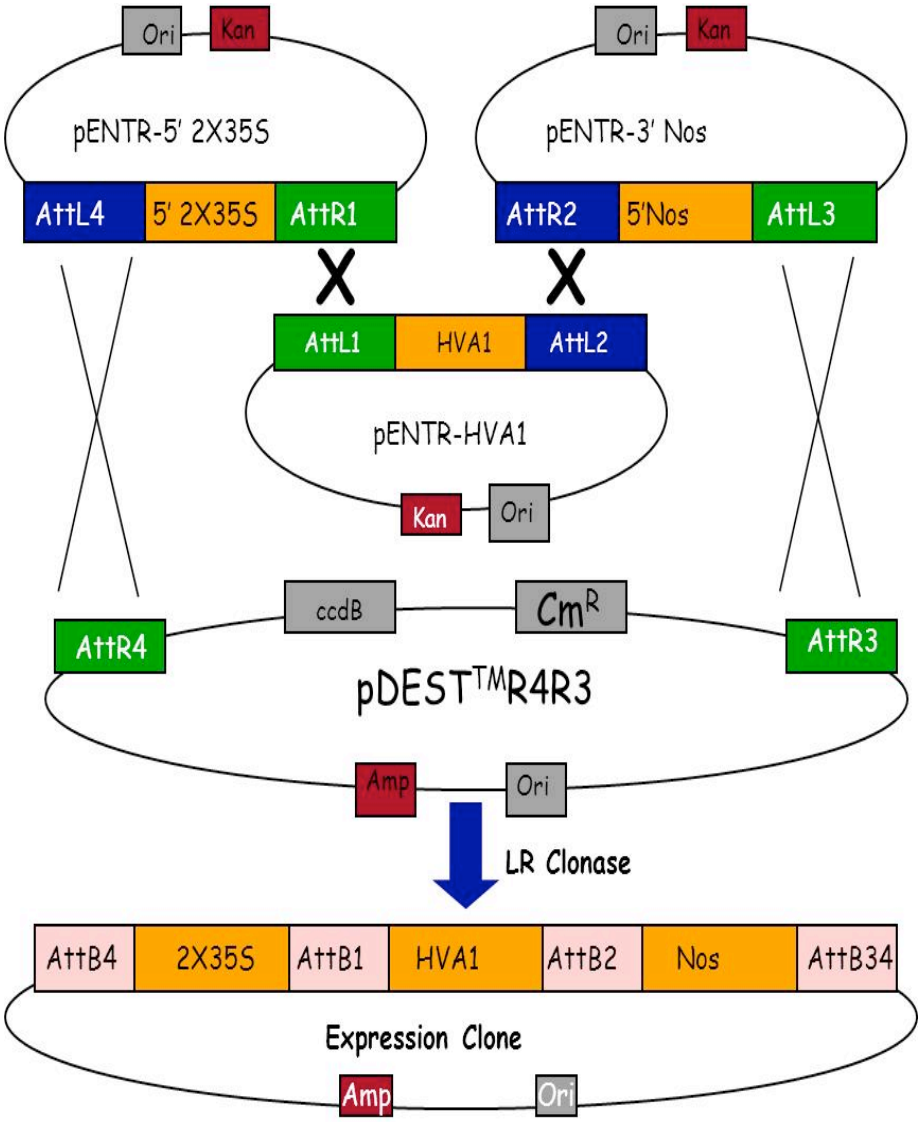

Fig.5. Multisite Gateway LR: Schematic drawing of LR recombination of three entry clones bearing the DNA fragments (b) "2x35S" promotor, (c) "HVA1" gene and (d) "Nos" terminator with the (e) MultiSite Gateway destination vector pTF 101.gw3 (pDEST TMR4R3).

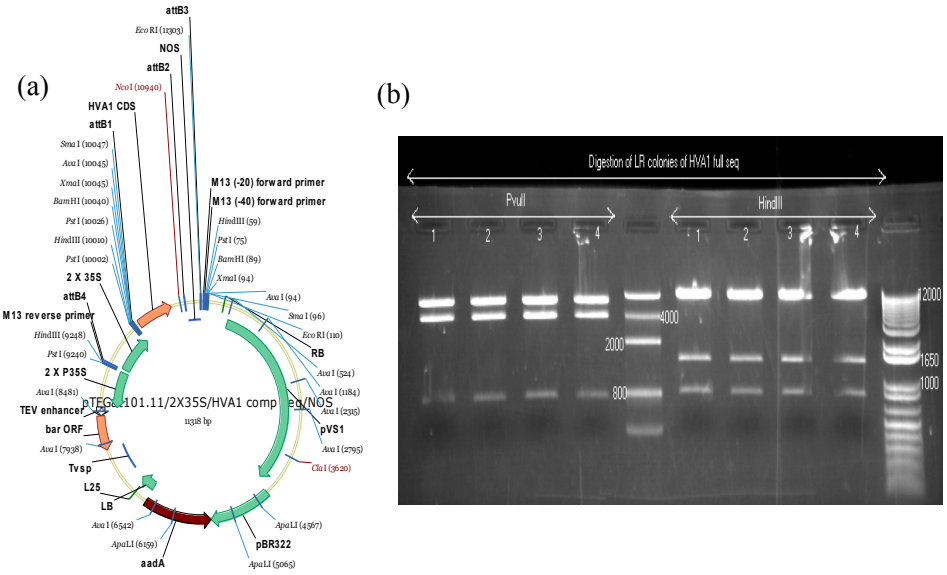

Fig.6. Cloning of HVA1 gene in a binary vector by gateway multisite: (a) regenerated pTFGat101.11 plasmid harboring new HVA1 construct. (b) Confirmation of HVA1gene integration in pTFGAT101.11 by digestion of LR colonies using HindIII and PvuII enzymes.

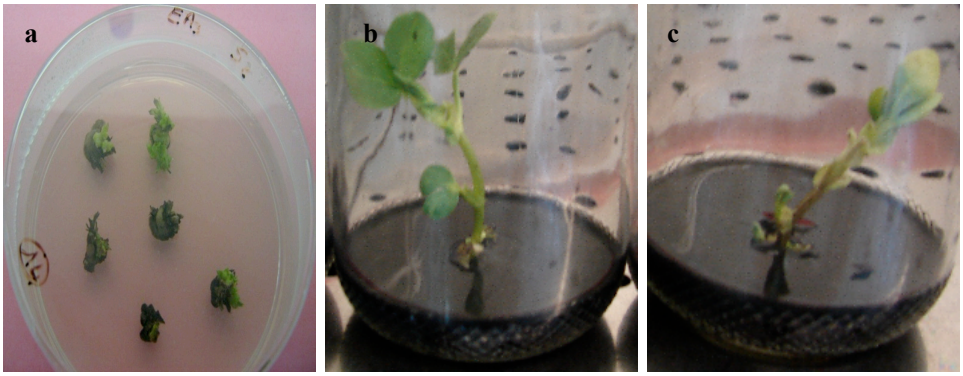

Fig.7: Faba bean genetic transformation by pTFGat101.11 containing new construct of HVA1: (a) using cotyledonary node as explants (b) basta resistant plant on selective media (c) non transformed plant on selective media.

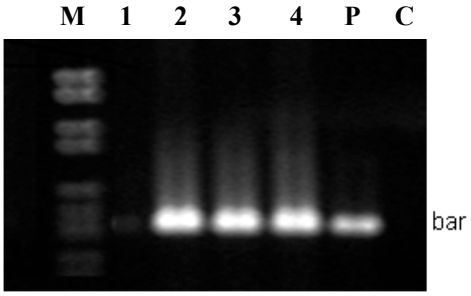

Fig. 8: Bar gene amplified by PCR. P: pTFGat 101.11 plasmid, L2, 3 and L4 : Transformed Plants , C: No transformed plant, M : Marker.

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