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Full Length Research Paper

Construction of PVX virus-expression vector to express enterotoxin fusion gene *LTB-ST* and transformation into tobacco

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Potato X potyvirus (PVX)-based vector has been comprehensively applied in transient expression system. In order to produce the heterologous proteins more quickly and stably, the *Cla*I and *Not*I enzyme sites were introduced into the Enterotoxin fusion gene *LTB-ST* by polymerase chain reaction (PCR) and the *LTB-ST* gene was introduced into the PVX-based vector in the site of the *Cla*I and *Not*I after digested incompletely by *Cla*I and *Not*I. Then the positive clone was picked and was sequenced and the clone with exactly same sequence was named PVX-LTB-ST vector. The recombinant plasmid was transformed into *Agrobacterium tumefacience* strain MOG101 and LBA4404, respectively. The fusion gene *LTB-ST* cannot be amplified stably in the MOG101, while it could be amplified stably in LBA4404. Of the two methods which were used to infect tobacco, the plasmid plaiting method could not make the leaves show mosaic symptoms, and plants which were infected using the agroinoculation method showed mosaic symptoms. One step reverse transcriptase (RT)-PCR analysis indicated the *LTB-ST* gene expressed in the inoculated tobacco plants with mosaic symptoms.

Key words: Enterotoxin fusion gene *LTB-ST*, PVX virus-expression vector, transient expression, tobacco.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is the major factor that causes diarrhea to infants and travelers in developing countries (DCs). Diarrhea is the most common health problem experienced by travellers to resource-poor regions of the world, occuring in 20-60% of travellers, and the ETEC causes more than 30% of travellers' diarrhoea (Torrell *et al*., 2009). When ETEC adhered to the surface of epithelia, it can excrete the heat labile enterotoxin (LT) and the heat-stable toxin (ST) that can make the person sick. So the LT and the ST are two important pathogenic factors in the pathogen. However, the protective role of specific immune responses and the antigens which elicit these responses are not well understood. LT is key vaccine target (McKenziea et al., 2008), while ST is a simple protein containing 19 polypeptides and it is so small that it has hardly any immunogenicity. In order to make them suitable for all kinds of bacteria study and get stronger immunogenicity, scientists often fused the *LT* and *ST* genes together; the *LT-ST* fusion gene could successfully express in *E. coli* (Zhang, 1994, 2000; Sack et al., 2007). The other research showed that the fusion *LT-ST* gene got high-level expression in recombinant *E. coli* and the fusion protein is nontoxic (Xu et al., 1999). Different systems have been developed for the synthesis of recombinant proteins so far (Jia et al., 2003). One way is to express the interest gene in plants in which the foreign protein is produced by permanent integration or transient expression with virus based vectors (Niu et al., 2009). The protein yields from the permanent integration of a foreign gene into plant

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Abbreviations: ETEC, Enterotoxigenic *Escherichia coli*; **LT,** heat labile enterotoxin; **ST,** heat-stable toxin; **PVX,** potato virus X; **CPMV,** cowpea mosaic virus; **RT-PCR,** reverse transcriptase-polymerase chain reaction.

genome are generally low (Mason and Arntzen, 1992), while the transient expression system can always results in high-level expression of either fusion protein or accumulation of free polypeptide (Yang et al., 2008). Especially with the development of molecular biotechnology, recombinant viral vector vaccine is one of the most promising approaches for vaccine research (Lu et al., 2008).The pharmaceutical proteins and vaccine were produced with viral expression vectors as bioreactors in recent years, which has become very successful in producing foreign proteins (Lomonossoff, 1995). At the same time, viral expression vector has many advantages over other production systems and it has become a hot point in plant genetic engineering.

Compared to the cultivation of transgenic plants, virus based expression in plants offers rapid production of diverse types of antigens (Wagner et al., 2004). Thus more and more antigens were expressed in plants using viral expression vectors, such as Hepatitis B (Mason et al., 1995) and Norwalk virus (Mason et al., 1996). The VP6 (Rotavirus major inner capsid protein) has been expressed in *Nicotiana benthamiana* plants using vectors based on potato virus X. The potato virus X (PVX) or cowpea mosaic virus (CPMV) containing the sequence of *HBcAg* could infect the host plants and remain stable during infection (Mechtcheriakova et al., 2006). The use of viral vectors could be considered a practical method for rapid production of assembled *HBcAg* particles in plants.

Here we report that the fusion gene *LTB-ST* could be expressed in plants using appropriate potato virus X vectors to produce LTB-ST fusion protein, and we expected that the functional LTB-ST fusion protein were expressed through PVX transient expression system, and probe the appropriate approach by which the diarrhea vaccine was produced in short time.

MATERIALS AND METHODS

Plasmid and strains

Nicotiana tabacum cv. *Samsun* which was provided by Dr. Mu Zhuang was used as the host plant. *Agrobacterium tumefaciens* strains MOG101, LBA4404, potato virus X (PVX) vector (*TV1*) were supplied from the biotechnology laboratory of the Institute of Vegetables and Flowers, China Academy of Agricultural Sciences. Expression vehicle PXZL01 (Xu et al., 1999) containing fusion gene, *LTB-ST*, was kindly provided by Professor Zhaoshan Zhang of Beijing Institute of Biotechnology. PVX_F (5'CAATCACAG TGTTGGCTTGC 3') and PVX_R (5' GACCCTATGGGCTGTGTTG 3') were a pair of primer that could amplify a 280 bp fragment from the PVX vector (*TV1*) (Zhuang et al., 2006).

Plasmid construction

All molecular manipulations were carried out following standard procedures (Sambrook et al., 2001). The fusion gene *LTB-ST* was polymerase chain reaction (PCR)-amplified from plasmid PXZL01 (Xu et al., 1999) using forward primer LTB-ST_F (5'GGG ATCGAT ATG AAT AAA GTA AAA TTT TAT GTT TTA T 3') and reverse primer LTB-ST_R (5'TCT GCGGCCGC GCA CCC GGT ACA AAG AGG AT 3'). To get the exact sequences of the fusion gene, Pfu hi-fi Taq polymerase (Takara) was used, the volume of reaction was 25 µL, and the PCR amplifications with the following cycling conditions: 94℃ for 3 min; 94℃ for 1 min, 58℃ for 2 min, 72℃ for 2 min, 35 cycles, 72℃ for 5 min. The *Cla*I and *Not*I recognition site was introduced into the PCR products, the PCR products was digested incompletely by *Cla*I (*Cla*I 1µL, 10×M buffer 2 µL, DNA 1µg, ddH_2O 16µL, 1~2 h) then digested by *Not*I. The *Cla*I/*Not*I digested PCR fragment was inserted into the PVX vector (*TV1*) between the *Cla*I/*Not*I restriction enzyme sites to create the recombinant plasmid. Then the positive clone was picked and was sequenced; the clone with exactly same sequence was named PVX-LTB-ST vector.

The inoculation of plasmid or agroinculation

The recombinant plasmid was transferred into *A. tumefaciens* strains MOG101 and LBA4404, respectively. After the *Agrobacterium* transformation was confirmed by PCR, the MOG101 clone harboring the recombinant plasmid was named as M-LTB-ST and the LBA4404 clone was named as L-LTB-ST. Then the positive *Agrobacterium* clone was inoculated over night with shaking (~180 rpm/min) in Luria-Bertani (LB) liquid medium with kanamycin at 50 µg/ml and rifampicin at 20 µg/ml (M-LTB-ST) or kanamycin at 50 µg/ml and rifampicin at 50 µg/ml (L-LTB-ST). The *Agrobacterium* was cultured to the $OD_{600} \approx 0.5$, and bacteria suspension picked with a toothpick was used as template for PCR detection (Chen et al., 2006). Also, the secondary culture of *Agrobacterium* clone was double checked with PCR in order to find out whether the foreign gene was stable or not.

N. tabacum cv. *Samsun* was grown in pots at 25℃ in a growth chamber under 16 h light/8 h dark cycles. One leaf of *tobacco* was inoculated with the plasmid of PVX-LTB-ST or the bacterial suspension of L-LTB-ST. Some carborundum was sprinkled on the leaves to be inoculated. The leaves were spread gently using the finger with the disposable glove, which would cause the imperceptible wound on the surface of the leaves. Then the 1 µL plasmid of PVX-LTB-ST or the 1 µL bacteria suspension of L-LTB-ST was inoculated onto the surface of the spread leaves with micropipettor, and also the plasmid or the bacteria suspension was spread with a gloved finger. The one piece of leaves was inoculated on each plant, and then the leaves were immediately covered with a plastic bag in order to keep the humidity for inoculation. The plasmid of PVX-LTB-ST was used to inoculate 30 plants, using the distilled water as the negative control. The bacteria suspension of L-LTB-ST and M-LTB-ST was used to inoculate 10 plants for each bacteria, using the distilled water as the negetive control. The symptom of tobacco was observed two weeks after inoculation.

Detection of systemically infected leaves

The inoculated leaves were checked with one step reverse transcriptase (RT)-PCR. The protocol used is as follows: The fresh post-inoculated leaf was weighted and grinded. 1 µg leaves was added into 10 µL virus extraction solution (0.5M pH 8.0 Tris-HCl, 2%PVP-40, 1%PEG 6000, 0.8% NaCl, 0.05%Tween 20), mixed, and 10 µL from the mixture diluted with 1000 µL distilled water. 0.5 µL diluted mixture was the template of the RT-PCR. The final reaction volume of RT-PCR was 25 µL, 1.5 mM $MgCl_2$, 200µM dNTPs, the forward and reverse primers were 5 pmol (final concentration) respectively , 5 U superscriptII, 4 U RNase inhibitor, 2.5 U Taq

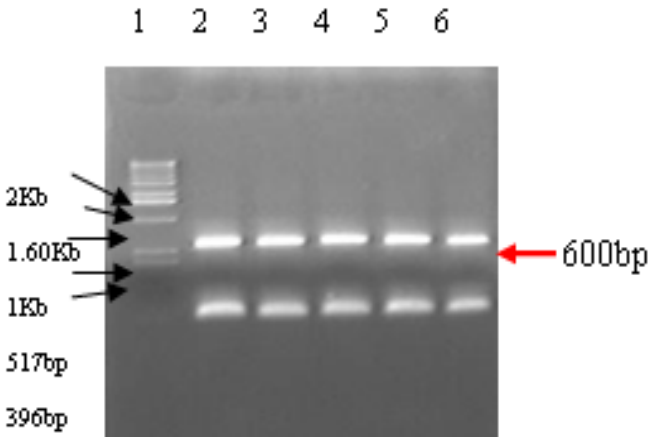

Figure 1. Agarose gel detection of the LTB-ST Pfu-PCR products. Lane 1, 1kb DNA ladder (from top to bottom: 8 kb, 7 kb, 6 kb, 5 kb, 4 kb, 3 kb, 2 kb, 1.60kb, 1 kb, 517 bp, 396 bp, 230 bp); lanes 2,-5, LTB-ST Pfu-PCR products.

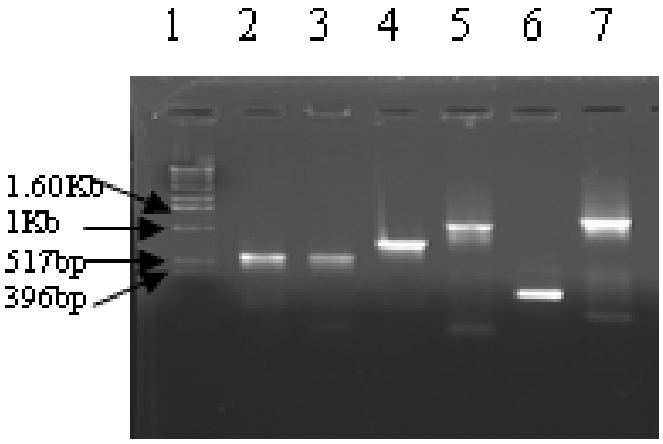

Figure 2. PCR analysis of recombinant plasmid. Lane 1, 1kb DNA ladder; lane 2, PXZL01 plasmids amplified by primer LTB-ST and LTB-ST-R; lane 3, recombinant plasmids amplified by primer LTB-ST-F and PVX-R; lane 5, recombinant plasmid amplified by primer PVX-F and LTB-ST-R; lane 6, TV1 plasmids amplifies by primer PVX-F and PVX-R.

DNA polymerase, 1× Taq PCR buffer. The first step is reverse transcription, 42℃ for 30 min; then 95℃ for 4 min, 94℃ for 20 s , 54℃ for 30 s , 72 ℃ for 40s, 30 cycles, followed by a final extension at 72℃ for 5 mins. The RT-SCR products were resolved by electrophoresis in 1.0% agarose gel.

RESULTS

The amplification of *LTB-ST* fusion gene

The recombination plasmid, PXZL01, carries the *LTB-ST* fusion gene. To reduce toxicity of the ST, the twelfth and the forteenth mononucleotide of the wild ST was changed from “T” and “G” into “G” and “C” separately, so Lys was substituted for Asn residue at position (nucleotide) 12 of ST by oligonucle-directed side mutagenesis. Leu was substituted for Ala at position (nucleotide) 14 of ST. So the LT-B/ST fusion peptides possessed no enterotoxic activity of *E. coli* heat-stable and heat-labile enter-toxins, and retained the antigen epitope (Xu et al., 2002). The LTB-ST_F and LTB-ST_R were used as primers; the products of PCR were LTB/Tyr-Pro-Gln-Asp-Pro-Ile-Ala-Asp- Pro-/Pro-ST (of which ST is a mutant). The Pfu hi-fi Taq polymerase possesses the 3’-5’ exonuclease activity, so the percentage of mismatching was dramatically decreased. The amplification products using Pfu hi-fi taq polymerase could be used in the construction of recombination clone after digestion. The size of PCR product was about 600 bp (Figure 1).

DNA recovery and incomplete digestion of *LTB-ST* fusion gene

The recovery DNA from gel was digested incompletely due to that fact that there was a *Cla*I restriction site in 5' Terminus of the *ST* gene. Then the digested product was deposited with ethanol and the precipitation was washed with 70% ethanol after it was dried. 1 µL digestion product was resolved by electrophoresis in 1.0% agarose gel.

The PCR confirmation of the recombination construct PVX-LTB-ST

The individual bacterial colony that grew on the plate was picked out with a toothpick and inoculated overnight at 37℃ with shaking (~200 rpm/min) in 50 ml LB liquid medium containing kanamycin at 50 µg/ml. The PCR confirmation of LTB-ST was carried out with bacteria suspension as template (Figure 2), the methods of PCR was based on Chen et al. (2006).

The endonuclease cutting of PCR products of recombination clone

There was a single restriction site of *BamH*I near to termination signal of the 3’ terminus of the *LTB* gene, and also a single restriction site *Cla*I near the 5’ terminus of the *ST* gene in the fusion gene. So the PCR product from LTB-ST_F and LTB-ST_R primer pairs was analyzed with restriction endonuclease enzyme *BamH*I or *Cla*I, respectively, to confirm whether the *LTB-ST* fusion gene was successfully cloned into the construct or not. Bands pattern expected from digestion with *BamH*I or *Cla*I should be different (Figure 3).

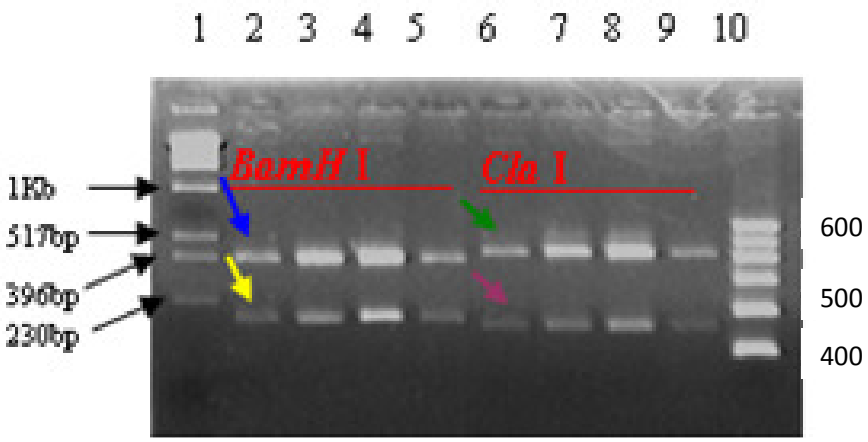

Figure 3. Registriction analysis of the PCR product of recombinant plasmids. Lane1, 1 kb DNA ladder; lanes 2-5, the PCR products of PXZL01, recombinant plasmid No 8, 15, 21 digested with *BamHI*; lanes 6-9, PCR products of PXZL01, recombination plasmid No. 8,15,21 digested with *Cla*I; lane 10, 100bp DNA ladder (from top to bottom).

Sequencing of the recombination plasmid PVX-LTB-ST

The recombinant plasmid was transformed into the *E. coli* Top10 competent cell and sequenced. The sequence was aligned to original one with DNAstar 5.0. There was no dislocation, frame-shift and nonsense mutation in the sequence of mononucleotide analyzed. The recombination clone containing the expected fusion gene was named as "PVX-LTB-ST". The construction process of recombination plasmid PVX-LTB-ST is illustrated in Figure 4.

The recombination plasmid was transformed into *A. tumefaciens* strain MOG101 and its stability tested

The recombination plasmid was extracted and transformed into *A. tumefaciens* MOG101, the PCR detection was carried out to verify whether the fusion gene was transferred into the MOG101 (Figure 5). The positive clone was named "M-LTB-ST". However, the positive recombination clone of bacterium was not stably reproduced during the subculture. The foreign gene could not be detected when the bacterium of subculture was checked by PCR. It is believed that the foreign gene is toxic to MOG101, so that the recombination fragment was prone to lose during the DNA replication of the bacterium. But the specific mechanism is still unknown.

The recombination plasmid was transformation into *A. tumefaciens* strain LBA4404 and its stability tested

The recombination plasmid was extracted and transformed into *A. tumefaciens* strain LBA4404, and expected positive colonies were checked with PCR (Figure 6). The positive clone was named as "L-LTB-ST". Contrast to the MOG101 transformation, the fusion gene *LTB-ST* was more stable in the LBA4404. The PCR result indicated that the foreign gene could replicate stably during the second round of inoculation.

Agroinoculation of tobacco

The positive clones of M-LTB-ST and L-LTB-ST were cultured to the OD_{600}≈0.5 before inoculated on the leaves of tobacco, respectively. Generally, the mosaic symptom will be visible on the inoculation leaves 10~14 days after inoculation and the mosaic symptom will aggravate gradually. So ten days after inoculation, the chlorosis mosaic symptom on the tobacco plants which were inoculated by L-LTB-ST was observed. However, the chlorosis mosaic symptom did not show up on the plants inoculated by PVX-LTB-ST or M-LTB-ST. So the method of agroinoculation was believed to be a effective method to inoculate plants and express foreign gene using PVX vector, and this phenomenon also show that the M-LTB-ST was unstable. The L-LTB-ST induced the same chlorosis mosaic symptom as the wild PVX virus on inoculated tobacco leaves. It indicated that the recombination virus still possessed the pathogenic ability, and the insertion of foreign gene did not shift the open reading frame (ORF) of PVX vector.

Detection of systemically infected leaves and the stability of the infectious vector

To address the transient expression of recombinant virus, systemically infected leaves were sampled and grinded for one-step RT-PCR after inoculated with L-LTB-ST (Figure 7). The expression of *LTB-ST* can be monitored in

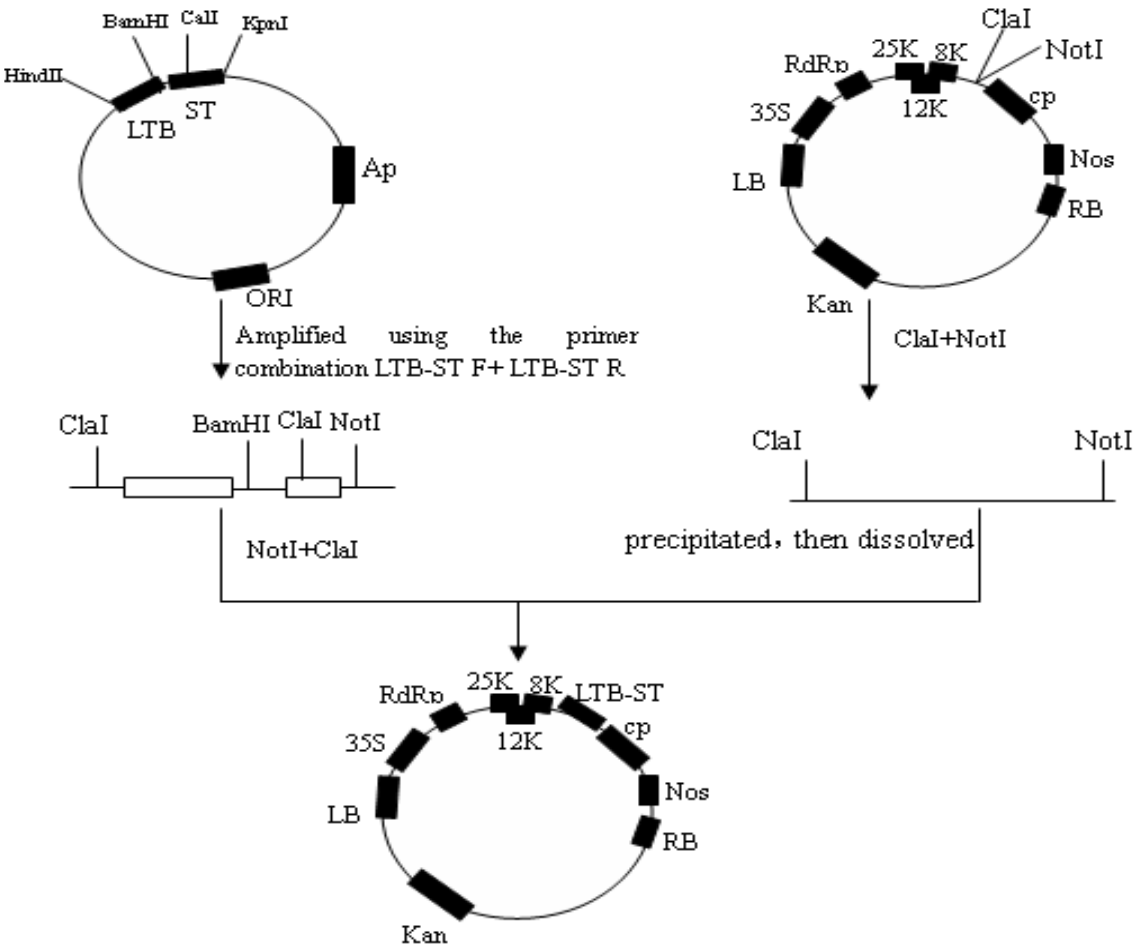

Figure 4. The construction of plant virus-based expression vector PVX-LTB-ST.

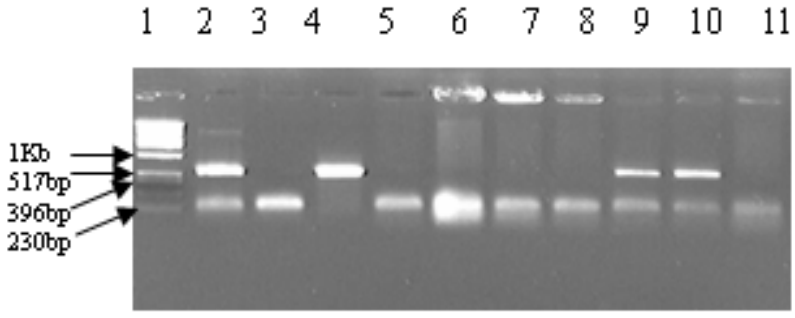

Figure 5. PCR analysis of MOG101 transformed by recombinant PVX-LTB-ST. Lane 1, 1 kb DNA ladder; lane 2, PXZL01 plasmids as positive control; lane 3, water as negative control; lanes 4, 9, 10: positive

the leaves inoculated using RT-PCR.

DISCUSSION

The foreign peptide and proteins was produced quickly with transient expression of virus-based construct. There are two systems for the transformation with plant virus-based vectors. One was to inoculate leaves directly using virus plasmid, in which the recombinant plasmid was extracted directly and inoculated on the leaves. The plasmid would be transformed into virus RNA by *in vitro*

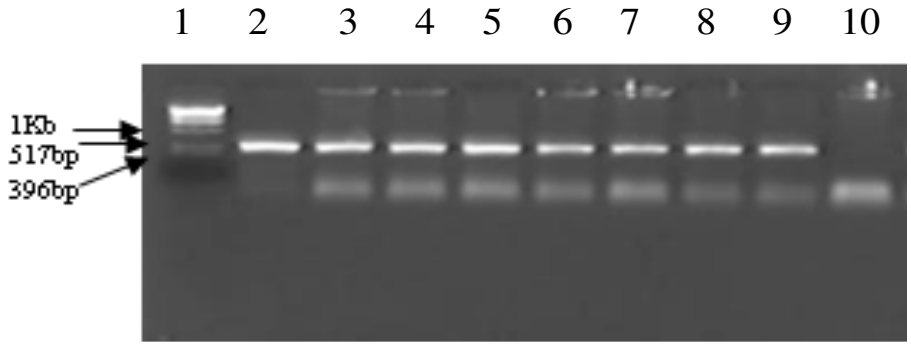

Figure 6. Colony PCR analysis of PVX-LTB-ST transformation in LBA4404. Lane 1, 1 kb DNA LADDER; lane 2, PXZLO1 plasmids (+CK); lanes 3-9, positive clones; lane 10, negative clone.

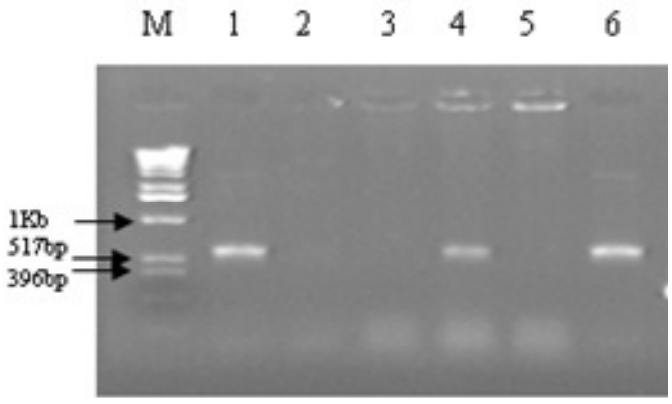

Figure 7. RT-PCR analysis of plants inoculated with L-LTB-ST. Lane M, 1 KB DNA ladder; lane 1, PXZL01 plasmids amplifies by primer LTB-ST F and LTB-ST R; lane 2, blank control; lane 3, negative control; lanes 4- 6, plants which caused chlorosis mosaic symtoms amplified by primer LTB-ST F and LTB-STR.

transcription and packed *in vitro*; this caused the plant to show chlorosis mosaic symptom on the leaves inoculated. Another is by adopting a technique called agroinoculation by which the recombinant plasmid containing foreign gene was inserted into the T-DNA promoted by the cauliflower mosaic virus (CaMV) 35S promoter. In this way, the foreign gene could be efficiently delivered into plant cells and initiate an infection (Jia et al., 2003). Generally, an *in vitro* transcription is required to produce infectious viral RNA prior to inoculation of host plants in the former system, which is a bottleneck and costly process. For the second one, the T-DNA could not be integrated into the host genome after it was transformed into the plant cell by agroinoculation. The foreign gene on the T-DNA would autonomously replicate at a great deal with the virus-mediated method, then amount of copies and products of foreign gene would be formed quickly in a plant cell.

In this study, the recombinant plasmid was transformed into the *A. tumefaciens* strain MOG101 by freezing-melting transformation method. We hoped that the foreign gene can be expressed in MOG101, but the gene *LTB-ST* did not replicate stably in the MOG101 after it was transformed. The foreign gene was lost during the sub-cultured process of bacterium and the strains of recombinant bacterium could not be reserved. Moreover, the chlorosis mosaic symptom was undetectable after agroinoculation.

Due to the poor stability of *LTB-ST* transformation in MOG101 during replication process, the recombinant plasmid was transformed into LBA4404. After subculturing several times, the LTB-ST could be replicated stably in the *Agrobacterium* strain LBA4404. The *LTB-ST* could be detected by one step RT-PCR with virus extraction from leaves inoculated by L-LTB-ST as template and also the chlorosis mosaic symptoms showed up after inoculation. In order to reveal the function of the foreign protein, enzyme-linked immunosorbent assay (ELISA) should be carried out for further research.

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