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人工合成的植物化猪α乳清蛋白基因在转基因拟兰芥的表达

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摘要:将一个 398 bp 的植物化的猪 α 乳清蛋白基因(Lactalbumin, LA)编码区克隆到带有花椰菜花叶病毒的 35S 启动子的质粒中。对它们进行 PCR 检测和序列分析,证实这些阳性克隆是实验预期的重组质粒。随即将 35S 启动子 - α- 乳清蛋白基因 - 终止子这一表达单元克隆到双元载体 pCG-CB 中，用该重组质粒双元载体 pCG-CB-Lact 转化农杆菌 V3101 后,以农杆菌法进行拟兰芥植物转化,用除草剂 Finale 对转化植物进行抗除草剂基因筛选,得到一些抗除草剂的转化植株。对这些抗除草剂植株进行猪 α 乳清蛋白基因 PCR 分析,成功筛选到带有猪 α 乳清蛋白基因并且可以在后代稳定遗传的转基因植物。Western blot 蛋白质表达分析,表明猪 α 乳清蛋白在拟兰芥中成功表达,并且猪 α 乳清蛋白被正确加工成天然蛋白。

关键词: 猪 α 乳清蛋白; 拟兰芥; 转化; 蛋白质表达
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Expression of Synthetic Porcine Alpha-Lactalbumin in Transgenic *Arabidopsis*

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Abstract: A 398-bp plantified encoding fragment of porcine α -lactalbumin, was cloned into plasmid under the control of the cauliflower mosaic virus 35S promoter. The expression cassette was introduced into *Arabidopsis* with *Agrobacterium* mediated transformation. Some herbicide-resistant lines of transformed *Arabidopsis* were selected. The gene integration of chromosomal DNA level were confirmed in regenerated plants. All of them are positive for porcine α-lactalbumin gene via genomic DNA PCR. The expression at the protein level could be confirmed only in the transgenic *Arabidopsis*. The arabidopsis-expressed lactalbumin (LA) migrated in SDS-PAGE with identical mobility to human LA prepared from human milk, indicating that the LA was correctly processed to yield a mature protein in plants

Key words: Porcine α -lactalbumin; *Arabidopsis*; Transformation; Expression

It has been recognized that proteins in seeds from the commonly grown crop species do not contain a nutritionally balanced amino acid content[1]. The seed proteins from cereals are generally deficient in the amino acids lysine and tryptophan, whereas those from legumes are deficient in the sulfur amino acids methionine and cystine[2,3].

Although seeds of legume species were the major food and oil source both for human and animal, their sulfur amino acids deficit of seed composition is dramatically constrained for nutrition value[2,3]. Because of these deficiencies, studies in many laboratories have focused on the genes that encode seed storage proteins[4-6]. Methods to improve the nutritional quality of seed

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proteins by genetic engineering are being evaluated. Up-to date, the technical development of plant transformation makes such experiments feasible. Successful transformation and modification of seed-specific proteins in plant seeds were broadly reported[7,8].

Therefore, alternation of legume seed composition especially to modify the sulfur level is a very import aspect for seed protein studies and need to be paid much more attention. Some animal genes were selected as transgenes to plants. Vodkin et al reported the introduction and expression of bovine casein gene into soybean seeds with Biolistic transformation[9]. In this project, Porcine was selected as suitable transgene because porcine α -lactalbumin is a major component of sow's milk and well-characterized amino-acid balanced proteins[8]. It is therefore ideally suited to swine nutrition. Furthermore, it is also a relatively small protein and the DNA that codes for it can be synthesized in the laboratory. The LA-expressed transgenic plants would be good food resource for pig industry. We constructed a synthetic porcine α-lactalbumin coding sequence containing plant-preferred codon and trans form plants in *Arabidopsis* model system. The resulted plants have been characterized by DNA level and western blotting.

1 Materials and Methods

Materials The vector (pGEM-4Z/lactal) containing plantified 398-bp DNA fragment gene of porcine alpha lactalbumin is kindly provided by Dr. Scott (USDA). The binary vector pCG-CB-ACLA is provided by Dr. Wurtele (Iowa State University). Rabbit anti-human LA is kindly provided by Dr. Scott (USDA). Human LA (h-LA, MW14.4 kD) was purchased from Sigma. The protein molecular weight markers were from Bio-Rad.

cDNA cloning and vector construction The plasmid DNA of plasmid pCG-CB-ACLA and plasmid pGEM-4Z/lactal were prepared by a modified alkaline lysis method[7]. The 398 bp DNA fragment in plasmid pGEM-4Z/lactal and a 6.5 kb large fragment in binary vector pCG-CB-ACLA, digested with *EcoR* I/ *BamH* I, were gel-purified using QIAEX II Gel Extraction Kit (QIAGEN Inc.). The 398 bp DNA fragment was ligated to pCG-CB binary vector to transform *E. coli* DH5α. The resulted plasmid with 35S promoter and target peptide sequence and bar gene selectable marker was named pCG-CB-alpha-lactalbumin (Fig. 1). The plasmid pCG-CB-alpha-Lact was introduced into *Agrobacterium tumefaciens* GV3101 by electroporation. The LA inserts were verified to have the correct sequence by PCR and DNA sequencing. The 5'primer for PCR and sequencing (Seq1) is 5' AAGCAGTTCACCAAGTGC. The 3'primer (Seq2) is 5'CGAGCCAGTCAATGC.

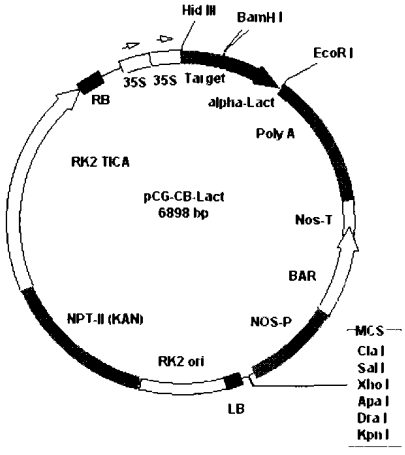

Fig.1 Binary vector with CaMV 35S promoter alpha lactalbumin gene expression cassette. The plasmid also contains a herbicide resistant gene (bar gene).

DNA sequencing The DNA mini preparation of plasmid DNA was prepared by QIAprep Miniprep Kit (QIAGEN Inc). A Perkin Elmer-Applied Biosystems 373A DNA sequencer was used in combination with the *Taq* DyeDeoxy Terminator Cycle Sequencing kit.

Transformation of *Arabidopsis* and screening of transgenic plant *Arabidopsis thaliana*(Columbia ecotype) transformation using floral dipping of *Arobacterium* culture to floral buds was according to the method described by Steve Clough et al[11], using the *Agrobacterium* binary vector system. Seeds collected from floral dipped plants were planted into soil. After about 1 to 2 days, spray the germinating seedling with Finale 100 mg L^{-1} (Bayer, Brand name). Transformants will survive and continue to grow, while the non- transformants will die within a few days. Each of

seeds collected from individual survived plants were planted into soil and were continuously selected with herbicide Finale (100 mg L^{-1}). This herbicide screening was carried through the next two generations. Those which have 100% resistant progency (the seedlings are all green and healthy after herbicide spraying) are stable homozygous seeds based on Mendel law.

Genomeic DNA extraction and PCR *Arabidopsis* leaf and seed DNA in regenerated plants were extracted with DNeasy Plant Mini K (QIAGEN Inc). The 5' primer for genomic DNA amplification (Seq1) is 5'AAGCAGTTCACCAAGTGC. The 3'primer (Seq2) is 5'CGAGCCAGTCAATGC. PCR was performed on MJ PC-200 with Taq DNA polymerase kit (Promega) for 30 cycles.

Protein extraction and analysis About 100 mg of fresh leaves and seeds were ground in 250 μl of extraction buffer (50 mmol/L MOPS pH 7.0, 0.2 mmol/L EDTA, 1 mmol/L PMSF, 1 mmol/L aminocaproic acid, 1 mmol/L benzamidine, 5 mmol/L DTT, and 1 mmol/L ascorbate), respectively. Ground tissue was transfered into microfugetube and centrifuge at 12 000×g for 5 min. The resulting supernants were collected and added four volumes of 80% isopropanol to pellet proteins at 4℃ for 3 hours. Tubes were centrifuge at 12 000×g for 10 min. The pellets were washed with 80% cold acetone twice. The total amount of proteins in extracts was estimated using the Bio-Rad Protein Assay kit with bovine serum albumin (Sigma) as a standard.

SDS-PAGE was performed according to Laemmli[12], with 12.5% gel. Western blot was performed according to Sambrook[10]. Proteins were blotted on a PVDF membrane (Immobilon-P, Millipore). The LA bands were visualized with anti-human LA (developed in rabbit) and ECL-Plus Immunodection kit (Amersham Pharmacia) (Refer to Amersham Pharmacia Kit manual).

2 Results

2.1 Porcine α-lactalbumin PCR analysis of genomic DNA in transgenic *Arabidopsis*

Two sets of PCR reaction of DNA template of the binary vector pCG-CB-Lact were set up. A 398-bp fragment encoding a porcine-lactalbumin and 436-bp bar gene were amplified, respectively, with primers of porcine α-lactalbumin and primers of bar gene (data not shown).

These two bands are the correct size desired fragments of porcine α-lactalbumin and bar gene. The DNA sequence of porcine α-lactalbumin confirmed the PCR result. This result showed that these primers of porcine α-lactalbumin and bar gene are highly specific for these encoding regions of the genes.

Therefore, the two pairs of primers were used to confirmed porcine α-lactalbumin and bar gene presented in transgenic plants. Genomic DNA of transgenic plants (transformation event Arab 06 and Arab15) were extracted and used for PCR template. A correct size of 398-bp fragment encoding a porcine-lactalbumin and 436-bp bar gene were amplified, respectively (Fig. 2).

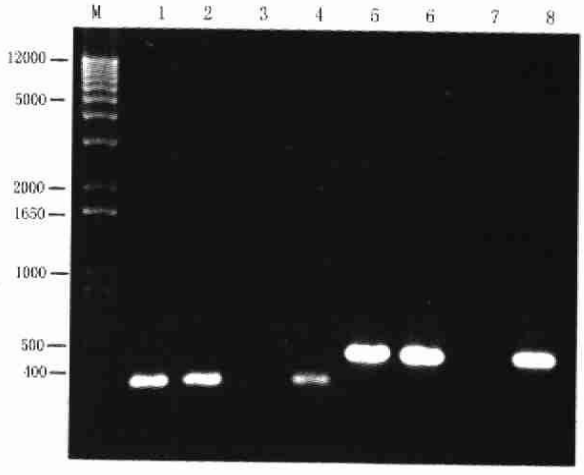

Fig. 2 Porcine α-lactalbumin and bar gene PCR analysis of genomic DNA in transgenic *Arabidopsis*

M: 1 kb plus DNA marker; 1–4: Porcine α-lactalbumin amplification with LactSeq; 5–8:Bar gene amplification with BarSeq; 1/5:Genomic DNA of Arab 06; 2/6:Genomic DNA of Arab15; 3/7:Genomic DNA of wild type, negative control; 4/8: Plasmid DNA of pCG-CB-LACT, positive control.

These results showed that porcine α-lactalbumin gene was successfully introduced in *Arabidopsis* and integrated into genomic DNA of transgenic plants with *Agrobacterium* mediated transformation. The gene integration and expression at the DNA level were also confirmed with Southern and Northern blot (Data published in different papers).

2.2 Protein expression analysis in regenerated plants

Protein expression would be the strong and directed evidence for successful plant transformation. In this research, the expression of plantified porcine α-lactalbumin gene at the protein level in a transgenic *Arabidopsis* was analyzed with SDS-PAGE and Western blot. Results showed the expression at the protein level could be detected both in seeds and leaves of transgenic events (Arab 06 and Arab15).

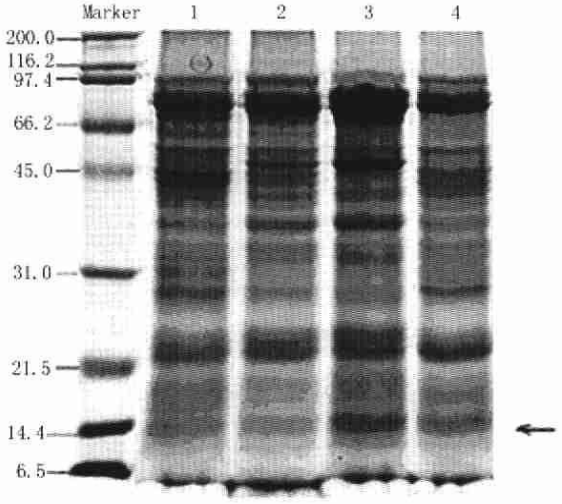

Fig. 3 Protein analysis of porcine α-lactalbumin in transgenic plants

The expressed lactualbumin in transgenic plants was indicated with arrow. Marker:The protein molecular weight markers were from Bio-Rad; 1: Leaves of Arab 06; 2: Seeds of Arab 06; 3: Leaves of Arab15; 4: Seeds of Arab15.

Furthermore, the results of protein analysis showed that the expression in transgenic plants is non-tissue specific expression. This reason is that cauliflower mosaic virus 35S promoter is universal promoter in engineered plants. The feature of cauliflower mosaic virus 35S promoter is its strong promoter effect in plants. But this universal promoter is poor spatial and tissue-specific expression of transgenic genes. 35S controlled protein expression in transgenic plants can not be occurred in the specific tissue or organs such as protein body in seeds. The arabidopsis-expressed LA migrated in SDS-PAGE with identical mobility to LA prepared from human milk, indicating that the LA was correctly processed to yield a mature protein in transgenic plants.

Fig. 4 Western blot of porcine α-lactalbumin in transgenic plants expression

Control: Seeds of wildtype *Arabidopsis*, negative control; 1: Leaves of Arab 06; 2: Seeds of Arab 06; 3: Leaves of Arab15; 4: Seeds of Arab15; h-LA: Human α-lactalbumin, positive control.

3 Discussion

Successful transformations in plant seeds were broadly reported and used in other researches [13-15]. Recently plant transformation was used for express system for some valuable animal genes, especially for development of edible vaccine.

Alternation of legume seed composition is very important for seed protein studies and need to be paid much more attention. One of such researches is modification of seed-specific proteins of plant seeds, especially to modify the sulfur level[8,9].

Porcine α-lactalbumin is a protein ideally suited to swine nutrition. The porcine α-lactalbumin DNA can be easily synthesized in the laboratory. Therefore, porcine α-lactalbumin was selected as a suitable transgene.

Generally speaking, it is much more difficult that original native porcine α-lactalbumin gene can be correctly transcribed and translated both at DNA level and protein level in transgenic plants [8]. The main reason is that there are different preferred codon for different species. Preferred codons in animal are much different from those in plants. The codon bias resulted in the transcription and translation problems of native porcine α-lactalbumin in plants.

Porcine α-lactalbumin gene could be plantified to be plant-preferred coding sequence of porcine α-lactalbumin with site-directed mutation. The plantified animal gene is a good way to overcome such transcription and translation problems in plants. In this research, a 398-bp plantified porcine α -lactalbumin, was cloned into a binary vector under the control of the cauliflower mosaic virus 35S promoter. Afterward, plant transformation of *Arabidopsis* model system was

successfully done with *Agrobaterium* mediated transformation. Seeds of all transgenic plants were harvested together and then do screening of transgenic plants containing porcine-lactalbumin encoding region with herbicide Finale application. The herbicide-resistant plants have been characterized by DNA level and Western blotting. All of transgenic events are PCR positive of porcine α-lactalbumin gene. The gene chromosomal integration and expression at the DNA level were confirmed in regenerated plants. Progeny analysis of herbicide selection and screening in putative transformants showed that the transformation of transgenic stability and heritability were occurred in regenerated plants. These results confirmed the hypothesis that plantification of animal genes is a feasible method to be correctly translated and expressed in plants.

The expression at the protein level could be detected in transgenic *Arabidopsis*. The arabidopsis-expressed LA migrated in SDS-PAGE with identical mobility to h-LA prepared from human milk, indicating that the LA was correctly processed to yield a mature protein in plants. Results of Western blot is coincident results that the arabidopsis-expressed LA was correctly expressed and processed in plants. The results suggested that synthetic transgene can be correctly translated and expressed in plants. The protein expression of transgenic plants was observed both in seeds and leaves of transgenic plants. It was showed that 35S promoter regualetd expression in plants is non-tissue specific. It was broadly reported that 35S promoter of cauliflower mosaic virus is a universal and highly strong promoter for its regulation in many species of plants. But 35S promoter is poor spatial and tissue-specific expression of transgenes.

Arabidopsis is an ideal model system for the transformation of plantified porcine α-lactalbumin due to its small genomic DNA and relative short life span. In this research, the arabidopsis transformation can only asset feasibility of our strategy to develop transgenic plants with plantified animal gene and has less application value. The most important part of the research is that we are also developing transgenic soybeans containing porcine α-lactalbumin in the seeds.

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