

Transgenic Expression and Identification of Recombinant Human Proinsulin in Peanut

Zheng Ling^{1,2,3}, Jiao Qi-Qing⁴, Wang Yu^{1,2,3}, Bian Fei^{2,3}, Qu Shu-Jie⁵, Wan Shu-Bo^{1,2,3,6}, Peng Zhen-Ying^{1,2,3}, Bi Yu-Ping^{1,3,6*}.

¹ School of Life Science, Shandong University, Jinan, P. R. China. ² Biotechnology Research Center, Shandong Academy of Agricultural Science, Jinan, P. R. China. ³ Shandong Provincial Key Laboratory of Genetic Improvement, Ecology and Physiology of Crops, Jinan, P. R. China. ⁴ Shandong Institute of Pomology, Taian, P. R. China. ⁵ Test Base Service Center, Shandong Academy of Agricultural Sciences, Jinan, P. R. China. ⁶ Center of Graduate Education, Shandong Academy of Agricultural Science, Jinan, P. R. China.

ABSTRACT

*The increased incidence of diabetes, coupled with the introduction of alternative insulin delivery methods that rely on higher doses, is expected to result in a substantial escalation in the future demand for affordable insulin. Plant-based systems offer a safe and economical method for producing pharmaceutical proteins. We used peanut (*Arachis hypogaea* L.) as bio-reactors to express biosafe, stable proinsulin. We designed two proinsulin analogues (FAIA and LAIA) with substitutions in their amino acid sequences. The fast-acting insulin analogue (FAIA) contains a Gly inserted between Cys19 and Gly20, as well as a Pro28Asp substitution, in the B chain. The long-acting insulin analogue (LAIA) contains a Gly inserted between Cys19 and Gly20 and two Arg residues inserted into the terminus of the B chain, as well as an Asn21Gly substitution in the A chain. Four plasmids were constructed: pROKII-Flag-FAIA, pROKII-Flag-LAIA, pCAMBIA2301-Oleosin-FAIA and pCAMBIA2301-Oleosin-LAIA. These plasmids were transferred into peanut to produce recombinant proinsulin. Western blot and GUS staining analysis indicated that some transgenic peanut successfully expressed exogenous proinsulin. Peanut seeds can act as insulin storage sites, which is the foundation for further production of recombinant proinsulin from peanut seeds.*

Key words: Proinsulin, peanut, diabetes, expression

*Authors for correspondence: yuping_bi@hotmail.com

INTRODUCTION

In recent years, the global incidence of diabetes has increased dramatically due to genetic variations, improper diet and lifestyle (Baeshen et al. 2014). The number of diabetic patients is estimated to reach 10 percent of the world population, skyrocketing to 592 million by 2035, with approximately 5.1 million deaths annually. Along with the skyrocketing patient numbers, the demand for insulin is increasing rapidly (more than 16 tons per year), with current insulin manufacturing technologies and production capacity unable to meet the growing market demand for high production in the future (Colagiuri 2010; Baeshen et al. 2014).

Early on, insulin was purified from the bovine or porcine pancreas. At present, *E. coli* and *Saccharomyces cerevisiae* are the most common hosts for the production of human insulin (Thim et al. 1986; Nilsson et al. 1996; Gellissen and Hollenberg 1997; Kjeldsen 2000; Porro et al. 2005; Huang et al. 2012; Ferrer-Miralles and Villaverde 2013; Baeshen et al. 2014). The large-scale manufacture of therapeutic insulin for humans has benefited tremendously from genetic engineering (Arakawa et al. 1998; Walsh 2005; Nykiforuk et al. 2006; Ferrer-Miralles et al. 2009; Boyhan and Daniell 2011; Qian et al. 2011). From 2004 to 2013, biopharmaceuticals were largely derived from *E. Coli* (24%), yeast (13%), mammalian cells (56%), transgenic animals and plant expression systems (3%) and insect cells (4%) (Gurramkonda et al. 2010; Qian et al. 2011; Walsh 2012; Nielsen 2013; Walsh 2013; Baeshen et al. 2014).

Transgenic plant expression systems have attracted attention due to advantages such as high-capacity production, safety, inexpensive investment, and fast and easy scale-up (Arakawa et al. 1998; Nykiforuk et al. 2006; Ruhlman et al. 2007; Xie et al. 2008; Boothe et al. 2010; Boyhan and Daniell 2011; Soltanmohammadi et al. 2014). Transgenic seeds and leaves exhibit long-term stability and can be used to stockpile insulin until it is needed. Nykiforuk et al. expressed an insulin-oleosin fusion in *Arabidopsis thaliana* seeds, and the insulin that accumulated in the transgenic seeds could significantly reduce glucose levels to a similar extent as commercially available insulin (Nykiforuk et al. 2006). Ruhlman et al. expressed a recombinant cholera toxin B-proinsulin fusion protein (CTB-Pins) in lettuce and tobacco chloroplasts, in which CTB-Pins accounted for almost 16% and 2.5% of the total soluble protein,

respectively (Ruhlman et al. 2007; Boyhan and Daniell 2011). Interestingly, the transformed tobacco leaves were able to lower blood and urine glucose levels when administered orally to non-obese diabetic mice (Ruhlman et al. 2007; Boyhan and Daniell 2011).

The preferred form of recombinant human insulin for heterologous expression is proinsulin, which has a B-C-A structure and possesses a longer in vivo half-life than mature insulin (Boyhan and Daniell 2011; Trabucchi et al. 2012; Aslam et al. 2013; Soltanmohammadi et al. 2014). To change the duration of insulin's effects, fast-acting insulin analogues (FAIA), medium-acting insulin analogues (MAIA) and long-acting insulin analogues (LAIA), such as Detemir, Glargine, Ultratard, Lispro and Glulisine, have been developed. These insulin analogues were obtained by changing several amino acids in the A-Chain and/or B-Chain (Martina et al. 1989; McKeage and Goa 2001; Chen and Yao 2002; Walsh 2005; Peterson 2006), for example, Lispro and Glargine.

Peanut is an important oilseed crop worldwide. China is the biggest peanut producer, accounting for approximately 41.5% of overall global production (<http://en.wikipedia.org/wiki/Peanut>). We have studied peanuts for many years and have made significant advances (Su et al. 2011; Peng et al. 2013; Xia et al. 2013). Peanut seeds are rich in protein and fat and can serve as a good reservoir for heterologously expressed proteins; thus, we sought to express the human insulin gene in peanut seeds. Because insulin is highly unstable and is prone to N-terminal degradation (Boothe et al. 2010), oleosin or flag tag was fused with proinsulin to protect the N-terminus from degradation. Otherwise the recombination protein purification cost will be reduced. Here, we share our experimental results with researchers in this field.

MATERIAL AND METHODS

Plasmid Design and Construction

In accordance with previous reports (Martina et al. 1989; McKeage and Goa 2001; Chen and Yao 2002; Walsh 2005; Nykiforuk et al. 2006; Peterson 2006), we designed two types of proinsulin analogues: a FAIA and a LAIA (Fig. 1). In the FAIA, Gly was inserted between Cys19 and Gly20, and Pro28 was replaced with Asp28, in the B chain. In the LAIA, Gly was inserted between Cys19 and Gly20 and two

Arg residues were inserted at the C-terminus of the B chain, and Asn21 was replaced with Gly21 in the A chain. We optimized the codon according to the plant codon preference. The two human proinsulin analogues were synthesized by Sangon Company (China). An eight-residue flag tag was fused at the N-terminus of the FAIA and LAIA. Flag-LAIA and Flag-FAIA fragments were amplified using the primers LAIAF and LAIAR or FAIAF and FAIAR and inserted downstream of the CaMV35S promoter in the binary expression vector pROKII, which carries the marker gene *Npt II*. The resulting plasmids were named pROKII-Flag-FAIA and pROKII-Flag-LAIA (Fig. 2).

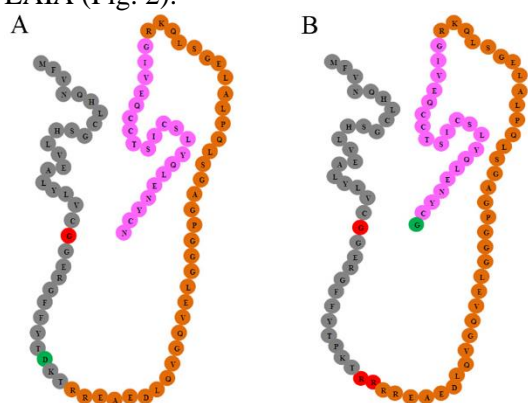


Figure. 1 Amino acid sequences of FAIA and LAIA. A: FAIA; B: LAIA. Grey circles represent the B-chain,

orange circles represent the C-peptide, pink circles represent the A-chain, red circles represent the inserted amino acids, and green circles represent the substituted amino acids.

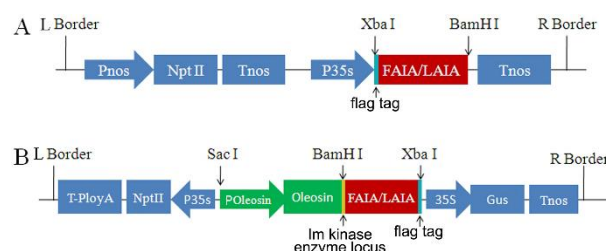


Figure. 2 The schematic diagram of the recombinant plasmids. A: pROKII-Flag-FAIA/LAIA plasmids; B: pCambia2301-Oleosin-FAIA/LAIA plasmids.

Total RNA was extracted from young seeds of the peanut cultivar ‘Luhua 14’ using the CTAB method (Albani et al. 1992) and transcribed to cDNA using a RevertAid First Strand cDNA Synthesis kit (Fermentas, Canada). The primers OLEOSINF and OLEOSINR (Table S1) were used to amplify the peanut oleosin gene fragment and its promoter (GenBank No. EF695400). PCR amplification was conducted as follows: one cycle of 95°C for 5 min, 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 5 min.

Table.S1 - Primers used in this paper

Primer	Sequence	Function
LAIAF	5'GCCTCTAGAATGGATTACAAGGATG ATGA 3' (<i>Xba</i> I)	Amplify and detect the LAIA fragment in pROKII-Flag-LAIA vector
LAIAR	5'GCCGGATCCTTACCCGCAATAATTC TCG 3' (<i>Bam</i> H I)	
FAIAF	5'GCCTCTAGAATGGACTATAAAGATG ATGA 3' (<i>Xba</i> I)	Amplify and detect the FAIA fragment in pROKII-Flag-LAIA vector
FAIAR	5'GCCGGATCCTTAATTGCAGTAGTTT TCAA3' (<i>Bam</i> H I)	
OLEOSINF	5'GCCGAGCTCAGGTCAACTACCATTC GT 3' (<i>Sac</i> I)	Amplify the peanut oleosin gene fragment and its promoter

OLEOSINR	5'GCCGGATCCAGTTCTCTTTGAATCC	
	TG 3' (<i>Bam</i> H I)	
O-LAIAF	5'GCCGGATCCGATGATGATGATAAG	Amplify the O-FAIA fragment in
	ATGTTTGTAAACCAGCACCTC 3'	pCAMBIA2301-Oleosin-LAIA vector
	(<i>Bam</i> H I)	construction
O-LAIAR	5'GCCTCTAGATTACTTATCGTCATCA	
	<u>TCCTTGTAATCCCCGCAATAATTCTCG</u>	
	AGTT 3' (<i>Xba</i> I)	
O-FAIAF	5'GCCGGATCCGATGATGATGATAAG	Amplify the O-LAIA fragment in
	ATGTTTGTGAACCAGCACCTC 3'	pCAMBIA2301-Oleosin vector construction
	(<i>Bam</i> H I)	
O-FAIAR	5'GCCTCTAGATTACTTATCGTCATCA	
	<u>TCTTTATAGTCATTGCAGTAGTTTTCA</u>	
	AGTTG 3' (<i>Xba</i> I)	
RTR	5'GCCCCGCAAACCGGAAAACAAACGT	Detect the pCAMBIA2301-Oleosin-
	TAAGT 3'	FAIA/LAIA transgenic lines
ACTINF	5'GGAAGTGGAAATGGTTAAGGCTGG 3'	Amplify the β -actin gene in RT-PCR
ACTINR	5'GTTTCAAACATGATCTGAGTCATC 3'	

The PCR products were separated by 1.0% agarose gel electrophoresis. O-LAIA and O-FAIA were amplified using the primer pairs O-LAIAF/O-LAIAR and O-FAIAF/O-FAIAR. In addition, an eight-residue flag tag was added to the C-terminus of O-FAIA and O-LAIA. The oleosin promoter and gene fragment were fused to the N-terminus of O-FAIA and O-LAIA via an enterokinase enzyme site. The fused O-FAIA and O-LAIA fragments were inserted into the binary expression vector pCAMBIA2301, which carries the marker gene *Npt* II. The resulting plasmids were named pCAMBIA2301-Oleosin-FAIA and pCAMBIA2301-Oleosin-LAIA (Fig. 2).

The four recombinant plasmids were transferred into *Agrobacterium tumefaciens* strain LBA4404

(Hoekema et al. 1983) for the genetic transformation of peanut.

Genetic Transformation of Peanut Axes

Mature embryo axes of peanuts were used to induce explants as described previously (Akasaka et al. 2000; Anuradha et al. 2006). Briefly, The axes were cut from the young seedlings (about 4-5 days old) of 'Luhua 14' (a Chinese peanut cultivar) and mixed with the transformed *A. tumefaciens* strain LBA4404 bacterial suspension for about 25 min, then put the axes on coincubate-medium (MS added 8 mg/L 6-BA and 0.7 mg/L NAA) under dark conditions for two days. The infected axes were transferred to induction-medium (MS added 50 mg/L Kan, 250 mg/L Cef, 8 mg/L 6-BA and 0.7 mg/L NAA) for sprouting for 3-4 weeks. The well-elongated green shoots were cut

off from the axes and put on the elongation-medium (MS added 100 mg/L Kan, 250 mg/L Cef, 2 mg/L 6-BA and 2.0 mg/L GA3) for 3-4 weeks, then transfer the green shoots on rooting-medium (MS added 0.5 mg/L IBA). About 3-4 weeks later, 5-8 strong roots will developed from the stem bases. The well-rooted plants were cultivated in pots in a greenhouse for production. The well-rooted plants were planted in pots with soil and cultivated in a controlled chamber at 28°C with a relative humidity of 50-60%.

The seeds from the positive transgenic peanut lines were grown in greenhouse (i.e., the T1 generation). All of the plants were identified using PCR. Positive plants from transgenic lines were kept for seed harvest. Such seeds (T2, T3 generation) were planted in greenhouse and identified. These T3 lines without character segregation were chosen for western blot analysis.

Molecular Identification of Transgenic and Peanut Plants

Genomic DNA was extracted from the young leaves of T0, T1 and T2 transgenic peanut lines (Wang et al. 2002). Four pairs of primers were used for the identification of all the transgenic plants. Primers LAIAF/LAIAR and FAIAF/FAIAR were used to amplify the Flag-LAIA and Flag-FAIA fragments. Primers O-LAIAF/RTR and O-FAIAF/RTR were used to amplify the O-LAIA/O-FAIA fragments. PCR amplification was conducted as follows: one cycle of 95°C for 5 min, 35 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The PCR products were separated by 1.5% agarose gel electrophoresis.

Histochemical Assay for GUS Activity

Both WT and positive transgenic peanut cotyledons were histochemically stained to test for GUS activity according to the method of Jefferson et al (Jefferson et al. 1987).

RT-PCR Analysis

Total RNA was extracted using the CTAB method from the leaves of T2 transgenic peanut lines. The two primer pairs LAIAF/LAIAR and FAIAF/FAIAR were used for RT-PCR analysis. β -actin was amplified as a reference gene using the primer pair ACTINF/ACTINR. PCR was conducted as follows: one cycle of 95°C for 5 min, 22 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The PCR products were separated by 1.5% agarose gel electrophoresis.

Extraction of Protein from Transgenic Peanut

Total proteins were extracted from pROKII-Flag-FAIA/LAIA T2 transgenic peanut leaves using the trichloroacetic acid (TCA)/acetone method. Plant leaves were crushed in liquid nitrogen, and 10% TCA acetone (supplied with 0.07% 2-mercaptoethanol and 1 mM PMSF) was added. The mixture was placed at -20°C for 1 h and microfuged at 12,000 rpm for 30 min in 4°C. The supernatant was discarded. Precooled acetone was added to the precipitate, which was placed at -20°C for 1 h and then microfuged at 12,000 rpm for 30 min at 4°C. This procedure was repeated twice. The final precipitate was vacuum dried, resuspended in 400 μ l lysis buffer (urea 7 M, thiourea 2 M, CHAPS 4%, DTT 65 mM, PMSF 1 mM), incubated for 20 min at room temperature, and then microfuged at 12,000 rpm for 30 min at 15°C. The supernatant contained the total protein extract.

Oleosin-proinsulin was extracted from T2 transgenic plants seeds using the method of Markley and Nykiforuk (Markley et al. 2006; Nykiforuk et al. 2006).

Western Blot Analysis

Protein content was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. A 40 μ l sample was taken and added to 10 μ l of sodium dodecyl sulfate (SDS) buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 0.5% bromophenol blue, 50% glycerol, 5% 2-mercaptoethanol) and boiled for 10 min. Western blot analysis was operated according the method described previously (Nykiforuk et al. 2006). The 0.22- μ m polyvinylidene difluoride membranes, monoclonal anti-insulin and alkaline phosphatase-conjugated goat anti-rabbit IgG were bought from AMRESCO (Canada), Abcam (America) and ZSGB-BIO (China).

RESULTS

Molecular Identification of Transgenic Plants

We designed two types of proinsulin analogues: a FAIA and a LAIA (Fig. 1), and constructed pROKII-Flag-FAIA/LAIA and pCAMBIA2301-Oleosin-FAIA/LAIA. The four plasmids were transformed into peanut mature embryo axes using *Agrobacterium*-mediated transformation. There were no phenotype difference between wide type and

transgenic lines. Finally several transgenic peanut lines were obtained (Fig. S1). The numbers of transgenic plants lines were in Table 1.

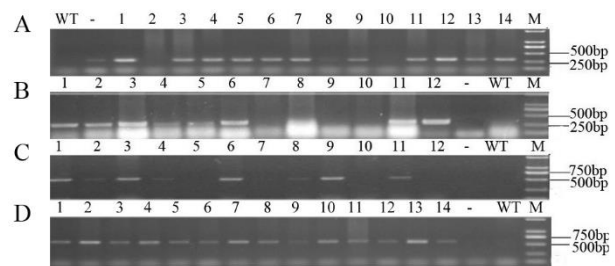


Table S1 Primers used in this study

Figure S1. PCR tests of transgenic peanut lines. A: pROKII-Flag-FAIA transgenic peanut lines; B: pROKII-Flag-LAIA transgenic peanut lines; C: pCAMBIA2301-Oleosin-FAIA transgenic peanut lines; D: pCAMBIA2301-Oleosin-LAIA transgenic peanut lines. M: DNA marker, -: negative control, WT: wild type 'Luhua14'.

Table 1 - The number of transgenic peanut lines

Plasmid	Transgenic peanut (lines)
pROKII-Flag-FAIA	20
pROKII-Flag- LAIA	7
pCAMBIA2301-Oleosin-FAIA	21
pCAMBIA2301-Oleosin- LAIA	14

T2 transgenic peanut lines were randomly chosen for RT-PCR analysis to examine LAIA and FAIA expression in the peanut. Total mRNA was extracted from young leaves of both WT and positive lines. Most of the transgenic peanut lines expressed high levels of LAIA or FAIA mRNA. LAIA-7 and FAIA-3 expressed highest proinsulin levels, but lines LAIA-3 and FAIA-5 which expressed little or no proinsulin (Fig. 3).

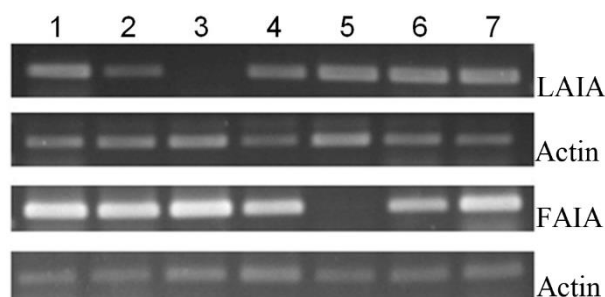


Figure. 3 RT-PCR analysis of T2 transgenic peanut lines. 1–7: T2 transgenic lines.

Because the pCAMBIA2301-Oleosin-FAIA/LAIA plasmids contain the GUS gene, we performed GUS staining to further identify the positive transgenic

peanut lines. All the cotyledons of the positive transgenic peanut were deeply stained and the WT showed no staining (Fig. 4), thus demonstrating the successful transformation of the peanut plants.

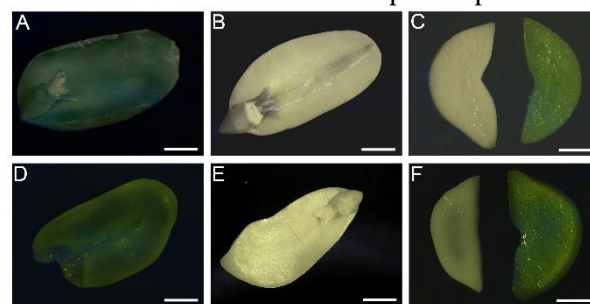


Figure. 4 GUS staining assay. A-C: pCAMBIA2301-Oleosin-FAIA transgenic peanut lines; D-F: pCAMBIA2301-Oleosin-LAIA transgenic peanut lines. A, D: GUS expression in all of the cotyledons. B, E: cotyledons of WT peanut without GUS activity. C, F: transverse sections of A, B, D and E. WT: wild type 'Luhua 14', Bars: 0.2 cm.

Western Blot Analysis in Transgenic Plants

Western blot analysis showed that some of the T2 transgenic peanut successfully expressed the exogenous proinsulin (Fig. 5). Of the pROKII-Flag-

FAIA/LAIA transgenic peanut lines, four lines expressed either FAIA or LAIA successfully (Fig. 5 A), but most of the peanut lines had more than one bands and had different size. In pCAMBIA2301-Oleosin-FAIA/LAIA transgenic peanut lines, six positive lines expressed Oleosin-FAIA/LAIA protein (Fig. 5 B).

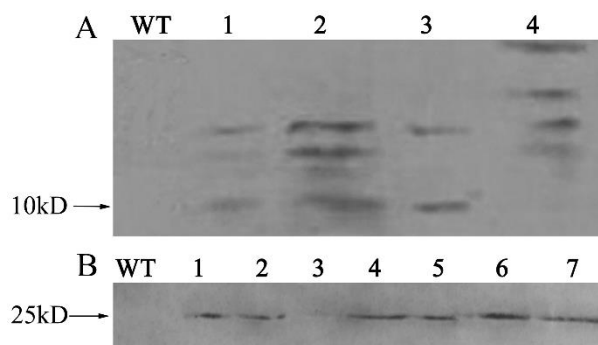


Figure. 5 Western blot analysis of T2 transgenic lines. A: pROKII-Flag-FAIA/LAIA transgenic peanut line leaves. 1-2: pROKII-Flag-FAIA transgenic peanut line leaves; 3-4: pROKII-Flag-LAIA transgenic peanut line leaves. B: pCAMBIA2301-Oleosin-FAIA/LAIA transgenic peanut line seeds. 1-4: pCAMBIA2301-Oleosin-FAIA transgenic peanut line seeds; 5-7: pCAMBIA2301-Oleosin-LAIA transgenic peanut line seeds.

DISSUSSION

Plant genetic engineering has been a very important technique for improving the economic characteristics of crops and enhancing their disease resistance. Recently, the use of plants as bioreactors to produce medical proteins such as antibodies and vaccines has emerged as a new trend. An obvious advantage is that the costs of plant-derived medical proteins are far lower than those of microorganism-derived or animal-derived medical proteins. Additionally, transgenic seeds exhibit long-term stability, enabling medical proteins to be stockpiled until they are needed.

Human insulin has been transformed into several plants, including Arabidopsis, lettuce, tobacco, and tomato (Nykiforuk et al. 2006; Ruhlman et al. 2007; Boyhan and Daniell 2011; Soltanmohammadi et al. 2014). Because insulin is highly unstable and is prone to N-terminal degradation (Boothe et al. 2010), it is often fused with a marker protein such as CTB or oleosin to protect the N-terminus from degradation (Nykiforuk et al. 2006; Boyhan and Daniell 2011). Here, we designed two strategies to produce human proinsulin in peanut, one strategy involved fusing a

flag tag to the N-terminus of FAIA/LAIA, and the other involved fusing peanut oleosin to the N-terminus of FAIA/LAIA, with a flag tag linked to the C-terminus. The flag tag and oleosin protect FAIA/LAIA from degradation and provide a good means of facilitating purification, and the Flag-FAIA/LAIA and Oleosin-FAIA/LAIA fusion proteins were successfully produced from peanut leaves and seeds (Fig. 5).

Insulin and proinsulin have been reported to form self-aggregates of monomers, dimers, tetramers and hexamers (Pekar and Frank 1972), which causes insulin and proinsulin to be more resistant to degradation. Boyhan et al. produced cholera toxin B-fused human proinsulin in tobacco and lettuce chloroplasts and found that the fusion protein aggregated in chloroplasts (Boyhan and Daniell 2011). In our experiments, exogenous proinsulin formed more than one bands and had different size in pROKII-Flag-FAIA/LAIA T2 transgenic peanut lines (Fig. 5 A). Maybe proinsulin aggregated and formed polymers. This may account for the high levels of FAIA/LAIA expression in the transgenic peanut leaves. In order to determine the proinsulin expression, we used ELISA (Ultra Sensitive Rat Insulin ELISA kit; Crystal Chemical Inc., Downers Grove, IL) (Nykiforuk et al. 2006) to detect pROKII-Flag-FAIA/LAIA T2 transgenic lines which were used in the western blot. The results revealed that all transgenic lines had considerably lower proinsulin expression levels (approximately 0.7% of total soluble protein) in stable transgenic plants than other researches previously. We speculate the reasons of low detection level may be that the ELISA kit we used is sensitive to insulin not proinsulin. We will further study the reason of this.

Currently, insulin products such as Detemir, Glargine, Ultratard, Lispro and Glulisine, etc., can be divided into different types based on their different rates of action. Lispro is one kind of fast-acting insulin that can act in 5-15 min after subcutaneous injection, the reason being that Lispro has two amino acid changes in the B-chain compared to native insulin. Glargine has a total of three different amino acids compared with native insulin, which extends the duration of its activity (McKeage and Goa 2001). Additionally, Chen et al. reported that inserting a Gly between B23 and B24 of the B-chain increases the activity of this insulin analogue compared with native human and pig insulin (Chen and Yao 2002). In accordance with these reports, we designed our own proinsulin analogues in the hopes that they will possess all the merits of Lispro and Glulisine, etc.

Now that we have successfully expressed proinsulin analogues in peanut seed, proinsulin will be purified and treated with enterokinase and trypsin and test the matured insulin activity in future studies.

CONCLUSIONS

We used peanut as bio-reactors to express proinsulin. Two proinsulin analogues (FAIA and LAIA) were designed and transferred into peanut to produce recombinant proinsulin. Western blot and GUS staining analysis indicated that some transgenic peanut successfully expressed exogenous proinsulin. This research lay a foundation for further production of recombinant proinsulin from peanut seeds, and promoted the development of molecular farming.

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