

High-level expression of modified gene encoding human adiponectin in transgenic rice

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ABSTRACT

Adiponectin is a polypeptide specifically secreted from human adipocytes, and its deficiency is closely linked to increased obesity and type II diabetes. There is an urgent demand for large-scale production of human adiponectin for pharmaceutical applications. Here, we report that we have successfully obtained a high-level of expression of modified genes encoding human adiponectin in transgenic rice. The 735 bp cDNA of the native human sequence was adopted to rice codon usage, fused to the translation initiation sequence in the N terminus and to the KDEL signal sequence in the C terminus. An amplification promoting sequence acting as an enhancer of transcription was also introduced to enhance gene expression. The presence of the transgene and mRNA transcripts was confirmed by PCR, Southern blot and RT-PCR. Western blot analysis revealed that a protein of approximately 30 kDa was produced in rice leaves. ELISA analysis was used to determine the amount of recombinant adiponectin in transformants with the modified gene in up to 0.32% of total soluble leaf protein. Our results establish the feasibility of high-level expression of recombinant human adiponectin in transgenic rice.

Key terms: human adiponectin, transgenic rice, modified gene, anti-diabetic.

INTRODUCTION

Diabetes has become a global health problem and is rapidly developing into a leading cause of human mortality and morbidity. It is estimated that the number of adults with diabetes worldwide will almost double over the next 25 years, from approximately 171 million in 2000 to 366 million by 2030 (Chaturvedi, 2007). Therefore, addressing this severe disease is of great importance for human health.

Adiponectin is a 244 amino acid polypeptide produced solely by adipose tissues (Scherer et al., 1995) and is also often called adipocyte complement-related protein of 30 kDa (Acrp30). Acrp30 plays a fundamental role in stimulating fatty-acid oxidation and reducing expression levels of molecules involved in gluconeogenesis in hepatocytes (Kadowaki et al., 2005). Obesity and insulin resistance are closely linked to decreased levels of plasma Acrp30 (Arita et al., 1999; Heilbronm et al., 2003) and lead ultimately to type two diabetes, which constitute 90% of all cases of diabetes. Since Acrp30 treatment of persons suffering from diabetes could ameliorate their symptoms, the large-scale recombinant production of this anti-diabetic pharmaceutical is highly desirable for therapeutic demands.

The expression of recombinant human Acrp30 has been exploited in several hosts like *E. coli*, *Pichia pastoris*, baculovirus and HEK293 cells (Fruebis et al., 2001; Liu et al., 2007; Carmo Avides et al., 2008). However, these systems have several disadvantages in terms of cost, stability, safety, etc (Fischer et al., 2004). Plants offer an alternative to microbial fermentation and animal cell cultures and are now gaining widespread acceptance as a general platform for the large-scale production of recombinant proteins (Streatfield, 2007). Plants are the most economical producers of biomass and allow for cost-effective production of recombinant proteins on an agricultural scale

(Fischer et al., 2004; Berberich et al., 2005). They avoid animal or microbial cell-culture contaminants, such as mammalian pathogens or bacterial components (Fischer et al., 2004; Ma et al., 2005; Ma et al., 2003). They are also capable of performing complex post-translational modifications, most importantly glycosylation, which is often crucial to the biological activity and stability of human therapeutic proteins (Ma et al., 2003). Up to now, there have been some pharmaceutical proteins expressed in plants involving antibodies and vaccines (Streatfield, 2007). Rice has been widely used as a model plant for the production of recombinant proteins. It is worth striving to engineer a novel anti-diabetic rice line that can produce large amount of human Acrp30. The objective of this study was to test the feasibility of developing transgenic rice plants to produce Acrp30.

Although there has been significant success in plant bioreactors, the wider use of plant-derived pharmaceuticals has been limited by low yields due to very poor expression of heterologous genes derived from different genetic backgrounds in plants (Gustafsson et al., 2004). So far, most recombinant pharmaceutical proteins have been produced in transgenic plants with a typical yield of less than 0.1% of the total soluble protein (TSP) of leaves (Ma et al., 2003). Thus, overcoming bottlenecks imposed by low yields in plant bioreactors is one of the key challenges for researchers working in the plant-derived pharmaceutical field. We therefore designed a modified gene encoding human Acrp30 with a combination of strategies for high-level regulated expression in rice, including partial codon-optimization, addition of an endoplasmic reticulum retention signal KDEL and of rice-biased translation initiation sequence, and insertion of an amplification promoting sequence (*aps*). The amount of recombinant protein with the modified gene reached a maximum yield of 0.32% of TSP of leaves. This is the first step toward high-level expression of

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modified genes encoding human Acrp30, an anti-diabetic protein in transgenic rice.

MATERIALS AND METHODS

Designing of modified Acrp30 gene

The 735 bp cDNA sequence of the human Acrp30 gene, cloned by our earlier experiments according to Maeda et al. (1996), was designed for high-level expression in rice plants. The codon usage frequency for each amino acid in rice plant species, obtained from CUTG (codon usages tabulated from GenBank) database, was calculated to distinguish the preferred codons of rice genes. The codons encoding the N- and C-terminal regions of Acrp30 that have low usage frequency in rice were substituted by rice-biased codons, based on a rare codon optimization approach. A rice-biased translation initiation nucleotide sequence GCCGCC (Nakagawa et al., 2008) was incorporated at the 5' end, while an endoplasmic reticulum retention signal KDEL was placed at 3' end. By using a recursive PCR strategy (Prodromou and Pearl, 1992), the modified gene with desired 5' and 3' flanking sequences was amplified in four individual parts, using four pairs of overlapping oligonucleotides AF1/AR1, AF2/AR1, AF3/AR2 and AF4/AR2 respectively (Table 1). All PCR cycles were as follows: 94°C, 5 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and a final cycle of 72°C for 10 min. The resulting complete nucleotide sequence of the modified gene was confirmed by an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems).

Vector construction and rice transformation

The modified Acrp30 gene was sub-cloned in a plant transformation vector pCAMBIA1301 between the *Bam*HI and *Sac*I sites, using standard recombinant techniques (Sambrook et al., 1995). The maize ubiquitin promoter (Ubi) was cloned into the *Hind*III-*Bam*HI site, and the nopaline synthase terminator (T-nos) was ligated into the *Sac*I-*Eco*RI site. The *aps* found to increase transcriptional activity of recombinant protein in plants (Borisjuk et al., 2000) was introduced into the *Hind*III site, upstream of the Ubi promoter. The binary plant vector containing the modified gene with the insertion of *aps*, under the control of Ubi promoter and T-nos, was named pM-*aps*-ADPN. Meanwhile, the vector carrying native Acrp30 gene

without any modifications was named pN-ADPN and was used as a comparison study (Fig. 1A).

The resulting two vectors were respectively transformed into the *Agrobacterium tumefaciens* strain EHA105, followed by co-culture with embryogenic calli of Taijing-9, a Japonica rice variety (*Oryza sativa* L.). Callus selection, maintenance and plant regeneration were performed following the procedure described previously (Liu et al., 2005). The hygromycin-resistant T₀ plantlets were well-developed and then transferred to pots with soil for growth and maturity.

PCR and Southern blot

Total Genomic DNA was isolated from rice leaf tissues using a plant genomic DNA extraction kit (Baitaike) and was analyzed by PCR amplification with specific primers 5'-GAGTCCTGCTTCCCCTGCCC-3' and 5'-CCATTACGCTCTCCTTCCCC-3' for the modified or native Acrp30 gene, and 5'-TGGCTCGCACCTGTCTAAC-3' and 5'-TTCGTCGGTCTTGCCT ATC-3' for the 18S rDNA used as a control. Southern blot analysis was carried out according to the standard procedures (Sambrook et al., 1995). Genomic DNA was digested with *Hind*III and then the resultant fragments were separated by electrophoresis in a 0.8% agarose gel and electroblotted onto a Hybond-N⁺ nylon membrane (Millipore) by Trans-Blot SD semi-dry electrophoretic transfer cell (BioRad). The membrane was hybridized with DIG labeled DNA probe (Fig.1A probe, a 592 bp cDNA fragment comprising most of the protein-coding sequence). Southern blot hybridization and probe labeling were conducted according to the instruction of DIG High Prime DNA Labeling and Detection Starter Kit I (Roche).

RT-PCR analysis

Total RNA was extracted from rice leaves using RNeasy Mini kit (Qiagen) and then quantified using a GeneQuant™ 1300 spectrophotometer (Biochrom). RNA (1 µg) was reverse transcribed using the PrimeScript™ 1st Strand cDNA Synthesis kit (Takara). PCR reactions were carried out and specific primers were 5'-GAGTCCTGCTTCCCCTGCCC-3' and 5'-CCATTACGCTCTCC TCCCC-3' for the target gene and 5'-GGAAGTGGTATGGTCAAGGC-3' and 5'-AGTCTCATGGAT ACCCGCAG-3' for rice actin1 gene. PCR reactions were amplified for 35 cycles with an optimal annealing temperature

TABLE 1
Primers designed for sequence optimization of Acrp30 cDNA

Primers	Sequences
AF1	5'-TCGCCCTCCCGGGCCATGACCAGGAAACCACGACTCAGGGCCCC-3'
AF2	5'-GCCGTGCTCCTCCTCCTCGCCCTCCCGGGCCATGACCAGGAAAC-3'
AF3	5'-ATGCTCCTCCTCGGCGCCGTGCTCCTCCTCCTCGCCCTCCCGGG-3'
AF4	5'-CGCGGATCCGCGCCATGCTCCTCCTCGGCGCCGTGCTCCTC-3'
AR1	5'-ATCTTTGTTGGTGTGTCATGGTAGAGAAGGAAGCCGGTGAAG-3'
AR2	5'-GAGCTCTTACTACAGTTCATCTTTGTTGGTGTGCTGTTAGAGGAG-3'

The restriction endonucleases recognition sites that were introduced into the primers are underlined (Bam HI recognition sequence, 5'GATCC3'; Sac I recognition sequence, 5' GAGCTC3').

of 56°C along with appropriate control reactions. All RT-PCR reactions were replicated five times.

Western blot and ELISA analysis

Extraction of TSP from rice leaves was performed according to Park et al (2007). Briefly, leaves were finely ground in liquid nitrogen and homogenized with the extraction buffer (50 mM potassium phosphate, pH 7.8, 5 mM EDTA, 0.05% β -mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). After centrifugation, the protein concentration was determined by Bradford assay using bovine serum albumin as standard (Bradford, 1976). An equal volume of SDS-PAGE loading buffer was added to each 25 μ l (25 μ g) protein sample. The mixture was heated at 95°C for 5 min and separated by SDS-PAGE using 12% polyacrylamide gels. Proteins were then electroblotted onto PVDF membranes using Trans-Blot SD semi-dry electrophoretic transfer cell (BioRad). A rabbit anti-human Acrp30 antibody (Bios) was used to detect the recombinant Acrp30. A HRP-conjugated mouse-anti-rabbit IgG (Bios) and the ECL system (Santa Cruz) were performed as described by the manufacturer.

The expression levels of recombinant Acrp30 in transgenic rice plants were determined by quantitative direct antigen coating-enzyme linked immunosorbent assay (DAC-ELISA).

Extraction and determination of TSP of rice leaves were performed as described above. The protein samples were diluted to 4 μ g/ml in coating buffer (15 mM Na_2CO_3 , 35 mM NaHCO_3 , pH 9.6). Subsequently, an enhanced protein-binding ELISA plate was coated and incubated with 100 μ l/well of coating buffer for 2 h at 37 °C. The wells of ELISA plate were washed three times with wash solution (10 mM Na_2HPO_4 , 3 mM KH_2PO_4 , 100 mM NaCl, 3 mM KCl, pH 7.4, 0.05% Tween-20) between each step. Then, the wells were blocked with 200 μ l of blocking buffer (5% Casein Hydrolysate in coating buffer) for 2 h, followed by incubation with the rabbit anti-human Acrp30 antibody (Bios) at 1:5,000 dilutions in blocking buffer for 2 h at 37 °C. The wells were again incubated with the HRP-conjugated mouse-anti-rabbit IgG (Bios) at 1:10,000 dilutions in blocking buffer for 2 h at 37 °C. After that 100 μ l TMB substrate were added to per well and the wells were incubated at RT for 30 minutes in the dark. The assay was read at 450 nm within 30 minutes in the microtiter plate reader (Tecan Genios Pro) after adding 100 μ l 2.0 M H_2SO_4 per well to stop the reaction. The concentrations of the samples tested were calculated using the absorbance values of the Acrp30 standard solutions (ProSpec) assayed at the same time. The results were analyzed statistically using the student *t*-test in the Excel program.

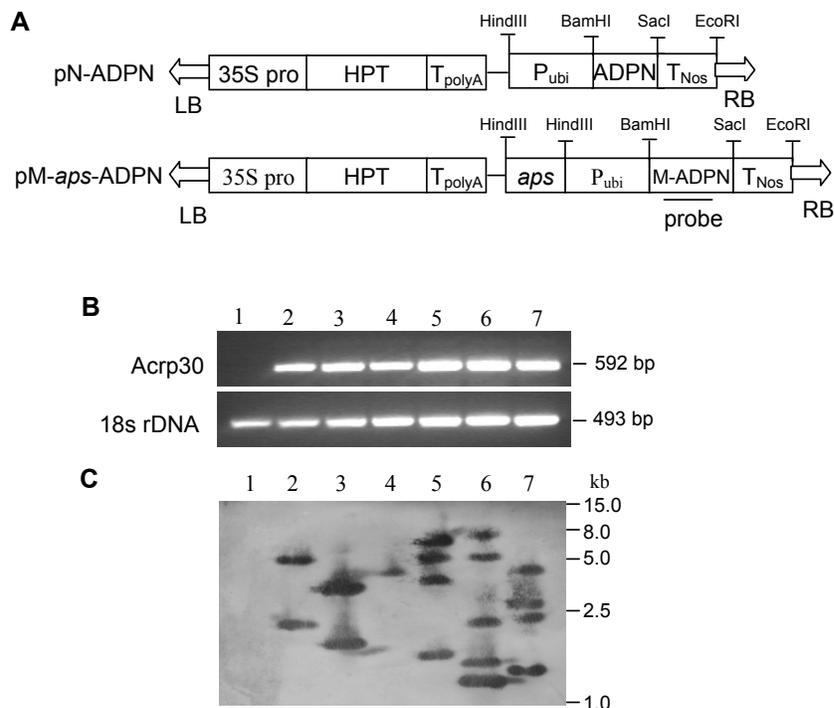


Figure 1: The schematic representation of the native and modified Acrp30 gene expression cassettes and its presence in transgenic rice. **(A) Schematic representation of pN-ADPN and pM-aps-ADPN expression cassettes used for rice transformation.** 35S pro, 35S promoter; HPT, hygromycin phosphotransferase gene, selectable marker gene; T_{polyA}, terminator of polyA; P_{ubi}, ubiquitin promoter; ADPN, cDNA sequence of native Acrp30 gene; M-ADPN, cDNA sequence of modified Acrp30 gene; T_{Nos}, terminator of nopaline synthase gene; RB, right border; LB, left border; *aps*, an amplification promoting sequence from tobacco rDNA. Probe used for southern hybridization analysis is a PCR-amplified fragment of Acrp30 gene with 592 bp in size. **(B) Detection of Acrp30 gene in control- and transgenic rice leaves by PCR.** A 592 bp fragment of native or modified Acrp30 gene was amplified and 18s rDNA was served as a control. Lane 1, untransformed control rice; Lane 2-4, the transformants with native gene (line RN1, RN3 and RN5); Lane 5-7, the transformants with modified gene (line RM3, RM7 and RM8). **(C) Southern hybridization analysis of control- and six randomly selected transgenic lines.** Rice genomic DNA digested with *Hind*III and hybridized with an amplified probe (See Fig. 1 A). Lane 1, untransformed rice plant; Lane 2-4, the transformants with the native Acrp30 gene (line RN1, RN3 and RN5); Lane 5-7, the transformants with the modified Acrp30 gene (line RM3, RM7 and RM8).

RESULTS

Designing of modified Acrp30 gene for high-level expression in rice

The aim of redesigning the human Acrp30 gene was to acquire a modified gene that could be efficiently transcribed and translated for high-level regulated expression in transgenic rice plants. Thus, the combination of alterations was introduced in the 735 bp cDNA sequence of the native Acrp30 gene. A total of 21 rare codons were replaced by rice-biased codons with replacement of 23 nucleotides in a modified cDNA sequence. Because codon optimization surrounding the N-terminus is particularly important for increasing recombinant protein production (Batard et al., 2000), codon substitution was emphasized in the N-terminal region where 17 rare codons were replaced by rice-biased codons. Furthermore, a rice-preferred translation initiation sequence GCCGCC was incorporated adjacent to the initiation codon ATG, which could enhance the efficiency of translation initiation (Nakagawa et al., 2008). Meanwhile, a 12-nucleotide sequence of endoplasmic reticulum retention signal KDEL was integrated at C-terminal end suitable for accumulation of the expressed recombinant protein in transgenic plant cells, which was followed by two stop codons to avoid translational read through (Agarwal et al., 2008). The complete modified gene with optimized flanking sequences on 5' and 3' end was obtained by recursive PCR amplification. The schematic drawing of gene modifications for high-level expression in rice is shown in Supplementary Fig. 1.

Molecular characterization of transgenic plants

Rice transformation was independently mediated by two *A. tumefaciens* EHA105 strains, one carrying pM-*aps*-ADPN and the other carrying pN-ADPN. The expression vectors containing either the native or modified gene were shown in Fig. 1A. Finally, 6 (named as line RN1-6 with the native gene) and 9 (named as line RM1-9 with the modified gene) promising T₀ transgenic rice plants were produced, respectively.

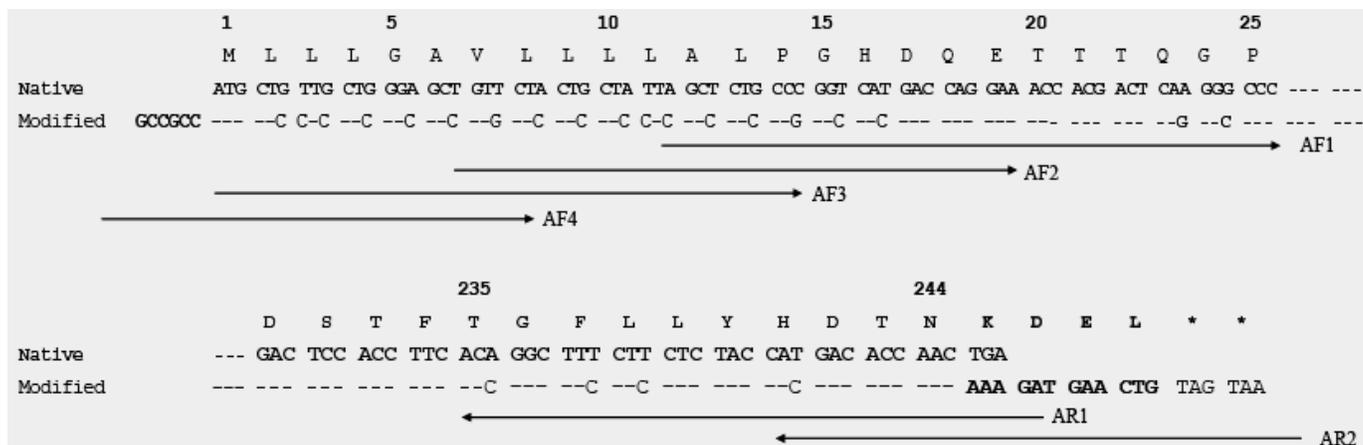
These promising T₀ transgenic rice plants were verified for the presence of the Acrp30 gene in the rice genome by PCR amplification. Fig. 1B shows the results of PCR amplification of six randomly selected T₀ transgenic plants. The expected 592 bp DNA fragment of the target gene was only determined in the transformants, whereas the 493 bp fragment of 18s rDNA was detected in both the transformants and untransformed rice.

Moreover, Southern blot analysis confirmed the integration of the target gene into the rice genome, although the hybridization patterns were different in the above six randomly selected T₀ transformant lines (Fig. 1C). It was previously reported that the presence of *aps* could be associated with 2-5-fold increase in the copy number of transgenic genes (Borisjuk et al., 2000). Similarly, as revealed in our study by comparative southern hybridization, the transformants with *aps* (line RM3, RM7 and RM8) seemed to contain many more multiple copies. No bands were observed in the untransformed rice.

To detect Acrp30 mRNA transcripts in transformant rice, semi-quantitative RT-PCR analysis was performed and rice actin1 was used as a loading control. The transformants were analyzed with oligonucleotide primers for the target gene and actin1 gene, resulting in fragments of 592 bp and 795 bp, respectively. However, the levels of Acrp30 mRNA expression varied differently among these transformant lines (Fig. 2A). The transformants with the modified Acrp30 gene and insertion of *aps* showed much higher transcription levels.

Expression of recombinant Acrp30 in rice plants

To examine whether Acrp30 was synthesized in transgenic rice and whether the protein had the expected size, we performed Western blot analysis. When analyzed on SDS-PAGE in reducing conditions followed by immunoblotting, the band of approximately 30 kDa band was detected in transformants (Fig. 2B). The higher amount of Acrp30 could be detected in line RM3, RM7 and RM8, whereas line RN1 and RN5 contained



Supplementary Fig. 1 The modified Acrp30 gene was amplified by recursive PCR strategy for high-level expression in rice plants. Based on the primary cDNA sequence of the native gene, six different PCR primers shown with arrows at their respective binding positions were designed for codons and flanking region optimization. The changed synonymous codons were labeled. The accuracy of the modified Acrp30 gene sequence was confirmed using the DNA sequencer.

less Acrp30. The expression levels of the protein correlated well with Acrp30 mRNA transcription activation. No such protein band was detected from untransformed control leaves.

Furthermore, the contents of recombinant Acrp30 expressed in transgenic rice leaves were quantified by DAC-ELISA analysis. As expected, the transgenic plants carrying modified genes produced more recombinant protein quantities than transformants with the native gene. The content of recombinant Acrp30 ranged from 0.19% to 0.32% of TSP in leaves of transgenic rice with the modified gene; while there was a range from 0.02% to 0.05% in leaves of transgenic plants with the native gene (see Supplementary Fig. 2A). The accumulation of Acrp30 in transgenic plants with the native gene reached an average level of $0.03 \pm 0.01\%$ (TSP). In contrast, the average amount of Acrp30 in the transgenic plants carrying the modified gene was $0.26 \pm 0.03\%$ (TSP) (see Supplementary Fig. 2B). Taken together, the data demonstrated that the modified Acrp30 gene could be transcribed and translated more efficiently than the native gene, and therefore much more protein was produced in transgenic rice plants carrying the modified gene.

DISCUSSION

Diabetes is a worldwide epidemic and is one of the most important risk factors leading to other health problems. The important metabolic effects of Acrp30 on blood glucose and lipid levels have been well documented in the last decade (Arita et al., 1999; Berg et al., 2001; Heilbronm et al., 2003; Zhou et al., 2005; Fu et al., 2005). Acrp30 is tremendously valuable in terms of basic research and clinical applications, due to its great potential for ameliorating insulin-resistance and treating type II diabetes mellitus. To engineer a novel anti-diabetic rice line producing large amount of human adiponectin is worth striving for.

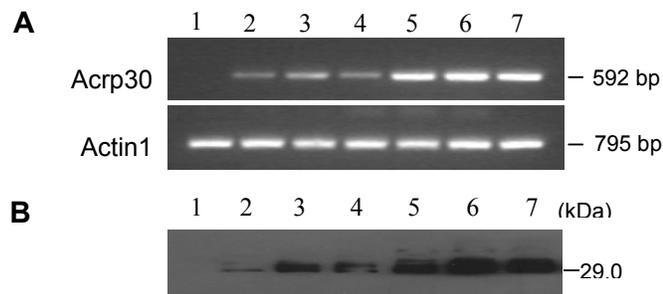
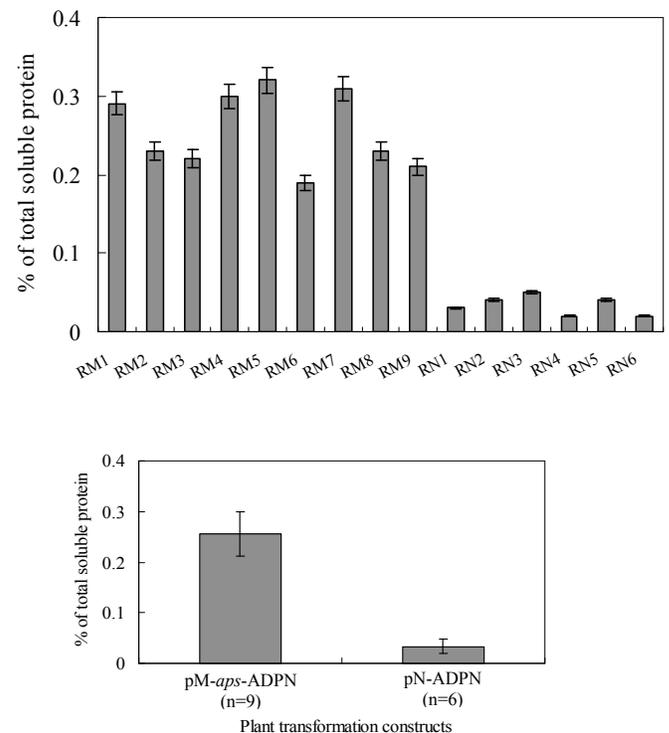


Figure 2 RT-PCR and Western analysis of Acrp30 in transgenic rice lines.

(A) RT-PCR amplification of Acrp30 gene in control- and transgenic rice leaves. The mRNA abundance was determined by RT-PCR analysis and rice actin1 gene was amplified as a loading control. Lane 1, untransformed rice plant; Lane 2-4, the transformants with the native Acrp30 gene (line RN1, RN3 and RN5); Lane 5-7, the transformants with the modified Acrp30 gene (line RM3, RM7 and RM8).

(B) Western immunoblotting of transgenic rice lines with a rabbit anti-human Acrp30 antibody and a HRP-conjugated mouse-anti-rabbit IgG. Lane 1, untransformed rice plant; Lane 2-4, the transformants with the native Acrp30 gene (line RN1, RN3 and RN5); Lane 5-7, the transformants with the modified Acrp30 gene (line RM3, RM7 and RM8).

Previous studies have shown that the use of transgenic plants for recombinant protein production often results in low yields (Kermode, 2006). For some pharmaceutical proteins expressed in plant systems, yields have varied from 0.01% to 1% of TSP (Hood et al., 2002), and have even been as low as 0.0001% of TSP (Chen et al., 2005). Thus, one of the key challenges for recombinant protein production in transgenic plants is the employment of effective strategies to enhance expression levels, which is a requirement for economic efficiency (Hood et al., 2002; Fischer et al., 2004; Stoger et al., 2005). Different organisms use synonymous codons with different preferences, and the codons in the target gene can be altered to adapt codon usage of host and subsequently improve heterologous protein expression. Currently there are two widely used codon optimization approaches: the full optimization of every codon (Holler et al. 1993; Gustafsson et al. 2004) and selective rare codon optimization (Deng, 1997). Full optimization has some potential disadvantages in comparison to selective rare codon optimization, such as introducing secondary mRNA structures that might hinder the translation process (Gustafsson et al., 2004; Kurland and Gallant, 1996). Indeed, there is no natural highly expressed gene that uses the full optimization of every codon. We therefore adopted the selective rare codon optimization method in our study. A total of 21 rare codons, 17 of which appeared in clusters or in the N-terminal part of the protein, were replaced by rice-biased codons. Furthermore, a rice-preferred translation initiation sequence GCCGCC was incorporated adjacent to the initiation ATG in order to enhance translational efficiency. Meanwhile, a 12-nucleotide signal sequence KDEL was integrated at the C-terminal end to ensure correct transport of recombinant protein into endoplasmic reticulum for glycosylation and accumulation. Moreover,



Supplementary Fig. 2

it was reported that an average 2.5 or 2-5 fold increase in mRNA expression levels, further resulting in distinct increase in recombinant protein by introducing *aps* into tomato and tobacco, respectively (Borisjuk et al., 2000; Yakoby et al., 2006). Therefore the *aps* was also inserted upstream of the Ubi promoter in the modification. The gene thus designed by our combination of modification strategies resulted in high-level expression of recombinant protein with a maximum of up to 0.32% of TSP in leaves of transgenic rice. As a comparative study, the native *Acrp30* gene has also been expressed in transgenic rice leaves ranging from 0.01% to 0.05% of TSP, which was substantially lower than the expression amounts of the modified gene. To summarize, the high-level regulated expression achieved in our study might be attributed mainly to the combined effects of different modifications incorporated within the coding sequence and flanking regions of the gene.

An important question arising from this study is whether stability of transgene expression would be maintained in rice cells, which is a critical concern. The stability of transgene expression is influenced by various factors including transformation events, background genotype, environmental conditions, etc. Instability of transgene expression in plants is often associated with complex multicopy patterns of transgene integration at the same locus, as well as position effects due to random integration (Koprek et al., 2001). Although the gene copy number is not always proportional to the mRNA expression level due to positional affect, methylation, and partial gene silencing, etc (Meyer and Saedler, 1996; Stam et al., 1998), our results from Southern blot and RT-PCR analysis suggested that *aps* is likely to promote transgenic expression by increasing both the target gene copy number and the transcription level in rice, similar to its effects previously reported in tobacco and tomato. Moreover, Borisjuk et al. (2000) demonstrated the effects of *aps* on the increase in the transgene copy number and the enhanced expression could be stably inherited in the progeny of the transgenic tobacco. Further experiments in our future study are still needed to observe if the offspring of transgenic rice carrying multiple copies of the *Acrp30* gene would stably retain a high level of expression.

Another concern that arises from this study is that the huge potential of production of pharmaceutical proteins in plants will only be realized if the products are provided with full biological activity. *Acrp30* is initially synthesized as a 30 kDa monomer and is then assembled into oligomeric isoforms that are secreted and circulate in plasma (Bodles et al., 2006). In the circulation, *Acrp30* is present predominantly as three oligomeric isoforms, including trimeric, hexameric and the high-molecular-weight (HMW) oligomeric complexes, which are stable and do not interconvert into each other (Wang et al., 2008). The monomers or a globular fragment of the *Acrp30* monomer produced by recombinant expression systems are biologically active, which has also been reported in some studies (Berg et al., 2001; Fruebis et al., 2001; Liu et al., 2007). However, it should be pointed out that in humans there is no evidence of the "physiological" presence of the monomer in the bloodstream (Wang et al., 2008). Our study only detected an electrophoretically 30-kDa band of *Acrp30* in Western immunoblot gel, which may have been due to analyzing under reducing conditions by SDS-PAGE. The post-translation modification of *Acrp30*, such as hydroxylation of multiple conserved proline and lysine residues and glycosylation

of hydroxylysines within the collagenous domain, have been shown to be crucial for *Acrp30* oligomerization *in vivo* (Wang et al., 2008). Whether the recombinant *Acrp30* produced by transgenic rice would be able to effectively form the native glycosylation pattern, the sugar chain structures of recombinant *Acrp30* by the reverse phase HPLC chromatogram, is still a question for the present study.

In conclusion, we established the feasibility of high-level expression of recombinant *Acrp30* in transgenic rice. The results indicated that genetically engineered rice containing a high-level expression of human *Acrp30* was successfully obtained. The functions of recombinant *Acrp30* are still being evaluated in both culture cell and mice models.

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REFERENCES

- AGARWAL S, SINGH R, SANYAL I, AMLA DV (2008) Expression of modified gene encoding functional human α -1-antitrypsin protein in transgenic tomato plants. *Transgenic Res* 17:881-896.
- ARITA Y, KIHARA S, OUCHI N, TAKAHASHI M, MAEDA K, MIYAGAWA J, HOTTA K, SHIMOMURA I, NAKAMURA T, MIYAOKA K, KURIYAMA H, NISHIDA M, YAMASHITA S, OKUBO K, MATSUBARA K, MURAGUCHI M, OHMOTO Y, FUNAHASHI T, MATSUZAWA Y (1999) Paradoxical decrease of an adipocyte specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 257:79-83.
- BATARD Y, HEHN A, NEDELKINA S, SCHALK M, PALLETT K, SCHALLER H, WERCK-REICHHART D (2000) Increasing expression of P450 and P450-reductase proteins from monocots in heterologous systems. *Arch Biochem Biophys* 379:161-169.
- BERBERICH T, TAKAGI T, MIYAZAKI A, OTANI M, SHIMADA T, KUSANO T (2005) Production of mouse adiponectin, an anti-diabetic protein, in transgenic sweet potato plants. *J Plant Physiol* 162: 1169-1176.
- BERG AH, COMBS TP, DU X, BROWNLEE M, SCHERER PE (2001) The adipocyte-secreted protein *Acrp30* enhances hepatic insulin action. *Nat Med* 7:947-953.
- BODLES AM, BANGA A, RASOULI N, ONO F, KERN PA, OWENS RJ (2006) Pioglitazone increases secretion of high-molecular-weight adiponectin from adipocytes. *Am J Physiol Endocrinol Metab* 291: E1100-E1105.
- BORISJUK N, BORISJUK L, KOMARNYTSKY S, TIMEVA S, HEMLEBEN V, GLEBA Y, RASKIN I (2000) Tobacco ribosomal DNA spacer element stimulates amplification and expression of heterologous genes. *Nat Biotechnol* 18:1303-1306.
- BRADFORD MM (1976) A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254.
- CARMO AVIDES M, DOMINGUES L, VICENTE A, TEIXEIRA J (2008) Differentiation of human pre-adipocytes by recombinant adiponectin. *Protein Exp Pur* 59:122-126.
- CHATURVEDI N (2007) The burden of diabetes and its complications: trends and implications for intervention. *Diabetes Res Clin Pract* 76 Suppl 1:S3-12.
- CHEN M, LIU X, WANG Z, SONG J, QI Q, WANG PG (2005) Modification of plant N-glycans processing: The future of producing therapeutic protein by transgenic plants. *Med Res Rev* 25: 343-360.
- DENG T (1997) Bacterial expression and purification of biologically active mouse c-Fos proteins by selective codon optimization. *FEBS Lett* 409:269-272.
- FISCHER R, STOGER E, SCHILLBERG S, CHRISTOU P, TWYMAN R (2004) Plant-based production of biopharmaceuticals. *Curr Opin Plant Biol* 7:152-158.
- FRUEBIS J, TSAO TS, JAVORSCHI S, EBBETS-REED D, ERICKSON MR, YEN FT, BIHAIN BE, LODISH HF (2001) Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci USA* 98: 2005-2010.

- FU Y, LUO N, KLEIN RL, GARVEY WT (2005) Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. *J Lipid Res* 46:1369-1379.
- GUSTAFSSON C, GOVINDARAJAN S, MINSHULL J (2004) Codon bias and heterologous protein expression. *Trends Biotechnol* 22:346-353.
- HEILBRONM LK, SMITH SR, RAVUSSIN E (2003) The insulin sensitizing role of the fat derived hormone adiponectin. *Curr Pharm Des* 9: 1411-1418.
- HOLLER TP, FOLTIN SK, YE QZ, HUPE DJ (1993) HIV1 integrase expressed in *Escherichia coli* from a synthetic gene. *Gene* 136:323-328.
- HOOD EE, WOODARD SL, HORN ME (2002) Monoclonal antibody manufacturing in transgenic plants-myths and realities. *Curr Opin Biotechnol* 13:630-635.
- KADOWAKI T, YAMAUCHI T (2005) Adiponectin and Adiponectin Receptors. *Endocrine Reviews* 26: 439-451.
- KARNAUKHOVA E, OPHIR Y, GOLDING B (2006) Recombinant human alpha-1 proteinase inhibitor: towards therapeutic use. *Amino Acids* 30:317-332.
- KARNOUP AS, TURKELSON V, ANDERSON WH (2005) O-linked glycosylation in maize-expressed human IgA1. *Glycobiol* 15:965-981.
- KERMODE AR (2006) Plants as factories for production of biopharmaceutical and bioindustrial proteins: lessons from cell biology. *Can J Bot* 84:679-694.
- KOPREK T, RANGE S, MCELROY D, LOUWERSE JD, WILLIAMS-CARRIER RE, LEMAUX PG (2001) Transposon-Mediated Single-Copy gene delivery leads to increased transgene expression stability in barley. *Plant Physiol* 125:1354-1362.
- KURLAND C, GALLANT J (1996) Errors of heterologous protein expression. *Curr Opin Biotechnol* 7: 489-493.
- LIU DG, LIU HL, SONG TJ, HUANG HY, LI X, TANG QQ (2007) Functional expression of the globular domain of human adiponectin in *Pichia pastoris*. *Biochem Biophys Res Commun* 363:769-775.
- LIU F, ZHAO Y, SU Y, XU M, CHEN L, ZHENG J (2005) Production of Marker-free transgenic rice with PEPC gene by *Agrobacterium*-mediated co-transformation. *Chin J Appl Environ Biol* 11:393-398.
- MA JKC, CHIKWAMBA R, SPARROW P, FISCHER R, MAHONEY R, TWYMAN RM (2005) Plant-derived pharmaceuticals-the road forward. *Trends in Plant Science* 10: 580-585
- MA JKC, DRAKE PMW, CHRISTOU P (2003) The production of recombinant pharmaceutical proteins in plants. *Nat Rev Genet* 4:794-805.
- MAEDA K, OKUBO K, SHIMOMURA I, FUNAHASHI T, MATSUZAWA Y, MATSUBARA K (1996) cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (Adipose Most abundant Gene transcript). *Biochem Biophys Res Commun* 221:286-289.
- MEYER P, SAEDLER H (1996) Homology-dependent gene silencing in plants. *Annu Rev Plant Physiol Plant Mol Biol* 47:23-48.
- NAKAGAWA S, NIIMURA Y, GOJOBORI T, TANAKA H, MIURA KI (2008) Diversity of preferred nucleotide sequences around the translation initiation codon in eukaryote genomes. *Nucl Acids Res* 36:861-871.
- PARK SY, YU JW, PARK JS, LI J, YOO SC, LEE NY, LEE SK, JEONG SW, SEO HS, KOH HJ, JEON JS, PARK YI, PAEK NC (2007) The Senescence-Induced Staygreen Protein Regulates Chlorophyll Degradation. *The Plant Cell* 19:1649-1664.
- PRODROMOU C, PEARL LH (1992) Recursive PCR: a novel technique for total gene synthesis. *Protein Eng* 8:827-829.
- SAMBROOK J, FRITSCH EF, MANIATIS T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- SCHERER PE, WILLIAMS S, FOGLIANO M, BALDINI G, LODISH HF (1995) A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 270:26746-26749.
- SCHINKEL H, SCHIERMEYER A, SOEUR R, FISCHER R, SCHILLBERG S (2005) Production of an active recombinant thrombomodulin derivative in transgenic tobacco plants and suspension cells. *Transgenic Res* 14:251-259.
- STAM M, VITERBO A, MOL JN, KOOTER JM (1998) Position-dependent methylation and transcriptional silencing of transgenes in inverted T-DNA repeats: implications for posttranscriptional silencing of homologous host genes in plants. *Mol Cell Biol* 18:6165-6177.
- STOGER E, MA JKC, FISCHER R, CHRISTOU P (2005) Sowing the seeds of success: pharmaceutical proteins from plants. *Curr Opin Biotechnol* 16:167-173.
- STREATFIELD SJ (2007) Approaches to achieve high-level heterologous protein production in plants. *Plant Biotechnol J* 5:2-15.
- WANG Y, LAM KS, YAU MH, XU A (2008) Post-translational modifications of adiponectin: mechanisms and functional implications. *Biochem J* 409:623-633.
- WANG Y, XU A, KNIGHT C, XU LY, COOPER GS (2002) Hydroxylation and Glycosylation of the Four Conserved Lysine Residues in the Collagenous Domain of Adiponectin. *J Biol Chem* 277: 19521-19529.
- YAKOBY N, GARVEY A, RASKIN I (2006) Tobacco ribosomal DNA spacer element elevates Bowman-Birk inhibitor expression in tomato plants. *Plant Cell Rep* 25:573-581.
- YAMAUCHI T, KAMON J, ITO Y, TSUCHIDA A, YOKOMIZO T, KITA S, SUGIYAMA T, MIYAGISHI M, HARA K, TSUNODA M, MURAKAMI K, OHTEKI T, UCHIDA S, TAKEKAWA S, WAKI H, TSUNO NH, SHIBATA Y, TERAUCHI Y, FROGUEL P, TOBE K, KOYASU S, TAIRA K, KITAMURA T, SHIMIZU T, NAGAI R, KADOWAKI T (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 423:762-769.
- ZHOU H, SONG X, BRIGGS M, VIOLAND B, SALSGIVER W, GULVE EA, LUO Y (2005) Adiponectin represses gluconeogenesis independent of insulin in hepatocytes. *Biochem Biophys Res Commun* 338:793-799.

