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Transformation of tobacco (*Nicotiana tabacum*) with antisense soybean (*Glycine max*) oleic acid desaturase gene

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ABSTRACT

Soybean (*Glycine max* L.) antisense oleic acid desaturase gene *FAD2-1* was cloned from soybean genomic DNA by PCR for its funtions to be determined. The amplicon was linked to pMD18-T vector and transformed into *E. coli* JM109. After sequencing, it was reversely inserted in pbt expression vector in order to construct plant antisense expression vector, which was introduced into *Agrobacterium tumefaciens* strains LBA4404 by freeze-thawing method. The modified strain LBA4404 was confirmed by double enzyme digestion and PCR detection. The antisense *FAD2-1* was transferred into tobacco (*Nicotiana tabacum*) by *Agrobacterium tumefaciens*-mediated leaf disc transformation, and 75 kanamycin-resistant tobacco plantplets were regenerated. PCR, PCR-Southern blot, RT-PCR methods and GC/MS analysis were used to detect *npt*-II gene, antisense *FAD2-1*, its transcript and oleic acid content to get the positive transgenic plants. The results indicated that the gene *FAD2-1* was 1196bp in length, bearing 96.7% identity with the published data in NCBI database,that antisense *FAD2-1* expression vector was successfully constructed and transformed into *Agrobacterium tumefacien* strains LBA4404 and tobacco cells, and that antisense gene *FAD*2-1 was successfully integrated into the genomes of tobacco cells, expressed in transgenic tobacco plant cells and improved tobacco fatty acids.

Key words: *Agrobacterium tumefaciens*, Antisense oleic acid desaturase gene, *Glycine max*, Transgenic tobacco

Soybean (*Glycine max* L.), one of the most important crops in the world, accounts for 56% of the global oilseed production. Soybean vegetable oil is composed predominantly of unsaturated 18-carbon fatty acid: the monounsaturated oleic (18:1) and polyunsaturated linoleic (18:2) and linolenic (18:3) acids. The relative composition of saturated and unsaturated fatty acids in seed is one of the major factors influencing the quality of edible oils. Some research results showed that the oils containing high monounsaturated (18:1) oleic acid and low polyunsaturated (18:2) fatty acids appear to have improved nutritional benefits and increased stability. High accumulation of oleic acid could be due to a lack of oleic acid desaturase (Δ12 fatty acid desaturase, *FAD2*) activity catalysing the desaturation of oleic acid to linoleic acid by inserting a double bond at the Δ12 position in fatty acid biosynthsis, which plays an important role in determining the ratio of total monounsaturased to polyunsaturased fatty acids in plants. Specialized fatty acid compositions desires for edible and industrial purposes have been produced in soybean crops through traditional breeding and selection alone or in combination with mutagenesis programs (Rani *et al*. 2007). But with the development of transgenic technology, the modification of seed oil composition is becoming possible (Kumar *et al*. 2011). Antisense technology has proven to be an effective means to reduce the level of specific enzyme in plants (Wen *et al*. 2011).

Here, we isolated a *FAD2-1* gene from soybean and analyzed its function by constitutively expressing its antisense genes in tobacco (*Nicotiana tabacum*), which led to the higher accumulation of monounsaturated (18:1) oleic acid in transgenic tobacco plants than control by its antisense inhibition. Antisense soybean *fad2* gene-expressing tobacco represents an alternative source of high oleic acid for food and non-food applications.

MATERIALS AND METHODS

Soybean seeds (Zhoudou12 cultivar,) were held in our laboratory. The leaf tissues were collected from soybean seedings which grew at 28°C for 12 days, and genomic DNA was isolated from leaf tissues using CTAB method. Tobacco seeds (cultivar Xanthi) were donated by Dr Meijuan

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The primers (Pf_1) 5'-TTTTTGTCGACACTAGG-CATGGGTCTAGCAAA-3' (forward) and (Pr_1) 5'-TTTTTGGATCCCCATCAATACTTGTTCCTGTACC-3' (reverse) were designed according to the known Δ12 fatty acid desaturase from NCBI. PCR amplification was carried out in a total volume of 30μl containing 0.3μg genomic DNA, 1μl *Taq* polymerase (Takara Bio, Tokyo, Japan), 3μl 10 × Taq buffer, 250μmol dNTP, 1.5mmol $MgCl_2$, 100pmol of each primer and the addition of PCR water to the final volume. PCR amplification was performed as follows: initial denaturalization at 94°C for 5min, followed by 35 cycles of 94°C for 1 min, 59°C for 1.5 min and 72°C for 3 min, with a final extension cycle of 72°C for 10 min. The amplified 1196-bp PCR product was harvested from agrose gel, purified with purified kit (JiKng, Shanghai, China), and cloned into the pMD18-T Vector (Takara Bio, Tokyo, Japan) to be sequenced by biotechnology company. DNA and deduced amino acid sequences were analyzed using BLAST-algorithm (Zhang and Hrazdina 2010) for sequence homology search and domain identification, and sequence organization and alignment was analyzed by DNASTAR.

The antisense expression vector of *FAD2-1* ($_p$anti-f-fad) was constructed which contained 2E-35s promoter::antisense *FAD2-1*::NOS and NOSP-NP II-nos, and introduced into *Agrobacterium tumefaciens* strain LBA4404 by freeze-thaw method. *Agrobacterium tumefaciens* harboring $_p$anti-f-fad was grown overnight in YEB medium (pH 7.0) containing 200μg/μl *Str* and 100μg/μl *Rif* at 28°C. Transformants were selected by plating cells in triplicates on solid YEB medium supplemented with 100μg/ml Str, 50μg/ml Kan after incubation for 2-3d at 28°C, and detected by double RE digestion and PCR. The transformed *Agrobacterium tumefaciens* cell pellet was suspended in YEB medium (pH 5.6) with 100μM Acetosyringone (Sigma). Tobacco seeds were germinated in pots with soil until two leaf-stage. *Agroinoculation* was done by leaf inoculation method, in which wounds were made by pricking it 5-6 times with 30-gaze needle. About 20μl *Agrobacterium* suspension were put on the wounds (Yadav *et al*. 2009). Plants were kept in green house at 26±2°C. In rooting MS medium containing 0.2mg/L NAA, 75 transformed tabacco plantlets rooted from the shoots, derived from the induced calluses on MS medium supplemented with 50μg/ml Kan, 500μg/ml Cef, 0.5mg/L 2,4-D and 1.0mg/L 6-BA,were obtained and used for *npt-II* gene PCR analysis.

Genomic DNA as template from transformed tobacco leaves, positive control ($_p$anti-f-fad) and two negative control (genomic DNA from non-transformants and water blank) were subjected to the amplification protocol of PCR with the following primers: Pf_2 (5'-GCTCCTACAAATGCCATCA-3') was designed according to sequence of *CaMV*35S promoter, Pr_2 (5'-TTTTTGTCGACACTAGGCATGGGT-CTAGC-3') was designed according to sequence of soybean antisense *FAD2-1*. The reaction was performed in a total volume of 20μl, containing 30pmol of either of primers, 5nmol dNTPs, 5μl 10 × *Taq* buffer, 1.5U *Taq* (TaKaRa) and 20ng template DNA. The mixture was initially denatured at 94°C for 2 min, and treated with 35 thermal cycles of 94°C for 30s (denaturation), 58°C for 60s (primer annealing) and 72°C for 90s (extension). The amplified products were analyzed by electrophoresis using 1.5% agarose gels, visualized and photographed under ultraviolet (UV) light. Subsequently, they were used for Southern blot (Kido *et al*. 2000), in which probe was made by Shanghai Dingguo, China based on soybean antisense *FAD2-1* and labeled using DIG.

Total RNA was extracted from leaf tissues of transformed tobacco plants and non-transformed tobacco plants using Trizols reagent (Invitrogen Co., USA) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNaseI (TaKaRa Biotechnology, China). Approximately 0.6μg total RNA of each sample was subjected to RT-PCR analysis according to manufacturer's instruction using one step RT-PCR kit (M-MuLV provided by Shanghi Sangon Co. Ltd, China), and the method described by Zhou *et al*. (2009) with the above primer pairs. The amplicons were separated using electrophoresis on 1.7% agarose gel with DNA marker and visualized with ethidium bromide staining and photographed under ultraviolet (UV) light.

GC/MS was performed on a 6890/5973N GC/MS instrument (Agilent, USA) under the following conditions: DB-5MS column (30 m × 0.25 mm × 0.25 μm), helium carrier gas, 1.0ml/min; injector, 260°C; detector, 280°C; column, 198°C, reference standards, including oleic acid and linoleic acid (ChemService, USA). The preparation of oleic acid and linoleic acid samples from young leaf tissues of tobacco plants and the determination of their contents were done according to the described method (Yang *et al*. 2008).

RESULTS AND DISCUSSION

Cloning and sequencing of gene FAD

Plant microsomal *delta*-12 fatty acid desaturase (*FAD2*) functions in the first committed step of the biosynthesis of polyunsaturated fatty acids via the desaturation of oleic acid to linoleic acid (Jung *et al*. 2011). In soybeans, *FAD*2-1 has been shown to be strongly expressed in developing seeds and likely plays a major role in controlling conversion of oleic acid to linoleic acid within storage lipids during seed development.

In the present work, using primers Pf1 and Pr2 designed according to the known Δ12 fatty acid desaturase from NCBI (GenBank accession number AY660024), one about 1.2kb fragment was amplified from soybean genomic DNA as

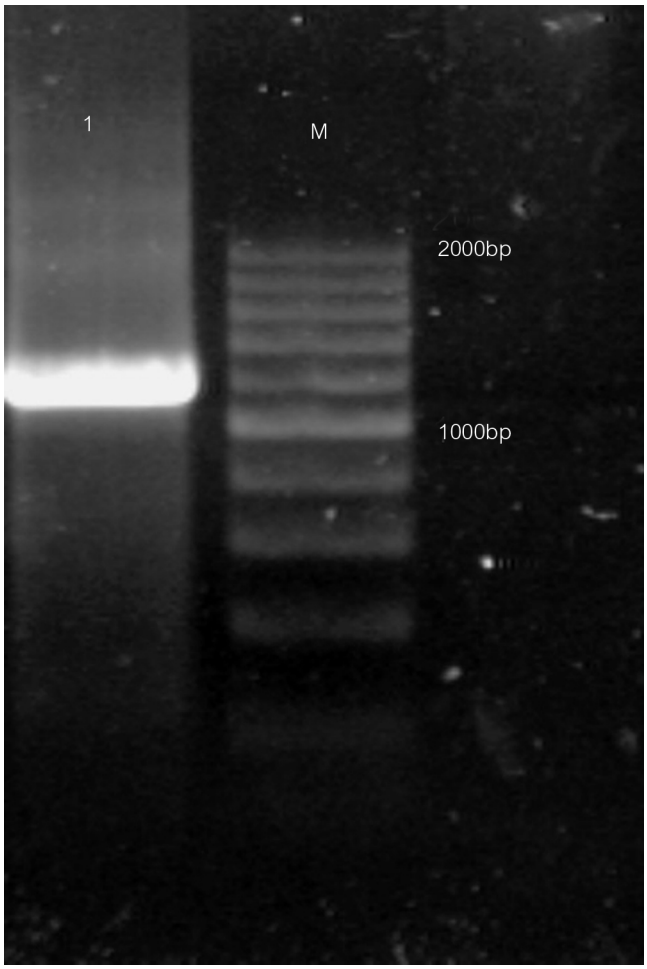

Fig 1 The fragment of *FAD2-1* gene in soybean amplified by PCR 1: *FAD2-1* from soybean genomic DNA. M: 200bp ladder marker composed of 2000bp, 1800bp, 1600bp, 1400bp, 1200bp, 1000bp, 800bp, 600bp, 400bp and 200bp.

showed in Fig 1, cloned into pMD18-T vector, and introduced into *E coli.* JM109 to obtain transformed bacteria, confirmed by PCR. pMD18-T vectors with 1.2kb fragment, extracted from the positive transformed clones, were sequenced (Fig 2). It is 1196bp in length, including a complete ORF consisting of 1164 bp, and coding for a putative protein of 387 amino acids with a molecular mass of 44.8 kDa or so. The homolog of the deduced amino acid sequence with that of 12 fatty acid desaturase from soybean *FAD2-1* from NCBI indicated that there were 17 amino acids different from the NCBI database, confirming that the cloned gene was soybean *FAD2-1* (Fig 3). In addition, three histidine cluster motifs, which are conserved among several 12 fatty acid desaturases and are considered to be essential for desaturase activity-acting as potential ligands for iron atoms-are highly conserved in the *Gmax FAD2-1* protein. The amino acd identity between these two proteins is 96.7%.

*Sal*I

TTTTTGTCGACACTAGGC ATG GGT CTA GCA AAG GAA ACA
ATA ATG GGA GGT GGA GGC CGT GTG GCC AAA GTT GAA
ATT CAG CAG AAG AAG CCT CTC TCA AGG GTT CCA AAC
ACA AAG CCA CCG TTC ACT GTT GGC CAA CTC AAG AAA
GCC ATT CCA CCG CAC TGC TTT CAG CGT TCC CTC CTC
ACT TCA TTG TCC TAT GTT GTT TAT GAC CTT TCA TTG
GCT TTC ATT TTC TAC ATT GCC ACC ACC TAC TTC CAC
CTC CTC CCT CAC CCC TTT TCC CTC ATT GCA TGG CCA
ATC TAT TGG GTT CTC CAA GGT TGC ATT CTT ACT GGC
GTG TGG GTG ATT GCT CAC GAG TGT GGT CAC CAT
GCC TTC AGC AAG TAC CCA TGG GTT GAT GAT GTT GTG
GGT TTG ACC GTT CAC TCA GCA CTT TTA GTC CCT TAT
TTC TCA TGG AAA ATA AGC CAT CGC CGC CAC CAC TCC
AAC ACG GGT TCC CTT GAC CGT GAT GAA GTG TTT GTC
CCA AAA CCA AAA TCC AAA GTT GCA TGG TAC ACC AAG
TAC CTG AAC AAC CCT CTA GGA AGG GCT GCT TCT CTT
CTC ATC ACA CTC ACA ATA GGG TGG CCT TTG TAT TTA
GCC TTC AAT GTC TCT GGC AGA CCC TAT GAT GGT TTT
GCT AGC CAC TAC CAC CCT TAT GCT CCC ATA TAT TCT
AAC CGT GAG AGG CTT CTG ATC TAT GTC TCT GAT GTT
GCT TTG TTT TCT GTG ACT TAC TCT CTC TAC CGT GTT
GCA ACC CTG AAA GGG TTG GTT TGG CTG CTA TGT GTT
TAT GGG GTG CCT TTG CTC ATT GTG AAC GGT TTT CTT
GTG ACT ATC ACA TAT TTG CAG CAC ACA CAC TTT GCC
TTG CCT CAT TAC GAT TCA TCA GAA TGG GAC TGG CTG
AAT GGA GCT TTG GCA ACT ATG GAC AGA GAT TAT GGG
ATT CTG AAC AAG GTG TTT CAT CAC ATA ACT GAT ACT
CAT GTG GCT CAC CAT CTC TTC TCT ACA ATG CCA CAT
TAC CAT GCA ATG GAG GCA ACC AAT GCA ATC AAG CCA
ATA TTG GGT GAG TAC TAC CAA TTT GAT GAC ACA CCA
TTT TAC AAG GCA CTG TGG AGA GAA GCG AGA GAG TGC
CTC TAT GTG GAG CCA GAT GAA GGA ACA TCC GAG AAG
GGC GTG TAT TGG TAC AGG AAC AAG TAT TGA
TGG GAGATCCAAAAA

*Bam*HI

Fig 2 DNA sequence of soybean gene *fad2-1* and the deduced amino acid sequence

Detection of positive transformants Agrobacterium tumefaciens *LBA4404 containing antisense* FAD2-1

The results of PCR amplification with primer pair of Pf1 and Pr2 and double enzyme digestion with *Sal*I and *Bam*HI showed that antisense *FAD2-1* was cloned into plant expression vector pbt, and transferred to *Agrobacterium tumefaciens* LBA4404 (Fig 4).

Generation and detection of transgenic tobacco plants expressing antisense FAD2-1 gene

The tobacco cells were transformed with vector $_p$anti-f-fad by *Agrobacterium tumefaciens*-mediated leaf disc transformation. Transgenic tobacco plants were obtained on MS medium containing kanamycin, different concentrations and kinds of plant hormones. Seventy five kanamycin-resistant tobacco plantplets were regenerated (T_0 plants, Fig 5, a-f), 67 of which contained *npt-II* gene detected by PCR (Fig 5, g). T_0 tobacco lines were then transplanted to soil for cultivation in greenhouse. There were no significant phenotypic difference between the control and transgenic plants. T_0 seeds were obtained from all antisense *FAD2*-1gene-transgenic plants. T-DNA integration in the transgenic

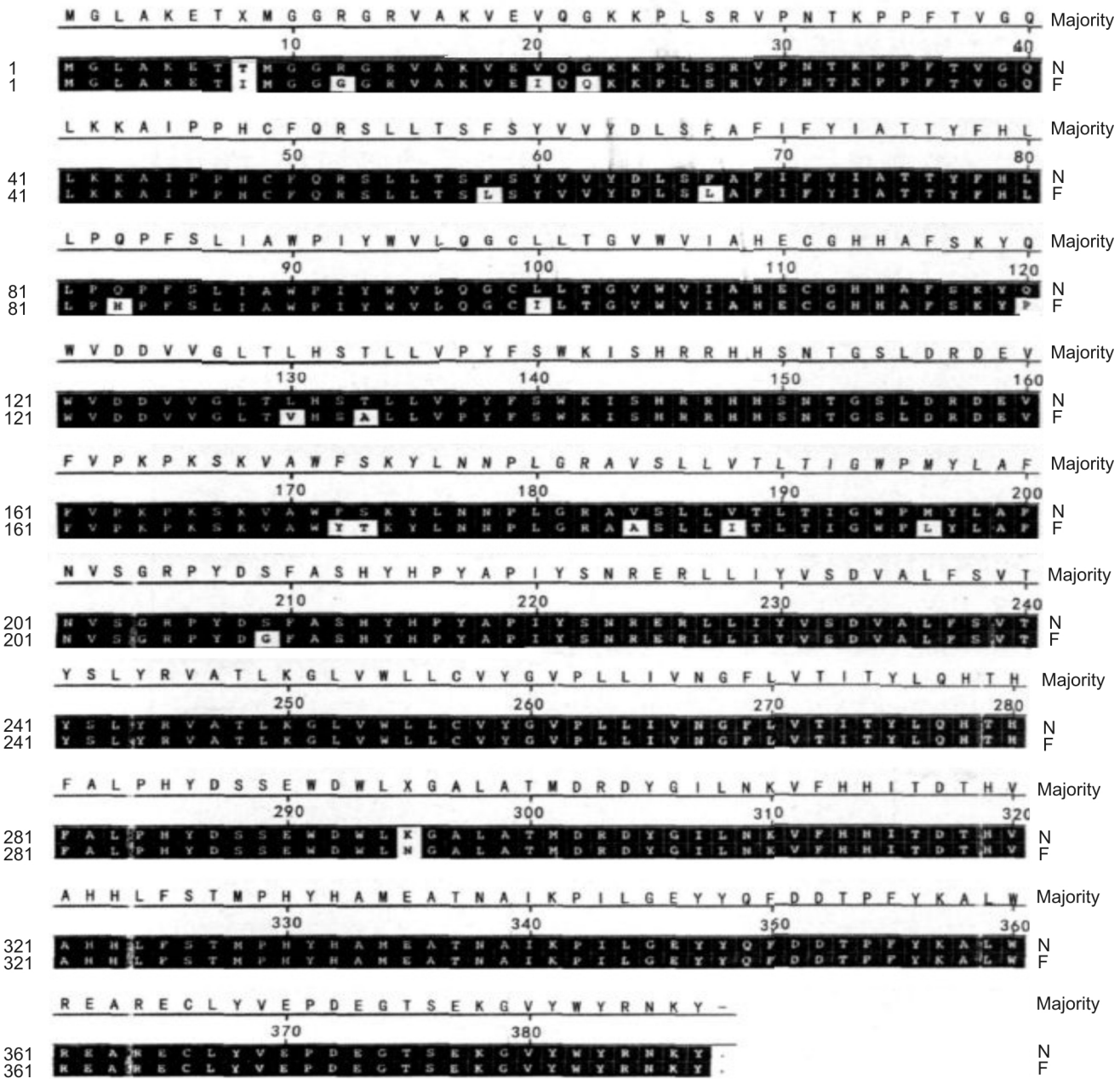

Fig 3 Alignment of the deduced amino acid sequence of *FAD*2-1 with soybean *FAD*2-1 in NCBI
N: the amino acid sequence of the soybean *FAD*2-1 in NCBI; F: the deduced amino acid sequence of cloned *FAD*2-1.

lines was confirmed by PCR-Southern blot detection of 35S promoter and antisense *FAD*2-1genes. They were present in three transformants, but none was found in the control (Fig 5, h). RT-PCR analysis indicated that antisense *FAD*2-1 mRNA was not expressed in the control plants but in the transgenic plants (Fig 5, i).

GC/MS analysis of three transgenic lines exhibited a certain decrease in oleic acid desaturase activity causing increased levels of oleic acid and decreased levels of linoleic acid, respectively, when compared to control plants. The proportion of oleic acid and linoleic acid was about 0.087% and 0.211% in the control plants, while it was 0.09%, 0.091% and 0.092, and 0.198%, 0.192% and 0.193% in the three transgenic tobacco lines, respectively.

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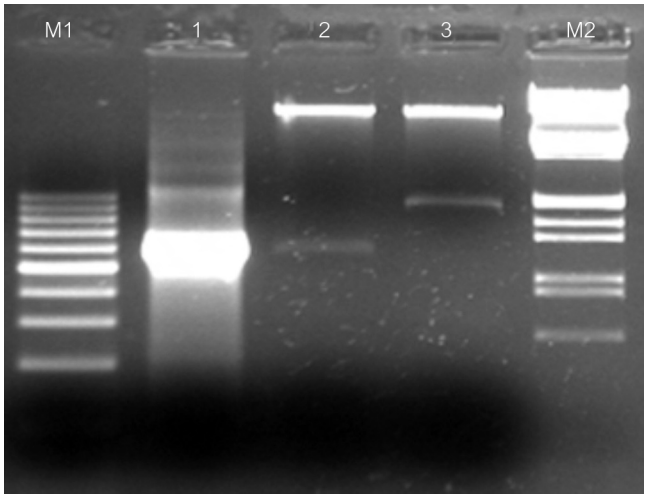

Fig 4 Agrose electrophoresis of PCR products and double RE digestion products of plant expression vector pbt harboring antisense gene *FAD2-1* in *Agrobacterium tumefaciens* LBA4404
M1: 200bp ladder, the same as shown in Fig 1; 1: PCR products of plant expression vector pBt harboring antisense gene *FAD2-1*; 2. double RE digestion products of plant expression vector pBt harboring antisense gene *FAD2-1*; 3: double RE digestion products of plant expression vector pBt harboring *Bt* gene. M2: λDNA/*Eco*RI+*Hin*dIII marker, the molecular weight is 21226bp, 3530bp,1904bp,1584bp, 1375bp, 947bp, 831bp, 564bp, respectively.

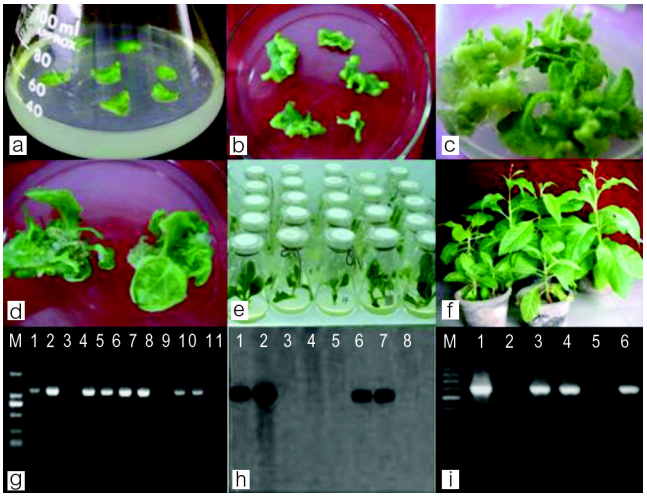

Fig 5 Tobacco leaf discs transformation, transformed plant regeneration and molecular detection
(a) Coculture of some tobacco leaf explants with *Agrobacterium tumefaciens* for 5d in MS medium with 1.0mol/L 6-BA and 0.05mol/L IBA. (b) Calluses induced from the above explants cocultured for 5d in MS medium with 1.0mol/L 6-BA, 0.05mol/L IBA, 80mol/L Kan and 500mol/L Cef. (c) Shoots from the calluses derived from the above explants cocultured for 20 days in MS medium with 1.0mol/L 6-BA, 0.05mol/L IBA, 80mol/L Kan and 500mol/L Cef. (d) Shoots cultured for 30d in MS medium with 80mol/L Kan and 500mol/L Cef. (e) Rooted plantlets cultured for 20d in MS medium with 80mol/L Kan and 500mol/L Cef. (f) Transformed plants cultured for 60d in green house after transplanted into flower pots. (g) Detection of *npt-II* gene in some transformed tabacco plantlets by PCR, M: 100bp Ladder; 1-7, 9: transformed tobacco plantlets; 8: PCR mix without DNA template; 10: $_{p}$anti-f-fad harboring soybean antisense *FAD2-1* gene; 11: Non-transformed tobacco plantlets (CK). (h) PCR-Southern blot detection of 35S promoter and antisense *FAD*2-1genes in transformed tobacco plants (To), 1 and 4-8: transgormed tobacco plants; 2: $_{p}$anti-f-fad harboring 35S promoter and antisense gene *FAD2-1*; 3: non-transgormed tobacco plant. (i) Detection of transgenic tobacco plants by RT-PCR, M: 200bp Ladder; 1: anti-f-fad harboring 35S promoter and antisense gene *FAD2-1*; 2: non-transgormed tobacco plant; 3-4, 6: transgenic tobacco plants; 5: PCR mix without tobacco RNA.

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