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Note

Transient expression of optimized and synthesized nattokinase gene in melon (*Cucumis melo* L.) fruit by agroinfiltration

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Abstract A synthetic gene (*sNK*) encoding Nattokinase (NK) was constructed by modifying its sequence based on the codon usage in plants. A version (*sNKi*) incorporating the first intron from the tomato E8 gene was also constructed. The synthetic *sNK* and *sNKi* genes were transiently expressed in melon (*Cucumis melo* L.) fruit. Quantitative real-time reverse transcription PCR (qRT-PCR) analysis and fibrinolytic activity assays showed that the expression level of recombinant NK controlled by E8 promoter was higher than that controlled by 35S promoter, with the maximum fibrinolytic activity of 79.30 U ml^{-1}. The intron could enhance the expression of *sNK* to an extent of 27.42%. Orthogonal tests determined the optimal agroinfiltration as: an acetosyringone concentration of 0.25 mM, an OD_{600}=0.6 and harvesting 5 days after infiltration.

Key words: Agroinfiltration, codon optimization, *Cucumis melo* L., intron, nattokinase.

Nattokinase (NK, Subtilisin NAT), an alkaline serine protease produced by *Bacillus subtilis* var. *natto*, was first found in a Japanese soybean fermented food—natto (Sumi et al. 1987). The enzyme is encoded by the *aprN* gene, which has an open reading frame of 1143 nucleotides (Nakamura et al. 1992). NK promotes thrombolysis by not only directly lysing thrombi *in vivo* (Sumi et al. 1990), but also by changing plasminogen to plasmin by increasing the production of thrombi-dissolving agents, such as tissue-type plasminogen activator (t-PA) and urokinase (Hara et al. 1996; Yuki et al. 1994), or by inactivating plasminogen activator inhibitor type1 (Urano et al. 2001). After oral administration, NK is stable in the gastrointestinal tract, can enter into the blood rapidly, and the high fibrinolytic activity is retained for more than 3 h (Tai and Sweet 2006). Additionally, NK is safe, convenient, low-cost, efficacious, and shows a preventive effect (Jovin and Müller-Berghaus 2004). These advantages made defined NK as a novel oral thrombolytic agent to prevent and treat cardiovascular diseases.

Plants have considerable potential for the mass production of recombinant pharmaceutical proteins, such as antibodies and enzymes. They provide safe and low-cost recombinant proteins, are easily transformed, and heterologous proteins can fold correctly to receive post-translational modifications (Fisher and Emans 2000; Giddings et al. 2004). Melon is one of the important economic crops and is cultivated worldwide. It has been a model plant for fruit ripening study (Ezura and Owino 2008) and will be a potential candidate crop for the production of oral proteins because of its edible character. Compared with stable transformation, transient expression provides a rapid method to assay the possible levels of transgene expression, promoter analysis and protein production (Wroblewski et al. 2005). In this study, we report the transient expression of a codon-optimized synthetic NK gene (*sNK*) in melon fruit. Using optimized agroinfiltration conditions, *sNK* interrupted with a plant intron and under the control of fruit-specific E8 gene promoter showed the highest expression level when detected by quantitative real-time reverse transcription-PCR (qRT-PCR) or by fibrinolytic activity assays.

The low level expression of exogenous genes in transgenic plants is a major factor restricting the large-

Abbreviations: NK, Nattokinase; *sNK*, Synthetic NK gene; *sNKi*, Synthetic NK gene with the first intron of the E8 gene inserted in; qRT-PCR, Quantitative real-time reverse transcription PCR.

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scale application of plants as bioreactors. Codon optimization is used frequently to improve the expression level of foreign genes in plants, such as LT-B (Mason et al. 1998), human lysozyme (Huang et al. 2002) and CTB (Kang et al. 2004). Here, we redesigned the coding sequence of the NK gene (GenBank ID: S51909.1) from *Bacillus subtilis* var. *natto* according to the codon usage frequency in plants, as displayed in the Codon Usage Database (http://www.kazusa.or.jp/codon), replacing rarely used codons with frequently used ones, usually by changing A/T at the codon position three to G/C; avoiding rare codons in plants, such as XCG and XTA; removing A/T rich sequences, such as AAT AAT, AAT TAA and AAC CAA, which are regarded as plant polyadenylation signals; and consecutive A/T strings (>5). The GTG initiation codon was replaced with the ATG triplet, and the three consecutive stop codons were changed to TAA TGA TAA. After modification, the overall G+C content in the *sNK* gene increased, but the sequence of the mature NK produced in plant cells was not altered. The new gene, named *sNK* (GenBank ID: KJ495732), with full-length of 1152 bp, was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and cloned into plasmid pUC57. The Kozak sequence "GCC ACC", a sequence at the end of the 5′ cap structure of eukaryotic mRNAs, may enhance the efficiency of transcription and translation of eukaryotic genes, was introduced upstream of the start codon (Kozak 1984). Thus the *sNK* gene would be translated more efficiently than the native gene and give high level expression in plants. A *Bam*HI restriction site were introduced at the 5′-end of the *sNK* gene and a *Sac*I restriction site was introduced at the 3′-end, to provide convenient restriction sites for cloning into the plant expression vector.

Overlap extension PCR can splice together fragments from different sources and is widely used to construct mutant genes, fusion genes, long fragment genes and for gene knockout (Kuwayama et al. 2002). We used this technique successfully to insert the first intron of the tomato E8 gene, which is specifically expressed in tomato fruit, into the *sNK* gene. The first intron of the E8 gene from tomato (*Lycopersicon esculentum* cv. Micro-Tom) is 423 bp-long and was amplified by PCR using primers P3 5′-GTA GTT CTC ACG GCA CAC AT GTA AGT ATT TTC ATT TCT TAG TCT GGA GAT TC-3′ and P4 5′-TGA GAG CGG CAA TAG TAC CTG CGAC CTG CAT TGA AAC ATG ACA TAT TTT TAT AT-3′ (the sequences overlapping the *sNK* gene are underlined). Total DNA extracted from a Micro-Tom tomato was used as the template. Overlap extension PCR was used to insert the intron into the *sNK* gene between nucleotides 519 and 520, which is the same position of the intron in the E8 gene. Two pairs of primers were designed to amplify the *sNK* gene in two fragments. Primer P1 5′-TAG GAT CCG CCA CCA TGA GG-3′ and P2 5′-TAAGAAATGAAAATACTTAC ATG TGC CGT GAG AAC TAC CG-3′, were used to amplify the preceding 519-bp fragment of *sNK*, P5 5′-AAA AAT ATG TCA TGT TTC AAT GCAG GTC GCA GGT ACT ATT GCC GCT-3′ and P6 5′-CAG AGC TCT TAT CAT TAC TGA GCG GC-3′ were used to amplify the following 633-bp fragment of *sNK*, respectively. In primer P2 and P5, the overlapping sequences of the E8 intron 1 are underlined. The overlapping sequence was at least 20 bp to ensure correct splicing of the subsequent amplification product. Prime STAR HS DNA Polymerase (Takara Bio Inc., Dalian, China) performed the PCR reactions. After the three DNA fragments were amplified, the 519-bp fragment of *sNK* and E8 intron 1 were fused together and used as the template to amplify ligation product, using P1 and P4 as primers. Finally, the 633-bp fragment of *sNK* was connected to sNK-intron1 fusion product to amplify full-length *sNKi* gene, used P1 and P6 as primers, which was cloned into the *pEASY*™-Blunt vector (Beijing TransGen Biotech Co., Ltd.), and sequenced by Sangon Biotech using universal M13 (+/−) primers. The sequencing results confirmed that correct splicing had occurred.

The plant expression vectors were constructed as below. The 1.1 Kb fruit-specific E8 promoter was amplified from *Lycopersicon esculentum* cv. Micro Tom using forward primer E8P1 5′-GGC AAG CTT CTA GAA GGA ATT TCA CGA AAT-3′ and a reverse primer E8P2 5′-GC GGA TCC CTT CTT TTG CAC TGT GAA TGA-3′ (the restriction sites *Hin*dIII and *Bam*HI are underlined), double digested with *Hin*dIII and *Bam*HI, and cloned into vector pPZP221 (Hajdukiewicz et al. 1994), which was predigested with *Hin*dIII and *Bam*HI. Plant binary expression vectors pPZP35S, pPZP35SN, pPZP35SNi, pPZPE8, pPZPE8N and pPZPE8Ni used in our study were assembled based on the pPZP221 vector. The *sNK* gene and *sNKi* gene were double digested with *Bam*HI and *Sac*I and cloned under control of either the cauliflower mosaic virus (CaMV) 35S promoter or the E8 promoter to construct vectors pPZP35SN, pPZPE8N, pPZP35SNi and pPZPE8Ni, respectively. Vectors pPZP35S and pPZPE8 were also constructed as the empty controls. The T-DNA regions of the six expression vectors are shown in Figure 1. All the vectors were transferred into *Agrobacterium tumefaciens* LBA4404 by the freeze-thaw method, and subsequently the *Agrobacterium* were used for agroinfiltration into melon fruit.

Growth and induction of agrobacterium was carried out according to the method of Orzaez et al. (2006). Five milliliters overnight grown cultures of agrobacterium LBA4404 were transfered to 50 ml induction medium [YEB medium plus 5 g l^{-1} sucrose,

0.02 mM acetosyringone, 10 mM 2-(*N*-morpholino) ethanesulphonic acid (MES), 100 mg l^{-1} rifampicin and 100 mg l^{-1} spectinomycin] and grown again overnight. When the optical density (OD_{600})=1.0, the cultures were centrifuged (5000 rpm×10 min) and resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES, 0.2 mM acetosyingone, pH 5.6) to make OD_{600}=0.9. The infiltration solutions were incubated at room temperature with gentle agitation (20 rpm) for at least 2 h.

Melon (*Cucumis melo* L. cv Hetao) planted in the field, was artificially self-pollinated at anthesis time, and tags were hung to mark pollination time as the start of the fruit development. Early-mature melon fruit [29–34 days after self-pollination (DAP)], the average size of which was about 116.17×136.32 mm, and mid-mature melon fruit (35–39 DAP), the average size of which was about 116.98×136.50 mm, were infiltrated using a sterile 1 ml syringe with a 0.7×30 mm needle. The needle was obliquely inserted into the mesocarp of melon fruit in the equatorial plane, and the infiltration solution was slowly pushed into the fruit while pulling out needle from it. Each fruit was injected with 1 ml of infiltration solution.

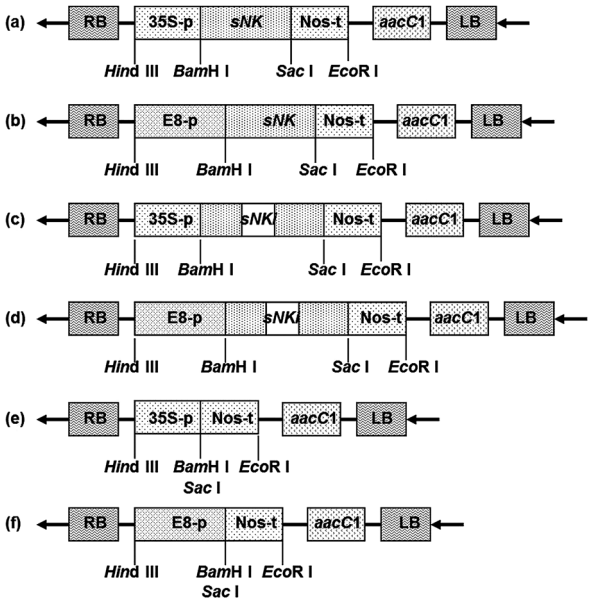

Figure 1. T-DNA regions of plant expression vectors. (a) pPZP35SN, (b) pPZPE8N, (c) pPZP35SNi, (d) pPZPE8Ni, (e) pPZP35S and (f) pPZPE8. 35S-p, CaMV 35S promoter; E8-p, fruit-specific E8 promoter from tomato; *sNK*, synthetic NK gene; *sNKi*, synthetic NK gene with the first intron of the E8 gene inserted in; nos-t, nopaline synthase gene terminator; *aacC*1, gentamycin acetyltransferase gene; RB, right border; LB, left border.

An orthogonal test was performed to determine the optimal conditions for agroinfiltration into melon fruit. The infiltration solution of agrobacterium LBA4404 harboring vector pPZP35SN was prepared according to the L9 (3^4) orthogonal test program (Table 1). Three factors were chosen and each factor had three levels as low, medium and high: acetosyringone concentration (0.2 mM, 0.25 mM, and 0.3 mM); OD_{600} (0.3, 0.6 and 0.9); fruit harvest time [3 days after infiltration (DAI), 5 DAI and 7 DAI]. The melon fruits at 3, 5 and 7 DAI were harvested, and RNA samples from 0.2 g mesocarp around the injection hole were prepared using RNAiso Plus (Total RNA) Kit (Takara). cDNA was reverse transcribed from 500 ng RNA using a *PrimeScript*™ RT Master Mix Kit (Takara). Reverse transcription reaction as follows: 37°C incubated for 15 min, 85°C for 5 s to inactivate the reverse transcriptase. The cDNA then diluted 5-fold and used as PCR template, with primers NKQP1 5′-CAC ATT ACC AGG AGG GAC TTA CG-3′ and NKQP2 5′-GCA GTG CTT TCC AAC CTA TCT CT-3′, producing a 141-bp fragment that was mixed with SYBR® *Premix Ex Taq*™ II Kit (TaKaRa) for quantitative real-time reverse transcription PCR (qRT-PCR) testing. A melon GAPDH 136-bp fragment (CGQP1 5′-AAA GAC TGG AGA GGT GGA AGA GC-3′, CGQP2 5′-CAA CGG TAG GAA CAC GGA AAG-3′) was used as an internal standard. The Option 3 Real-time PCR system (Bio-Rad) amplified the sequences and the MJ Option Monitor Analysis software analyzed the sequences. The PCR analysis condition was: denaturation at 95°C for 10 s, followed by 39 amplification cycles of denaturation at 95°C for 5 s and annealing and extension at 60°C for 20 s while doing melting curve analysis. Using the method $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001) for data analysis, gene expression of non-infiltrated melon fruit was set at 1. The Ct mean and standard deviation were

Table 1. The program of orthogonal test

Test No.	Factors		
	AS concentration (mM)	Agrobacteria concentration (OD_{600})	Fruit harvest time (days after infiltration)
1	0.2	0.3	3
2	0.2	0.6	5
3	0.2	0.9	7
4	0.25	0.3	5
5	0.25	0.6	7
6	0.25	0.9	3
7	0.3	0.3	7
8	0.3	0.6	3
9	0.3	0.9	5

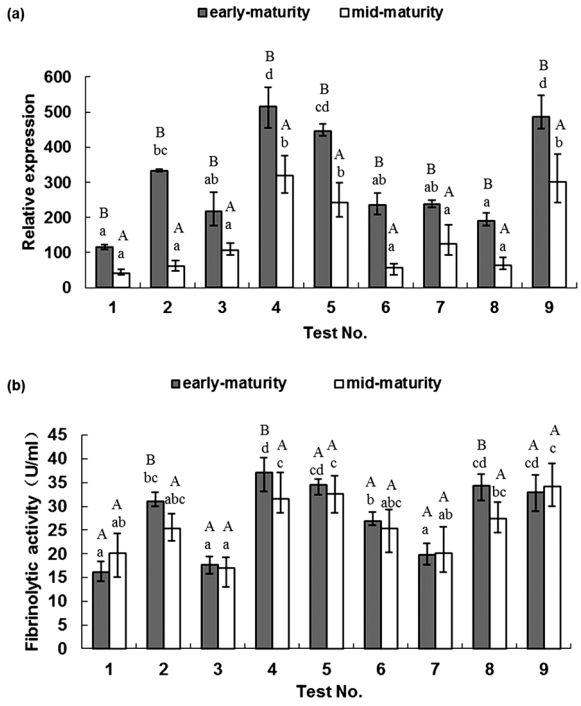

Figure 2. Transient expression analysis of the *sNK* gene in melon fruit in an orthogonal test. (a) Expression of *sNK* gene by quantitative real-time reverse transcription PCR (qRT-PCR). Using the method $2^{-\Delta\Delta CT}$ for data analysis, gene expression of non-infiltrated melon fruit was set at 1. (b) Fibrinolytic activity assay. Fibrinolytic activity was determined according to the area of the lysis zone which linearly related to the urokinase units of fibrinolytic activity. The results are presented as the means of three independent determinations ±SDs. Results for *sNK* gene expression in each test group, on different maturity stage, significantly different from each other ($p \leq 0.05$) if the upper case letter on the top of the columns is not the same. Results for *sNK* gene expression in 9 test groups, significantly different from each other ($p \leq 0.05$) if the lower case letter on the top of the columns is not the same.

calculated from three biological and three technical replicates. The statistical analysis was performed using the software SPSS17.0. qRT-PCR analysis results showed that tests number 4, 5 and 9 produced higher levels of expression of nattokinase (Figure 2a). The levels of transient expression of nattokinase in early-mature melon fruit were higher than in mid-mature one (ANOVA, $p \leq 0.05$). The software Orthogonality Experiment Assistant (Sharetop Software Studio. Copyright 2001–2002) was used to measure the optimal conditions with reference to the level of gene expression. The optimal level of three factors were that an AS concentration of 0.25 mM, an $OD_{600}=0.6$ and a time of harvest of 5 DAI.

Crude nattokinase samples were prepared as follows: 0.2 g mesocarp around the injection hole of each melon fruit mentioned above was ground in liquid nitrogen, 0.4 ml phosphate-buffered saline (PBS, pH 7.4) was added and placed at 4°C for 4 h after mixing. The samples were then centrifuged (12000 rpm×30 min) at 4°C and the supernatants were used as crude enzyme samples. The fibrin plate method (Astrup and Müllertz 1952), with some modifications, was used to determine the fibrinolytic activity, using urokinase as a standard. Briefly, 10 ml 0.15% (w/v) of bovine fibrinogen solution (in barbital buffer, pH 7.8), and 10 ml 1% (w/v) agarose solution were added to 0.15 ml 100 U ml^{-1} thrombin solution and mixed in a 90-mm petri dish. Holes with 2 mm diameter were made on the fibrin plate and 10 μl of each crude enzyme sample was then added. The plate was incubated at 37°C for 18 h. Two perpendicular diameters of the lysis zone on the fibrin plate were measured, and the fibrinolytic activity was determined according to the standard curve of urokinase (the area of the lysis zone was linearly related to the urokinase units of fibrinolytic activity in the range of 0 to 100 IU ml^{-1}) Urokinase, fibrinogen and thrombin were purchased from Sigma. The results are presented as the means of three independent determinations ±SDs. Fibrinolytic activity assay results showed that tests number 4, 5, 8 and 9 produced higher levels of expression of nattokinase (Figure 2b). The fibrinolytic activity in most of the test groups (Nos. 2–6 and 8) in early-mature melon fruits were higher than in mid-mature ones, but the difference was not significant ($p>0.05$), which had the same tendency as the expression of RNA. The fibrinolytic activity in three groups (Nos. 1, 7, 9) in mid-mature melon fruits were slightly higher than that in early-mature ones, which showed the opposite trend to the expression of RNA, but there was no significant difference between each other ($p>0.05$). The inconsistency could due to the difference of physical and biochemical status between early-mature stage and mid-mature stage. Melon (*Cucumis melo* L. cv Hetao) is a climacteric fruit with short ripening period in which many ripening related genes are expressed. In early-mature stage, gene expression is given priority to transcription, but in mid-mature stage, translation and post-translational modification may be dominant which could express high fibrinolytic activity.

The promoter is the key regulatory element in transcription, and plays an important role in achieving high-level expression of a gene. Unlike constitutive promoters, such as the CaMV 35S promoter and the ubiquitin promoter, tissue-specific promoters control gene expression in specific tissue or at specific developmental stages. Thus a transgenic product driven by such a promoter will concentrate in certain organs, like seeds or fruits, to limit any possible negative effects on plant growth and improve the harvesting efficiency. The fruit-specific E8 promoter from tomato has been used for the expression of Cholera toxin B protein (Jiang et al. 2007). In this study, the expressions of the *sNK* gene controlled by 35S promoter and E8 promoter were different (Figure 3a, b). qRT-PCR analysis and fibrinolytic activity both indicated that the expression

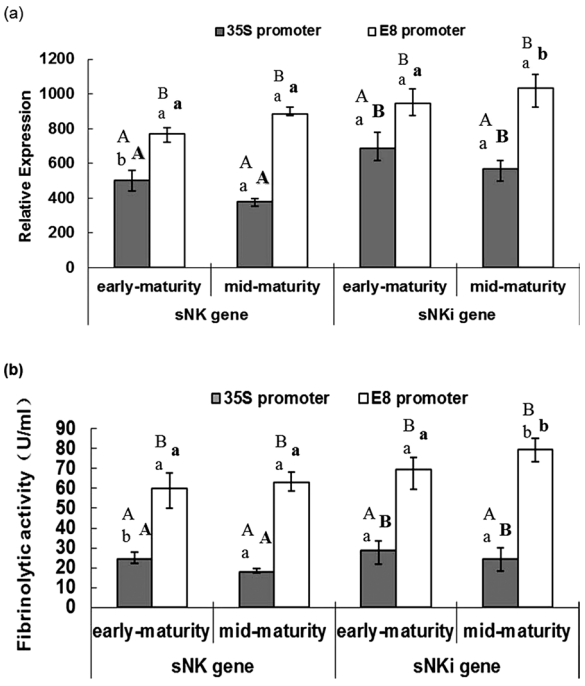

Figure 3. Transient expression of the *sNK* gene and *sNKi* gene in different melon fruit at different stages of maturity under the control of 35S promoter or E8 promoter. (a) qRT-PCR analysis. (b) Fibrinolytic activity assay. The results, means of three replicates, were compared using LSD test. Results for 35S promoter and E8 promoter controlled *sNK* or *sNKi* gene expression, significantly different from each other ($p \leq 0.05$) if the upper case letter on the top of the columns is not the same. Results for *sNK* or *sNKi* gene expression, on different maturity stage, do not differ from each other ($p > 0.05$) if the lower case letter on the top of the columns is the same. Results for *sNK* and *sNKi* gene expression, under the control of 35S promoter, significantly different from each other ($p \leq 0.05$) if the bold upper case letter on the top of the columns is not the same. Results for *sNK* and *sNKi* gene expression, under the control of E8 promoter, significantly different from each other ($p \leq 0.05$) if the bold lower case letter on the top of the columns is not the same.

of the gene in melon fruit infiltrated by agrobacterium harboring vectors pPZPE8N and pPZPE8Ni was higher than those harboring vectors pPZP35SN and pPZP35SNi (significant test by LSD, $p \leq 0.05$). The maximum fibrinolytic activity expressed by the *sNKi* gene under the control of the E8 promoter reached 79.30 U ml^{-1}, while that controlled by the 35S promoter is only reached 24.37 U ml^{-1}. Similar results were obtained for the expression of the *sNK* gene. The fruit-specific E8 promoter enhanced the nattokinase expression level in mid-mature of melon fruit, while the constitutive 35S promoter had not the same effect.

Introns play an important role in the regulation of gene expression, such as maintaining the structure of specific chromosomes and the function of specific genes (Niu and Yang 2011). Introns, therefore, have become important elements to improve the expression of foreign genes. In this case, the expression level of *sNKi* gene, which was interrupted with an intron from the E8 gene of tomato, was higher than that of the *sNK* gene whether under the control of 35S promoter or E8 promoter, the average enhanced extent was 27.42% (Figure 3a, b). Significant test by LSD, $p \leq 0.05$. This proved that an intron can improve the expression level of *sNK* genes in melon fruit. In monocotyledonous plants, the enhanced extent was much higher. *adh1* and *sh1*, introns of maize, increased the expression level of reporter gene 10–100 fold higher in transgenic maize (Callis et al. 1987; Vasil et al. 1989).

Nowadays, cardiovascular disease has become the second largest disease (only after the cancer disease), which seriously threatened human health and life (Datar et al. 1993). Enzymes such as urokinase (UK) and tissue-type plasminogen activator (t-PA) have been widely used in the treatment of thrombosis. However, compared with these enzymes, nattokinase, a new thrombolytic enzyme from the traditional fermented food—natto, has advantages in several aspects. Firstly, NK with oral administration was more security than injections. Secondly, NK is a single-chain protein and the molecular weight is far less than UK or t-PA, so it can easily absorbed by the body (Nakajima et al. 1993). Thirdly, the fibrinolic effect of NK can last for 8 to 12 h, longer than other thrombolytic enzymes (Milner and Makise 2002). Nattokinase has the potential to become a new oral thrombolytic agents in the treatment and prevention of thrombosis, thus it needs more inexpensive production system.

The current expression system of NK was mostly prokaryotic secretory expression system. NK was overexpressed in *Escherichia coli* as an insoluble recombinant protein linked to the C terminus of oleosin by an intein fragment to form a artificial oil (Chiang et al. 2005). Chen et al. (2010) constructed a chromosomally located T7 expression system in *Bacillus subtilis* and the secretion of NK up to 10860 CU ml^{-1}. Li et al. (2007) expressed the active NK authentically in *Spodoptera frugiperda* cells with the modified Bac-to-Bac system and the recombinant NK retained fibrinolytic activity of 60 U ml^{-1}.

The use of transgenic plants for the large-scale production of heterologous proteins is gaining worldwide recognition. Plant tissues such as leaves, fruits, seeds and vegetables are also used as vehicles for the edible vaccines (Mishra et al. 2008), which can avoid downstream processing to purify and deliver the vaccines.

In this study, A modified NK gene based on the codon usage of plants was transient expressed in melon fruit. The highest fibrinolytic activity was evaluated as 79.30 U ml^{-1} while the fruit-specific E8 promoter and plant intron both play important roles in the expression of *sNK* gene.

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