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Construction and functional analysis of nattokinase-producing cucumber obtained by the CRISPR-Cas9 system

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ABSTRACT

Nattokinase (NK) is effective in the prevention and treatment of cardiovascular disease. Cucumber is rich in nutrients with low sugar content and is safe for consumption. The aim of this study was to construct a therapeutic cucumber that can express NK, which can prevent and alleviate cardiovascular diseases by consumption. Because the *Bitter fruit* (*Bt*) gene contributes to bitter taste but has no obvious effect on the growth and development of cucumber, so the NK-producing cucumber was constructed by replacing the *Bt* gene with *NK* by using CRISPR/Cas9. The pZHY988-Cas9-sgRNA and pX6-LHA-U6-NK-T-RHA vectors were constructed and transformed into *Agrobacterium tumefaciens* EHA105, which was transformed into cucumber by floral dip method. The crude extract of NK-producing cucumber had significant thrombolytic activity *in vitro*. In addition, treatment with the crude extract significantly delayed thrombus tail appearance, and the thrombin time of mice was much longer than that of normal mice. The degrees of coagulation and blood viscosity as well as hemorheological properties improved significantly after crude extract treatment. These findings show that NK-producing cucumber can effectively alleviate thrombosis and improve blood biochemical parameters, providing a new direction for diet therapy against cardiovascular diseases.

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KEYWORDS

cardiovascular diseases, CRISPR/Cas9, nattokinase, cucumber, *Bt* gene

1. INTRODUCTION

Cardiovascular diseases (CVDs), encompassing a wide range of disorders including coronary heart disease, hypertension, cardiac hypertrophy, and heart failure, are the main cause of death and disability worldwide (Wu et al., 2020). Nattokinase (NK), a serine protease secreted by *Bacillus subtilis* var. *natto* found in the Japanese traditional food natto (Suzuki et al., 2022), possesses a variety of key beneficial effects on cardiovascular system due to its strong anti-thrombus and thrombolytic activity (Wu et al., 2019). However, the smell and viscous texture of natto are not accepted by most people, and other components in natto limit the function of NK (Nishinari et al., 2018; Liu et al., 2021).

The expression of recombinant proteins in plant systems has been promoted as a cost-effective production platform (Schillberg et al., 2019), e.g., the production of tissue plasminogen activator (Abdoli Nasab et al., 2016), LTB-VP6 (Jin et al., 2022), and HBc-based virus-like particles (Moradi Vahdat et al., 2021). Cucumber is a common economic crop plant grown around the world (Rajab et al., 2023). Because cucumber is rich in vitamins, protein, inorganic salt and low in sugar (Liu et al., 2022), it is safe for patients with CVDs. The *Bt* gene plays an important role in the formation of bitterness in cucumber (Shang et al., 2014). The knockout of *Bt* has no effect on the growth and development of cucumber and could improve fruit texture. CRISPR/Cas9-mediated gene editing has been successfully applied to bacterial, yeast, plant, and mammalian cells (Schmidt et al., 2023). This approach can be used to edit the genome at a fixed point, avoiding the destruction of endogenous genes caused by the random insertion (Wen and Zhang, 2022). Therefore, the *NK* gene was inserted into the cucumber genome by using CRISPR/Cas9 to stably express NK and evaluate its physiological function. This study is expected to provide a new strategy for the adjuvant prevention and treatment of CVDs.

2. MATERIALS AND METHODS

2.1. pZHY988-Cas9-sgRNA vector preparation

According to the sequence of the cucumber *Bt* gene, single-guide RNAs (sgRNAs) were designed using BioTools (http://biootools.com/). The *Bsa*I site was added to the sgRNA and two complementary sgRNA oligos were synthesised. pZHY988-Cas9 vector was linearised by *Bsa*I (Takara, Japan). The sgRNA was cloned into the linearised pZHY988-Cas9 vector under the action of T4 DNA ligase (Takara, Japan).

2.2. Point mutation in the *NK* gene

B. subtilis natto (BNCC 185324) was used to obtain the *NK* gene. Since the start codon of *NK* in the *B. subtilis natto* genome is GTG, it must be replaced with ATG for expression in eukaryotes. Therefore, the primers *NK-M1* and *NK-M2* were designed to mutate the start codon of *NK*.

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2.3. Construction of the PX6-LHA-U6-NK-T-RHA vector

The donor gene *LHA-U6-NK-T-RHA* was constructed by overlap PCR and connected to the pMD-19T vector. All primers used in this study are shown in Supplementary Table 1. (Supplementary files are available on the server of the Publisher). The LHA-U6-NK-T-RHA and (Marker-free) PX6 vectors were digested by *Xho*I and *Pac*I. The purified *LHA-U6-NK-T-RHA* and the linearised pX6 fragments were ligated by T4 DNA ligase.

2.4. Transformed pZHY988-Cas9-sgRNA and pX6-LHA-U6-NK-T-RHA vectors into *Agrobacterium tumefaciens* EHA105

The freezing and thawing method was used to transfer PZHY988-Cas9-sgRNA and pX6-LHA-U6-NK-T-RHA vectors into *Agrobacterium tumefaciens* EHA105. The transformed cells were cultured on LB solid medium containing 50 mg mL^{-1} rifampicin and 50 mg mL^{-1} kanamycin at 28 °C for 48 h. The transformants were randomly selected for PCR identification.

2.5. Genetic transformation of cucumber by the floral dip method

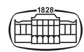
Cucumber seedlings were quickly obtained by soaking the South China D9302 seeds in water (1:4, v/v) at 55 °C and stirring continuously. All female flowers were selected as genetic transformation receptors for the transformation. Female flowers 5–6 days before bloom were selected, and the *A. tumefaciens* EHA105 infection solution was dipped on the bud and ovary of these female flowers. It was ensured that the smear amount was large enough to drop the infection solution. The procedure was repeated again after 3 days. One day before flowering, the male flowers and infected female flowers were bagged and isolated, and the artificial pollination was conducted the next day. Cucumbers were picked and seeds were collected when the cucumbers were ripe (T0). Primers were designed according to the target sequences for PCR detection. The positive control (CT) was the PCR product obtained using pZHY988-Cas9 as template, and the negative control (WT) was the PCR product obtained using the wild-type cucumber genome as the template. The positive transgenic cucumber seeds of T0 were continuously planted according to the above method and T1 fruits were obtained for functional analyses.

2.6. Extraction and analysis of the crude extract of NK-producing cucumber

The fresh cucumber was cut into small pieces and ground into powder. The powder was mixed with PBS and centrifuged at 12,000 r.p.m. at 4 °C for 30 min. NK produced by cucumber was purified by hydrophobic chromatography and gel-filtration chromatography. The crude extract was precipitated using 60% $(NH4)_2SO_4$ and collected by centrifugation. Then, the crude extract was passed in PBS through a Sephadex G-75 column (Yuanye, Shanghai, China). Finally, the crude extract was transferred into a dialysis bag (molecular weight 8,000–14,000 Da, Yuanye, China) and put into distilled water at 4 °C for 24 h. The samples after dialysis were freeze-dried and subjected to SDS-PAGE to evaluate the purity. The activity of the crude extract was detected using the Botany Nattokinase ELISA Kit (Xinyu Biology, Shanghai, China) according to the manufacturer's instructions.

2.7. Thrombolytic effects of the crude extract of NK-producing cucumber *in vitro*

The thrombolytic effect of NK was measured as described previously (Choi et al., 2014). Fresh blood of a pig was allowed to clot spontaneously. The blood clot was added to the crude extract



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solution with different concentrations and incubated in a thermostat water bath for 5 h or 10 h at 60 r.p.m. and 37 °C. Normal saline was used as the control group. The blood clot was removed from the water bath and weighed. Dissolution of blood clot in each sample was calculated as follows:

$$\text{Dissolution}/\% = \frac{m_a - m_b}{m_a} \times 100 \quad (1)$$

Note: m_a is the mass before dissolution/g; m_b is the mass after dissolution/g.

2.8. Thrombus induction and NK-producing cucumber crude extract treatment *in vivo*

Male Kunming (KM) mice, 4 weeks of age, specific pathogen-free, were purchased from the Laboratory Animal Center of Xuzhou Medical University. After acclimatised for 1 week, the KM mice were randomly divided into four groups: (1) normal control (NC, $n = 5$), normal mice treated with saline, (2) negative group (NG, $n = 5$), intraperitoneal injection of carrageenan (50 mg kg^{-1}) and treated with saline, (3) positive group (PG, $n = 5$), intraperitoneal injection of carrageenan (50 mg kg^{-1}) and treated with nattokinase (8,000 FU kg^{-1}), and (4) cucumber treatment group (CG, $n = 5$), intraperitoneal injection of carrageenan (50 mg kg^{-1}) and treated with crude extract (8,000 IU kg^{-1}). The time of appearance and length of the thrombus tail within 24 h were recorded. The blood was collected and the thrombin time was measured after 48 h of treatment. Fibrinogen was measured using ELISA kits (TIANGEN, Beijing, China) according to the manufacturer's instructions. The mouse tail was cut and embedded in paraffin. HE staining was used to assess the anti-thrombotic ability of the crude extract. The animal study protocol was approved by the Experimental Animal Ethics Committee of Xuzhou Medical University (approval number: L20210226457).

2.9. Statistical analysis

Values are presented as means ± standard deviation (SD). The data analysis was performed using SPSS program version 17. Student's *t*-test was used to determine differences between two groups. Multiple groups were compared by ANOVA with Dunnett's pair-wise comparisons. A value of $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. pZHY988-Cas9-sgRNA vector construction

According to the cucumber *Bt* gene, 12 potential sgRNAs were designed. Considering the target site on the CDS of the *Bt* gene, the second sgRNA was selected for further analyses (Supplementary Table 2). The *Bsa*I site was added to both ends of sgRNA by oligo synthesis. The pZHY988-Cas9 vector and sgRNA were simultaneously digested with *Bsa*I and then connected with T4 DNA ligase. Colony PCR revealed that the length of amplification product was consistent with the expected fragment size (Fig. 1a). Sequencing results also supported the successful construction of the pZHY988-Cas9-sgRNA vector (Fig. 1b).

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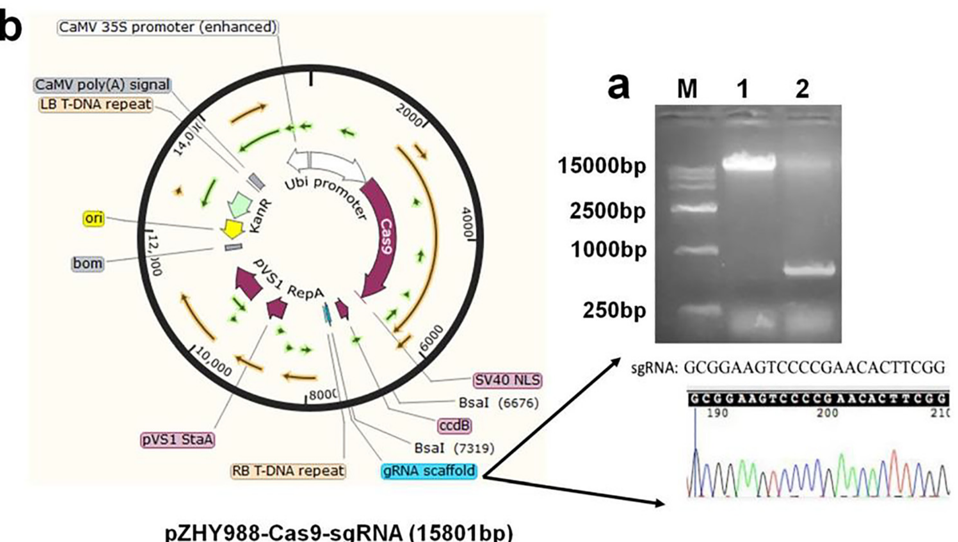

Fig. 1. Construction of the pZHY988-Cas9-sgRNA vector. a. Colony PCR of the pZHY988-Cas9-sgRNA vector. Lane M: DL15000 DNA marker; Lane 1: plasmid of pZHY988-Cas9-sgRNA; Lane 2: Colony PCR products to verify pZHY988-Cas9-sgRNA construction. b. Diagram of the pZHY988-Cas9-sgRNA vector

3.2. Point mutation in the *NK* gene

A single colony was randomly selected and genomic DNAs were extracted for PCR detection. The PCR product lengths were consistent with the length of the *NK* gene (1146 bp) (Supplementary Fig. 1a). The positive colonies were sequenced, and the sequences were consistent with the *NK* sequence obtained in a search against the NCBI database. The initiation codon *GTG* was successfully mutated to *ATG* (Supplementary Fig. 1b).

3.3. Construction of the pX6-LHA-U6-NK-T-RHA vector

Overlap PCR results showed that the sizes of *LHA-U6* (557 bp), *T-RHA* (580 bp), *LHA-U6-NK* (1702 bp), and *LHA-U6-NK-T-RHA* (2282 bp) were consistent with the expected sizes (Supplementary Fig. 2). The purified *LA-U6-NK-T-RA* and linearised pX6 fragments were digested by *Xho*I and *Pac*I and ligated by T4 DNA ligase. LHA-U6-NK-T-RHA and pX6 were successfully connected, as determined by *Xho*I and *Pac*I digestion (Fig. 2a). The PCR products of full-length *LA-U6-NK-T-RHA* (2282 bp) were also verified by agarose gel electrophoresis (Fig. 2b).

3.4. Transformation of pZHY988-Cas9-sgRNA and pX6-LHA-U6-NK-T-RHA vectors into *A. tumefaciens* EHA105

Transformants showing double resistance were randomly selected for colony PCR detection. The lengths of the PCR products were nearly 400 bp and 2600 bp, consistent with the expected

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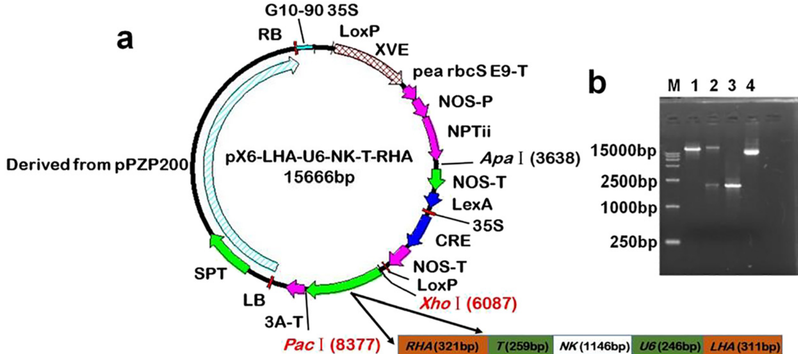

Fig. 2. Construction of the vector of pX6-LHA-U6-NK-T-RHA. a. Diagram of the pX6-LHA-U6-NK-T-RHA vector. b. Verification of the pX6-LHA-U6-NK-T-RHA vector. Lane M: DL15000 DNA marker; Lane 1: Recombinant vector of pX6-LHA-U6-NK-T-RHA; Lane 2: Enzyme digestion of the pX6-LHA-U6-NK-T-RHA vector by *Xho*I and *Pac*I; Lane 3: Colony PCR products to verify pZHY988-Cas9-gRNA construction; Lane 4. pX6 vector

lengths (Supplementary Fig. 3). These results indicate that the pZHY988-Cas9-sgRNA and pX6-LHA-U6-NK-T-RHA vectors were successfully transformed into *A. tumefaciens* EHA105.

3.5. Genetic transformation of cucumber and genotype analysis

The cucumber cultivation and transformation processes are shown in Supplementary Fig. 4. As many female flowers were chosen as possible to improve the success rate of floral dip transformation. The fruits were picked and seeds were harvested when the cucumbers were ripe. A total of 13 cucumber fruits were harvested and 83 cucumber seeds were obtained. All harvested seeds were cultivated, and 68 seedlings survived (T0). Cucumber genomic DNA was extracted and primers were designed according to the Cas9 sequence for PCR verification. As shown in Fig. 3a (Lanes 2, 4, 5, and 6), NK-producing cucumbers were obtained.

3.6. Purification of NK

NK produced by cucumber was purified by hydrophobic chromatography and gel-filtration chromatography. As shown in Fig. 3b, the crude extract yielded many bands after purification by $(NH_4)_2SO_4$ precipitation (Lane 1). The bands representing impurities were reduced after purification by gel-filtration chromatography (Lane 2), and the molecular weight of the target protein was approximately 28 kDa, consistent with that of NK (Fig. 3b).

3.7. Thrombolytic activity of the crude extract of NK-producing cucumber *in vitro*

NK activity of the crude extract was 202.29 ± 30.70 IU mg^{-1}, equivalent to 7.86 ± 1.10 IU g^{-1} (fresh cucumber). Different doses of NK were added to blood clots and dissolution was detected. As shown in Table 1, the thrombolytic effect of the crude extract increased as the concentration increased, and there was significant difference between NK groups and the control group ($P < 0.001$). In addition, with the extension of the reaction time, the dissolution rate for each

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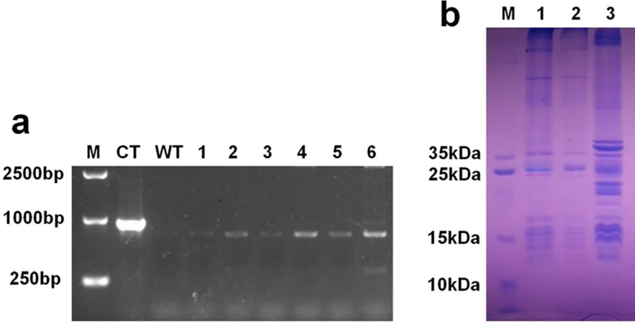

Fig. 3. PCR detection of T0 plants and purification of crude extract. a. Lane M: DL2500bp DNA Marker; Lane CT: PCR products of *NK*; Lane WT: Wild-type cucumber; Lanes 1-6: NK-producing cucumber. b. SDS-PAGE of purified crude extract. Lane M: protein Marker; Lane 1: $(NH4)_2SO_4$ precipitation of the crude extract; Lane 2: chromatography of the crude extract; Lane 3: crude extract, unpurified

Table 1. Thrombolytic effects of the crude extract *of* NK-producing cucumber *in vitro*

Group	Crude extract (mg)	m_a (g)	m_b (g)	Dissolution (%)
Control (5h)	0	0.49 ± 0.015	0.36 ± 0.033	25.69 ± 7.79
Control (10h)	0	0.52 ± 0.033	0.32 ± 0.023	37.17 ± 7.0
Crude extract (5h)	10	0.51 ± 0.042	0.20 ± 0.019	59.51 ± 4.47***
Crude extract (5h)	20	0.48 ± 0.063	0.14 ± 0.016	70.59 ± 5.58***
Crude extract (10h)	10	0.50 ± 0.047	0.19 ± 0.022	62.35 ± 1.52***
Crude extract (10h)	20	0.52 ± 0.072	0.076 ± 0.011	85.05 ± 3.04***

m_a: the mass before dissolution/g; m_b: the mass after dissolution/g; ***: $P < 0.001$ vs. control (5 h) or control (10 h).

concentration increased and was higher than that of the control group ($P < 0.001$). These results showed that the crude extract had significant thrombolytic activity *in vitro*.

3.8. Thrombolytic effects of NK-producing cucumber crude extract *in vivo*

Thrombin activity is an important factor causing thrombosis, and the thrombin time reflects the level of thrombin activity. In this study, the crude extract could significantly delay the appearance of the thrombus tail, and the thrombus length was significantly shorter than that of the control group (2.61 ± 0.40 cm vs. 5.22 ± 0.30 cm, $P < 0.001$) (Fig. 4a). After treatment with the crude extract of NK for 48 h, the thrombin time of mice was much longer than that of normal mice (12.71 ± 0.50 s vs. 7.99 ± 0.31 s, $P < 0.001$) (Fig. 4b). However, there was no significant difference in fibrinogen levels among the groups (Fig. 4c). Histomorphology of the thrombus tail showed that the arterial intimal became thicker, the lumen diameters increased, and the vessels were filled with blood clots in NG group compared with corresponding parameters in the NC group. Consistent with the changes in the PG group, crude extract treatment significantly reduced the degrees of coagulation and blood viscosity, inhibited intimal thickening, and improved hemorheological properties (Fig. 4d).

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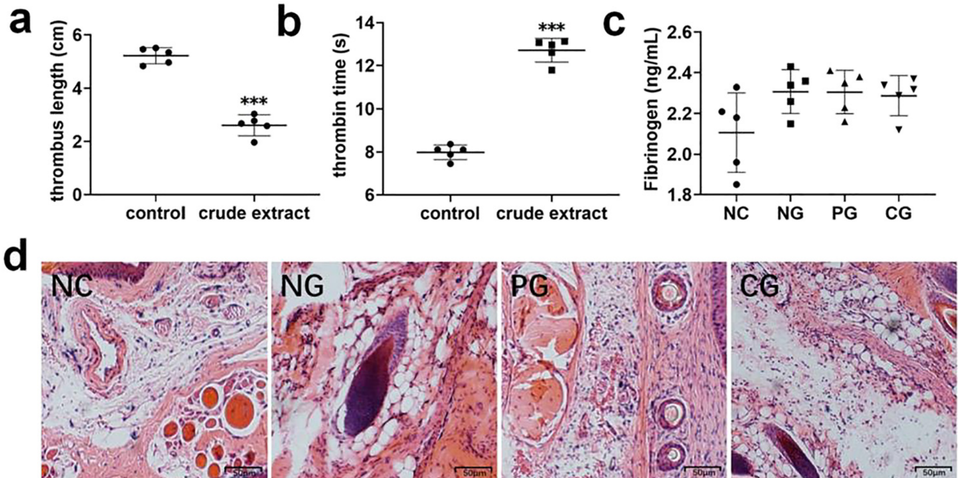

Fig. 4. Effects of the crude extract on the thrombus length (a), thrombin time (b), fibrinogen levels (c), and histopathological characteristics (d) of the thrombus tail. Blood was collected to determine the thrombin time and fibrinogen concentration after treatment with the crude extract of NK for 48 h. The tails of mice were collected for a histopathological analysis after carrageenan injection for 24 h. ***: $P < 0.001$ vs. control. Bars correspond to 50 μm

3.9. Discussion

Nattokinase is a thrombolytic drug with advantages over urokinase, streptokinase, and tissue plasminogen activator, including a low price, high efficiency, and lack of toxicity (Zhou et al., 2021). However, its taste limits its consumption. In this study, an adjuvant treatment scheme for CVD through diet therapy was designed by replacing the *Bt* gene with the *NK* gene by using CRISPR/Cas9, a simple, efficient, and versatile system in cucumber (Figs 1–2 and Supplementary Figs 1–3). Because NK can be delivered orally, cucumber as a bioreactor to produce NK can exert an adjuvant therapeutic effect by direct consumption (Wu et al., 2019). This avoids the difficulty of protein purification in plant bioreactors and the immunogenicity of plant protein injection.

"Off-target" effects are an important limitation of CRISPR/Cas9. However, these effects can be reduced by optimising gRNAs (Aquino-Jarquin, 2021). In this study, 12 sgRNAs that targeted the *Bt* gene of cucumber were designed, of which 9 sgRNAs had a very low probability of "off-target" effects, and one sgRNA targeting the first exon of *Bt* was selected in this study (Supplementary Table 2). After target gene editing using the CRISPR/Cas9 system, the homologous recombination repair system was used to replace *Bt* with *NK*. The homologous recombinant donor genes were constructed by overlap PCR, which is a PCR known to allow assembly of multiple DNA fragments into one construct (Tran et al., 2021). The extension efficiency was better when the left homologous arm was fused to the U6 promoter (*LHA-U6*) and the terminator was fused to the right homologous arm (*T-RHA*). However, the extended fusion efficiencies of larger fragments (*LHA-U6-NK* and *LA-U6-NK-T-RHA*) were reduced, and even the electrophoretic bands of PCR products were dispersed. It is possible that the overlapping region of the fusion gene was not appropriate or the fused DNA had a complex secondary structure.

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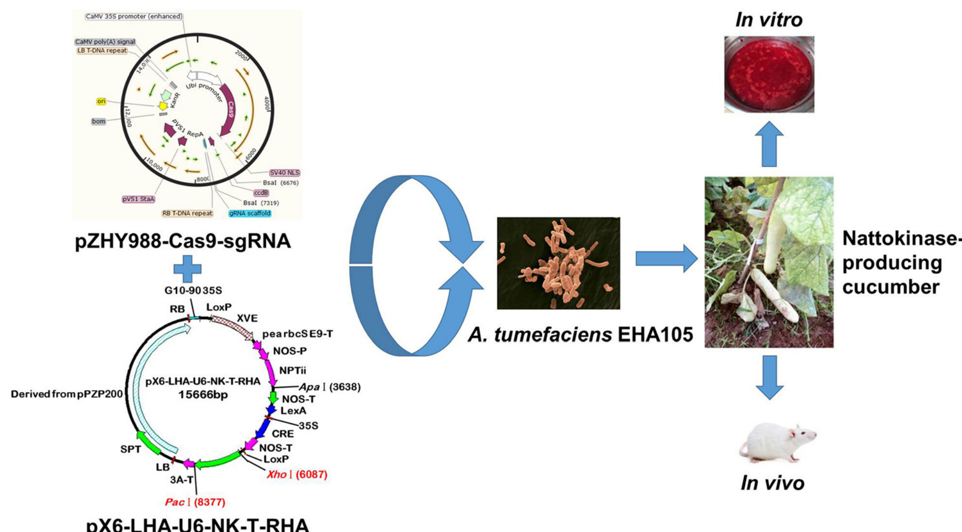

Fig. 5. NK-producing cucumber was constructed using CRISPR/Cas9 and the function of its crude extract was analysed *in vitro* and *in vivo*

The length of homologous arm in eukaryotic cells greatly affects the probability of homologous recombination (Vinette and Petitclerc, 1999). However, the effect of the homologous arm length on homologous recombination efficiency in cucumber has not been reported.

4. CONCLUSIONS

In this study, 12 sgRNAs were designed using BioTools and one sgRNA was selected to connect with pZHY988-Cas9. The donor gene *LHA-U6-NK-T-RHA* was constructed by overlap PCR and connected with pX6. PZHY988-Cas9-sgRNA and pX6-LHA-U6-NK-T-RHA vectors were transformed into *A. tumefaciens* EHA105, and with the transformants the cucumbers were infected by the floral dip method. The crude extract of NK-producing cucumber has both thrombolytic and anti-coagulation functions *in vivo* and *in vitro* (Fig. 5). Therefore, NK-producing cucumber might be an ideal functional food option for the prevention and treatment of thrombosis. However, only one sgRNA for gene editing was obviously insufficient to establish the optimalised CRISPR/Cas9 system. In future studies, we will further examine the remaining sgRNAs to establish a more efficient and specific CRISPR/Cas9 gene editing system.

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SUPPLEMENTARY MATERIALS

Supplementary data to this article can be found online at https://doi.org/10.1556/066.2022.00231.

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