Research Square

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Nuclear localization signal peptides enhance genetic transformation of Dunaliella salina

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Abstract

Dunaliella salina (*D. salina*) expression system shows a very attractive application prospect, but it currently has a technical bottleneck, namely the low or unstable expression of recombinant proteins. Given the characteristics of cell-penetrating peptides (CPPs) or/and nuclear localization signal (NLS) peptides, this study firstly attempted to improve the transformation rate of foreign gene with trans-activating transcriptional (TAT) protein or/and NLS peptides. Using salt gradient method, exogenous plasmids were transferred into *D. salina* cells with TAT or TAT/NLS complexes simultaneously. The β-glucuronidase (GUS) gene expression was identified by means of histochemical stain and RT-qPCR detection. Through observation with light microscope, TAT-mediating cells exhibit an apparent cytotoxicity even at ratios of 0.5, no significant toxicity was noted in the TAT/plasmid/NLS complex group. It is obvious that with the addition of peptides the toxicity decreases significantly. Histochemical staining showed that the transformants presented blue color. under light microscope, but the negative control and blank control are not. Furthermore, based on a TAT/plasmids ratio of 4 with 10 μg NLS peptides mediation, RT-qPCR results demonstrated that the transcripts of target gene were increased by 269 times than that of control group. Collectively, this study demonstrated that combination of TAT and NLS peptides can significantly improve the transformation rate and expression level of foreign gene in *D. salina* system. It offers a promising way for promoting the application and development of *D. salina* bioreactor.

1. Introduction

Currently, *Dunaliella salina* (*D. salina*) system was mainly used to produce the recombinant proteins in many fields, like the human canstatin, shrimp virus VP28 protein, and soybean kunitz trypsin inhibitor etc [1–3]. But the unstable or low expression of products is presently considered a technological bottleneck, associated closely with the transformation process of *D. salina*. Although several transformation approaches have been developed [4, 5], exogenous genes expression in *D. salina* remains unsatisfactory. So, improving the transformation efficiency of target gene in *D. salina* cells is an imperative aspect for current research.

Cell-penetrating peptides (CPPs) can enhance the ability of cellular uptake of various cargoes cross the cytomembrane, and then form complexes or nanoconjugates with DNA molecules that facilitate the insertion of exogenous genes into host genome [6, 7]. And now, they have been widely used in the range of pharmaceutical industry, genetic engineering, and biomedical fields, etc [7–9]. Trans-activating protein (TAT) is derived from the HIV protein trans-activator of transcription, which is the most commonly used and well-understood CPPs. Through covalent or noncovalent attachment to cargo molecules, TAT peptides can efficiently mediate intracellular delivery in a receptor-independent manner [13, 14]. Except that, the nuclear localization signal (NLS) peptides can surmount the nuclear membrane barrier and then deliver DNA to nucleus [15]. The most common form of DNA moleculars binding to NLS peptides or proteins is the electrostatic binding [16]. As to NLS sequences, SV40 large-T antigen is the most extensively studied, especially in the form of PKKKRKV [17]. But the function of NLS to enhance gene

delivery is controversial. Therefore, deep study of NLS function for delivering the target genes is still needed.

So far, CPPs have been applied in transformation of *D. salina*, *P. tricornutum*, *Chlorella vulgaris*, and *C. reinhardtii*, which including types of R9, pVEC, TRA, PEN, and TAT [18–22]. However, genetic transformation of *D salina* mediated by NLS peptides has not yet been studied. To further improve the transformation rate of foreign genes, the combination of NLS peptides and CPPs be firstly explored in this study. Under the various ratio condition, the complexes of TAT/plasmids non-covalently bound to NLS to enhance characteristics of intracellular trafficking performance. After treatment by TAT/plasmids/NLS complexes, their potential cytotoxicities were assessed through observation of cell morphology. In addition, the transformants were identified by means of histochemical staining and RT-qPCR detection.

2. Materials And Methods

2.1 Algal strain and culture conditions

The algal strain was purchased from the Guangyu Biotech Co., Ltd (Wenzhou, China), and cultured in the modified PKS liquid medium [23]. Through 2–3 weeks' culture with the 12h/12h light/day cycle at 26℃, *D. salina* cells were collected by centrifugation when cells grow to the exponential stage. Through three times washing with fresh liquid medium, the final *D. salina* density was adjusted to 10^7-10^8 cells/ml for future use.

2.2 Preparations and detection of different complexes

The pCAMBIA1303 plasmids bear a selective gene (Kan^+) and a report gene (β-glucuronidase, GUS). TAT (YGRKKRRQRRR-NH_2, $C_{64}H_{119}N_{33}O_{13}$) peptides and NLS (PKKKRKV, $C_{40}H_{78}N_{14}O_8$) peptides were synthesized with a purity of 96% from Nanjing Peptide Biotech Co. Ltd (Jiangsu, China). They were diluted to 10 mg/ml with sterile water and kept at -80℃. By mixing particular volume of peptide solution with 1 μg plasmids solution, the TAT/plasmids complexes were prepared at various weight ratios 0.25:1, 0.5:1, 1:1, 2:1, 4:1, 8:1). And then, the mixture were adjusted to a total volume of 20 μl with sterile deionized water. After a brief vortex, the mixtures were incubated for 30 min at 37 ℃ for stable complex formation. On the basis of formation of TAT/plasmids complexes, the NLS peptides (10 μg) are added to the complexes. Mixture was continually incubated for another 15 min and then used for subsequent experiments. The complex stability was assessed by 1% (w/v) agarose gel electrophoresis. After that, the bands were visualized and analyzed by a gel minigel imaging analysis system (Sege, Beijing, China). Meanwhile, the particle size and zeta potential of all complexes were measured in triplicate by a Nano-ZEN3600 zetasizer (Malvern, UK). The pCAMBIA1303 plasmid was used as the positive control in this experiment.

2.3 CPPs mediated the plasmids delivery

CPPs R9 could successfully transport dsRNA into *D. salina* by incubating their complexes [20]. Compared with the R9, transportan, TAT, and penetratin peptides, pVEC indicated a more better performance in transporting exogenous proteins into algal cells [22]. In this paper, we studied the effect of TAT to mediate the plasmids delivery through the salt gradient method [5]. The experimental groups were divided into five groups as follows: plasmid group, TAT/plasmid group, TAT/plasmid/NLS group, negative control group (*D. salina* cells transformed without plasmids), and blank control group (wild type *D. salina* cells). The translocation of complexes into *D. salina* cells was performed following the described method with slight modifications [5]. Briefly, approximately 10 ml exponentially growing *D. salina* cells were harvested by centrifuging at 3000 rpm for 2 min, and then washed twice with fresh liquid medium. Subsequently, 1 ml cell suspension (10^7-10^8 cells/ml) was centrifuged and re-suspended in 200 μl of 0.2 M fresh medium containing complexes of different ratios. The TAT or NLS peptides/plasmids complexes were prepared as previously described. All of the above operations were carried out in 1.5 ml EP tube. Finally, the cells were centrifuged at 3000 rpm for 2 min and cultured for 48–72 h in fresh medium before assaying for expression of GUS genes.

2.4 Cytotoxicity assessment of peptides/plasmids complexes

To determine the cytotoxicity of TAT or NLS peptides, the treated *D. salina* cells with different peptides/plasmids complexes were observed under light microscope. At 24 h after treatment, the number of surviving cells in experimental and blank groups were counted separately. As described by Jefferson et al, histochemical staining was conducted in transformed *D. salina* cells at 48 h after transformation [23].

2.5 RT-qPCR analysis of GUS gene expression

To compare the delivery efficiency of different peptides/plasmids complexes, the real time fluorescent quantitative PCR (RT-qPCR) was carried out as following steps. Firstly, the total RNAs were extracted from *D. salina* cells using trizol reagent (CWbio, China) in different groups. The purity and integrity of RNAs was confirmed by spectrophotometry and 1% agarose gel electrophoresis. For the reverse transcription reaction, first-strand cDNA was synthesized using a reverse transcription kit (CWbio, China) and then stored at -80°C for further use. RT-qPCR was carried out with a ABI 7500 PCR system using UltraSYBR Mixture (CWbio, China). The primers used for RT-qPCR are forward primer 5'-AAGCGTGGTGATGTGGAGTA-3' and reverse primer 5'-ATCGCTGATGGTATCGGTGT-3'. At the same time, the β-actin gene was used as an internal reference. RT-qPCR was performed in 50 μl reaction mixture, which containing 2 μl cDNAs template, 0.2 μM each primer, 25 μl 2×Ultra SYBR mixture, and the right amount of nuclease-free water. Dissolution and amplification curves were first observed, and then the right curve was chosen. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression of GUS gene. In the experiments, each sample was repeated three times independently.

2.6 Statistical analysis

All experiments in this study were repeated independently at least three times. The data were analyzed using the paired t-test or one-way analysis of variance (ANOVA) with GraphPad Prism 7.0 (San Diego,

USA) and expressed as mean ± standard. For all statistical analyses, $P < 0.05$ was considered statistically significant.

3. Results

3.1 Interaction of peptides with plasmids

Generally, electrostatic interaction-induced DNA-binding capacity is a prerequisite for gene carriers. Through agarose gel electrophoresis, the interaction of peptides with plasmids was evaluated. As shown in Fig. 1a, there are seven channels in gel plate. The first channel represented the alone plasmids, and the channels 2–7 represented the TAT/plasmids complexes at the different ratios (0.25:1, 0.5:1, 1:1, 2:1, 4:1, 8:1). At a ratio of 2:1 or higher (w/w), the plasmids band completely disappeared which signifies the strong binding of TAT with plasmids. However, NLS has poor ability to bind non-covalently to plasmids. As shown in Fig. 1b-c, the plasmids were not completely blocked in gel pores even at very high ratios of 384:1. TAT peptides exhibited a stronger binding ability to DNA, which may due to the higher content of arginine. As for effect of NLS peptides to the binding of TAT and plasmid the results demonstrated that the bands intensity gradually became weaker at ratio of 0.25 to 1, while did not change significantly when the ratio over than 2:1 (shown in Fig. 1d). It can be seen that the NLS peptides have an evident effect on stability of TAT/plasmids complexes, which may correlate with the relative affinity of each component. Although the specific effect of NLS peptides on the spatial structure of complexes is not fully understood, we speculated that the addition of NLS peptides could affect the surface charge distribution of TAT/plasmid complex and decrease its potential.

3.2 Size and zeta potential measurements of complexes

After measurement of particle sizes, their showed a trend of gradual increasing when ratio ranged from 0.25 to 0.5 (shown in Fig. 2a). The diameter of complex was larger than 300 nm at the ratio of 0.25 to 1 and smaller than 300 nm at the ratio of 2 to 8. When the particle size is less than 300 nm, the peptide/plasmid complex is considered suitable for cellular uptake. For another, because the cell membrane is negatively charged, the positive charge of complex is crucial for complexes to enter cells. After measurement of zeta potential of each TAT/plasmid complex, the results showed that as the TAT/plasmid ratio increases (from 0.25 to 8), their zeta potential value increases correspondingly (Fig. 2b). When TAT/plasmid ratio is 2, their zeta potential is positive, which is consistent with the result of gel mobility shift assay.

When the ratio of NLS/plasmid complex is higher than 32, the complex showed a larger particle size, some even exceed 1000 nm, which is not beneficial for endocytosis (Fig. 2c). Their zeta potential remained negative until the ratio of 256:1 (Fig. 2d). The results are consistent with those obtained by agarose gel electrophoresis. When NLS peptides (10 μg) were added to TAT/plasmids complex with different ratios, the resulting complexes have the particle size of more than 1000 nm (Fig. 2e). Meanwhile, introducing NLS to the complex can significantly decrease the zeta potential (Fig. 2f). The reason for

change of particle size and zeta potential in TAT/plasmids/NLS complex maybe due to the repulsive force between TAT and NLS peptides.

3.3 Cytotoxicity analysis of peptides/plasmids complexes

By microscopic examination, the cytotoxicity of peptides/plasmids complex to *D. salina* cells was evaluated. When the amount of plasmids is less than 1 μg, no significant cytotoxicity was observed in treated *D. salina* cells with TAT/plasmids or TAT/plasmids/NLS complexes. Simultaneously, the groups transformed with TAT/plasmids and TAT/plasmids/NLS complexes showed the highest relative GUS expression at the ratio of 2 and 4, respectively. In all TAT/plasmids transformation groups, cell ruptured in different degrees along with the increasing of plasmid concentration after salt gradient transformation. However, the NLS/plasmids group did not show significant toxicity to cells. Through adding NLS peptides to TAT/plasmid complex, it can significant alleviate the cytotoxicity of TAT/plasmids complex and further improve the cell integrity. The main cause came from the increasing of zeta potential of TAT/plasmids complexs with NLS peptides [14]. The cells disruption showed a dose-dependent manner with TAT content. With increasing of TAT content, the protective effect of NLS peptides to cells was more obvious. Although NLS peptides did not exhibit any cell-penetrating activity by itself, they play a crucial role in assisting TAT/pasmid complex into *D. salina* cells, which enhanced the transformation efficiency of foreign gene to host cells.

3.4 RT-qPCR analysis of transformants

Through RT-qPCR analysis, the expression level of GUS gene was evaluated and the results converted to relative expression values using $2^{-\Delta\Delta Ct}$ method. In all of the plasmids-bearing experimental groups, the expression of GUS gene was detected with salt gradient method. Through salt gradient transformation, the *D salina* cells were transformed by TAT/plasmids complex at the ratio of 0.25 to 8 (shown in Fig. 3). When the ratio is 2, the complex presented the highest expression level of GUS gene, which exhibites about 20-fold enhancement as compared with control group (Fig. 3a). After adding 10 μg NLS peptides to the complex, the highest expression level of GUS gene was achieved at the ratio of 4 (Fig. 3b). Target gene expression is depicted as 269-fold expression compared to the alone plasmid group. As shown in Fig. 4, the positive cells had the blue color by histochemical staining (Fig. 4c), but not in either blank control group (Fig. 4a) or negative control group (Fig. 4b). These results were consistent with the results of RT-qPCR that means the foreign genes not only were highly transformed to *D. salina* system, but also their expression levels were improved with the form of peptides/plasmids complex.

4. Discussion

Untill now, several methods have been used in *D. salina* transformation, like glass beads [23], electroporation [24], and bombardment particle [25] etc. They have some disadvantages of low transformation rate, poor cell viability and transient expression, which greatly limit the actual practical transformation of *D. salina* [23, 26, 27]. Given this, using osmotic pressure difference, we successfully established a novel transformation method with the features of fast, simple, minimal damage, and high-

efficient [5]. To further improve the transformation rate, this study firstly attempted to combine TAT and NLS peptides to deliver plasmids into *D. salina* cells. With the distinct advantages of CPPs, like the efficient transmembrane potency and carrying cargo molecules into host cells without affecting their biological activity, TAT/plasmid complex combined with salt gradient method achieved a much more high transformation rate for *D. salina* system. At the transcription level of target gene, it can nearly reach to more than 20-fold in particular range of weight ratios. In addition, this approach offers the advantages of rapid, economic, and high reproducibility etc.

By salt gradient method, the transformation efficiency of TAT/plasmid complex was significantly improved with addition of NLS peptides. When TAT/plasmids ratio is 4 (with 10 μg NLS), the expression of GUS genes is increased by 269 times compared with the the plasmid group. It is speculated that the NLS may be involved in the nuclear internalization of plasmids or dissociation of complex after entering the cell. Meanwhile, when NLS peptides mediate more complexes entry into the nucleus, there are still many obstacles in the process of gene delivery, such as promotion, transcription and translation, etc. Therefore, it is necessary to overcome these issues in the further research of NLS peptides.

In this study, the transformed *D. salina* cells were only identified at the level of cells and nucleic acid. But there were no visible western blot results in many experiments. It may be mainly caused by random integration of gene into the genome, low expression level of target gene, or protective mechanism of host cells. The next step will be to further study the expression and bio-activities of recombinant proteins. And also, to achieve higher transformation efficiency, the effects of different concentration of NLS peptides need to be deeply studied in the next works. Collectively, such an easy, efficient and high-efficient protocol has the potential to be a major tool for genetic operation of *D. salina*.

Declarations

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Competing interests

The authors declare no conflicts of interest in relation to this research and its publication.

Author Contributions

LN, SY wrote the manuscript. LN, SY, AF drew the pictures. CL, BY, YL, SX, YX conduct RT-qPCR and histochemical staining analysis. T polished the paper. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Availability of data and materials

The data of this article is included within the article. And also, the data and materials can also be requested from the corresponding author and the first author.

References

1. Feng SY, Li XB, Xu ZS, Qi JJ (2014) *Dunaliella salina* as a novel host for the production of recombinant proteins. Appl Microbiol Biot 98(10):4293–4300. https://doi.org/10.1007/s00253-014-5636-4
2. Feng SY, Feng WP, Zhao L, Gu HH, Li QH, Shi K, Guo SX, Zhang NN (2014) Preparation of transgenic *Dunaliella salina* for immunization against white spot syndrome virus in crayfish. Arch Virol 159(3):519–525. https://doi.org/10.1007/s00705-013-1856-7
3. Chai XJ, Chen HX, Xu WQ, Xu YW (2013) Expression of soybean kunitz trypsin inhibitor gene SKTI in *Dunaliella salina*. J Appl Phycol 25(1):139–144. https://doi.org/10.1007/s10811-012-9847-8
4. Poungpair O, Bangphoomi K, Chaowalit P, Sawasdee N, Saokaew N, Choowongkomon K, Chaicumpa W, Yenchitsomanus PT (2014) Generation of human single-chain variable fragment antibodies specific to dengue virus non-structural protein 1 that interfere with the virus infectious cycle. Mabs 6(2):474–482. https://doi.org/10.4161/mabs.27874
5. Song GN, Wang W, Hu LN, Liu Y, Feng SY (2019) An exploration of the rapid transformation method for *Dunaliella salina* system. AMB Express 9(1):181. https://doi.org/10.1186/s13568-019-0905-3
6. Peraro L, Kritzer JA (2018) Emerging methods and design principles for cell-penetrant peptides. Angew Chem Int Edit 57(37):11868–11881. https://doi.org/10.1002/anie.201801361
7. Thagun C, Chuah J, Numata K (2019) Targeted gene delivery: targeted gene delivery into various plastids mediated by clustered cell-penetrating and chloroplast-targeting peptides. Adv Sci 6(23):1902064. https://doi.org/10.1002/advs.201970142
8. Wu Y, Yao X, Chen Y, Li YP, Tian WQ (2017) Advance of DNA and CCPs-based nanocarriers in drug delivery systems. Bio-Med Mater Eng 28(s1):S255–S261. https://doi.org/10.3233/bme-171648
9. Fu K, Lu, Lin C (2020) Cell penetrating peptide as a high safety anti-inflammation ingredient for cosmetic applications. Biomolecules 10(1):101. https://doi.org/10.3390/biom10010101
10. Ruseska I, Zimmer A (2020) Internalization mechanisms of cell-penetrating peptides. Beilstein J Nanotechnol 11:101–123. https://doi.org/10.1002/9783527626830.ch7
11. Liu J, Afshar S (2020) In vitro assays: friends or foes of cell-penetrating peptides. Int J Mol Sci 21(13):4719. https://doi.org/10.3390/ijms21134719
12. Kardani K, Bolhassani A (2021) Cppsite 2.0: An available database of experimentally validated cell-penetrating peptides predicting their secondary and tertiary structures. J Mol Biol 433(11):166703. https://doi.org/10.1016/j.jmb.2020.11.002

13. Klabenkova K, Fokina A, Stetsenko D (2021) Chemistry of peptide-oligonucleotide conjugates: a review. Molecules 26(17):5420. https://doi.org/10.3390/molecules26175420
14. Sakamoto K, Morishita T, Aburai K, Sakai K, Abe M, Nakase I, Futaki S, Sakai H (2020) Key process and factors controlling the direct translocation of cell-penetrating peptide through bio-membrane. Int J Mol Sci 21(15):5466. https://doi.org/10.3390/ijms21155466
15. Bogacheva M, Egorova A, Slita A, Maretina M, Baranov V, Kiselev A (2017) Arginine-rich cross-linking peptides with different SV40 nuclear localization signal content as vectors for intranuclear DNA delivery. Bioorg Med Chem Lett 27(21):4781–4785. https://doi.org/10.1016/j.bmcl.2017.10.001
16. Yi WJ, Yang J, Li C, Wang HY, Liu CW, Tao L, Cheng SX, Rx ZC, Zhang XZ (2011) Enhanced nuclear import and transfection efficiency of TAT peptide-based gene delivery systems modified by additional nuclear localization signals. Bioconjug Chem 23(1):125–134. https://doi.org/10.1021/bc2005472
17. Kanazawa T, Yamazaki M, Fukuda T, Takashima Y, Okada H (2015) Versatile nuclear localization signal-based oligopeptide as a gene vector. Biol Pharm Bull 38(4):559–565. https://doi.org/10.1248/bpb.b14-00706
18. Hyman JM, Geihe EI, Trantow BM, Parvin B, Wender PA (2012) A molecular method for the delivery of small molecules and proteins across the cell wall of algae using molecular transporters. P Natl Acad Sci USA 109(33):13225–13230. https://doi.org/10.1073/pnas.1202509109
19. Suresh A, Kim Y (2013) Translocation of cell penetrating peptides on *Chlamydomonas reinhardtii*. Biotechnol Bioeng 110(10):2795–2801. https://doi.org/10.1002/bit.24935
20. Wei Y, Niu J, Li H, Huang A, He L, Wang G (2015) Cell penetrating peptide can transport dsRNA into microalgae with thin cell walls. Algal Res 8:135–139. https://doi.org/10.1016/j.algal.2015.02.002
21. Gadamchetty P, Mullapudi P, Sanagala R, Markandan M, Polumetla AK (2019) Genetic transformation of *Chlorella vulgaris* mediated by HIV-TAT peptide. 3 Biotech 9(4):139. https://doi.org/10.1007/s13205-019-1671-2
22. Kang S, Suresh A, Kim YC (2017) A highly efficient cell penetrating peptide pVEC-mediated protein delivery system into microalgae. Algal Res 24:360–367. https://doi.org/10.1016/j.algal.2017.04.022
23. Feng SY, Xue LX, Liu H, Lu P (2009) Improvement of efficiency of genetic transformation for *Dunaliella salina* by glass beads method. Mol Biol Rep 36(6):1433–1439. https://doi.org/10.1007/s11033-008-9333-1
24. Sun Y, Yang ZY, Gao XS, Li QY, Zhang QQ, Xu ZK (2005) Expression of foreign genes in *Dunaliella* by electroporation. Mol Biotechnol 30(3):185–192. https://doi.org/10.1385/mb:30:3:185
25. Tan CP, Qin S, Zhang Q, Jiang P, Zhao FQ (2005) Establishment of a micro-particle bombardment transformation system for *Dunaliella salina*. J Microbiol 43(4):361–365. https://doi.org/10.1128/CMR.00001-08
26. Feng SY, Hu LN, Zhang QH, Zhang FQ, Liu Y (2020) CRISPR/Cas technology promotes the various application of *Dunaliella salina* system. Appl Microbiol Biot 104(20):8621–8630. https://doi.org/10.1007/s00253-020-10892-6

27. Hu LN, Feng SY, Liang GF, Du JX, Niu CL (2021) CRISPR/Cas9-induced β-carotene hydroxylase mutation in *Dunaliella salina CCAP19/18*. AMB Express 11(1):83. https://doi.org/10.21203/rs.3.rs-302435/v1

Figures

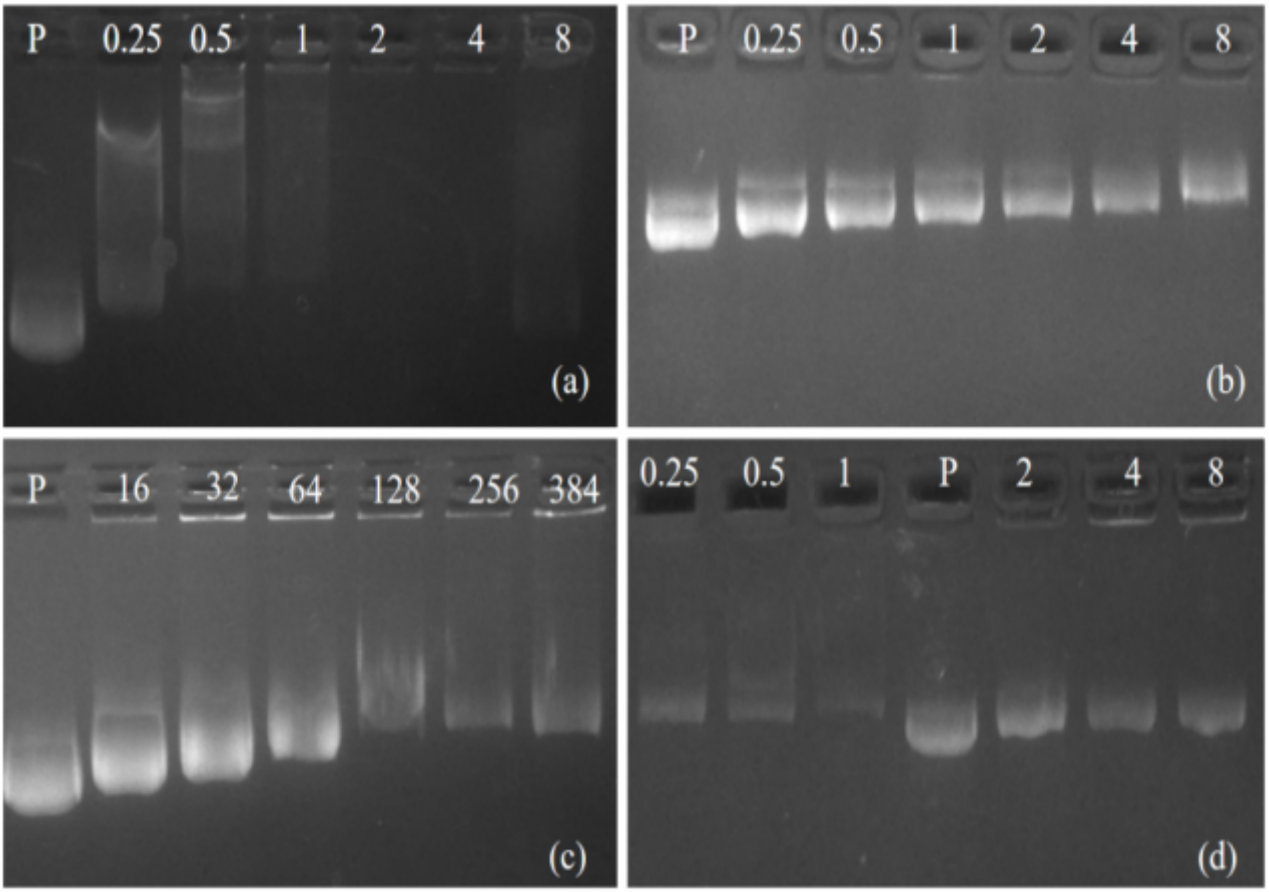


Figure 1

Agarose gel analysis of peptide/plasmid complex at various weight ratios. P indicates the alone plasmid

(a) TAT/plasmid ratio is from 0.25 to 8; **(b)** NLS peptide/plasmid ratio is from 0.25 to 8; **(c)** NLS peptide/plasmid ratio is from 16 to 384; **(d)** At the ratio of 0.25 to 8, NLS peptides were added to complexes contaning plasmid and TAT to form the ternary complexes.

Figure 2

Average particle size and zeta potential of peptide/plasmid complexes at the different ratios

(a) and **(b)** represent the Z-average and zeta potential of TAT/plasmid at the ratio of 0.25 to 8, respectively; **(c)** and **(d)** represent the Z-average and zeta potential of NLS peptide/plasmid at the ratio of

16 to 382, respectively; **(e)** and **(f)** represent the Z-average and zeta potential of TAT/plasmid with NLS peptide at the ratio of 0.25 to 8, respectively.

Figure 3

Relative expression level of GUS gene in transformants through RT-qPCR analysis

W, wild type *D. salina* cells; B, blank control group; P, alone plasmid group; The 0.25T represents that the ratio of TAT/plasmid for this experimental group is 0.25.Others indicated transformed groups with different peptide/plasmid complex; **(a)** and **(b)** represented the relative GUS expression level of transformants, which mediated by salt gradient transformation with the complex of TAT/plasmid and TAT/plasmid/NLS peptide at the ratios of 0.25 to 8, respectively. Data represent mean ± standard deviation, n=3. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 compared with control group, ns represents no statistical difference.

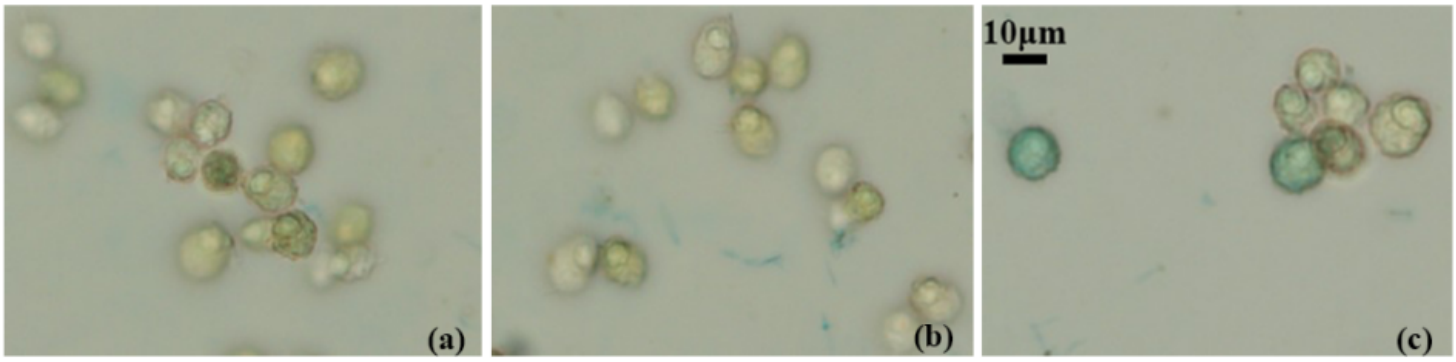


Figure 4

Histochemical staining of transformed cells with the TAT/NLS peptide/plasmid complexes

(**a)** Blank control, wild type *D. salina* cells were untransformed and directly stained; (**b)** Negative control, *D. salina* were transformed without plasmid; (**c)** Positive experimental group, *D. salina* were transformed with the TAT/NLS peptide/plasmid complexes. Arrow represents the positive transformants with the blue color.