Production of transgenic *Allium cepa* by nanoparticles to resist *Aspergillus niger* infection

Eman Tawfik1 · Ibtisam Hammad1 · Ashraf Bakry2

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**Abstract**

**Background** Transgenic plants are becoming a more powerful tool in modern biotechnology. Genetic engineering was used in biotech-derived products to create genetically modified (GM) plants resistant to diseases. The onion (*Allium cepa*, L.) is a common, important perennial vegetable crop grown in Egypt for food and economic value. Onions are susceptible to a variety of fungal infections and diseases. *Aspergillus niger* is a common onion phytopathogen that causes diseases such as black mould (or black rot), which is a major issue, particularly when exporting onions. *A. niger* grows between the bulb’s outer (dead, flaky) skin and the first fleshy scales, which become water-soaked. Thionin genes produce thionin proteins, which have antimicrobial properties against a variety of phytopathogens, including *A. niger*. Chitosan nanoparticles act as a carrier for the thionin gene, which allows *A. cepa* to resist infection by *A. niger*.

**Methods and results** Thionin gene (Thio-60) was transformed into *A. cepa* to be resistance to fungal infection. The gene was loaded on chitosan nanoparticles to be transformed into plants. Transgenic *A. cepa* had a 27% weight inhibition compared to non-transgenic one, which had a 69% inhibition. The expressed thionin protein has a 52% inhibitory effect on *A. niger* spore germination. All these findings supported thionin protein's antifungal activity as an antimicrobial peptide. Furthermore, the data presented here demonstrated the efficacy of chitosan nanoparticles in gene transformation.

**Conclusion** The present study describes the benefits of producing transgenic onion resistance to black rot diseases via expression of thionin proteins.

**Keywords** *Allium cepa* · *Aspergillus niger* · Chitosan nanoparticles · Thionin · Transformation

**Introduction**

The onion (*Allium cepa*, L.) belongs to the Alliaceae family and is indigenous to the Middle East and Asia. It’s a seasonal crop, a bulbous biennial herb with bulbs that grow underground. Essential minerals, vitamins, carbohydrates, and amino acids in *A. cepa* give it food and medical properties. Because of its high content of fructans, flavonoids, macro- and micronutrients, the onion is used as a potherb and contains an essential component of the human food chain. The onion plays an important role in folk medicine because it helps to prevent heart disease and other illnesses [1, 2].

Onions can be infected by several post-harvest diseases such as black mold rot caused by *Aspergillus niger*. The most destructive disease of storage in the field is onion black mould rot, which is a major problem when exporting. The pathogen is spread by infected seeds or soil. The infection usually starts with the germination of onion seeds and can last for months. Visual external and internal symptoms of black mold are noticed on bulbs. However, these symptoms are not visualized on seeds. *A. niger* produced several enzymes and toxins in the bulb and other tissues [3, 4].

To defend themselves against pathogens, plants produce a variety of antimicrobial peptides, including thionins. Thionins are known to have antimicrobial properties and are a part of the plant defence system. Thionins are short peptides of about 5 kDa with antimicrobial activity and found in plants only. They are expressed in many monocotyledons and dicotyledon plants, as about one hundred thionin sequences have been described in fifteen plant species. Regarding pathogen attack, the expression of thionin
genes is regulated by methyl jasmonate hormone which has an essential role in defense reactions [5–11]. Asano et al. [12] explained the mechanism of thionins inhibition of pathogenic fungi: thionins’ antimicrobial properties come from their ability to cause pore formation on phytopathogen cell membranes, allowing potassium and calcium ions to escape the cell.

The transformation of gene of interest identified is by different methods which were classified into two types (indirect and direct). The indirect method involves the use of a vector to deliver gene. The direct method is directly by various chemical and physical ways of gene transfer. The genetic diversity exists in available species are analyzed to identify the gene of interest to develop an elite cultivar [13]. Chitosan nanoparticles provide an efficient and rapid direct method for gene transformation. Biodegradable polymers like chitosan can detect an advantageous charge on the DNA conjugate nanomaterial surface. Chitosan is a polymer that has been widely used in nucleic acid delivery and tissue engineering packages [14, 15].

Gene delivery using chitosan-based polysaccharides have gained significant attention in recent years as new functional biomaterials with potential applications in various fields. Chitosan chemical properties are insoluble in most solvents but slightly soluble in diluted organic acids such as acetic, lactic, malic, formic, and succinic acids. The degree of deacetylation (DA) of a chitosan biomaterial is the actual molarity of the glucosamine residue in the polymer chain to indicate the cationic charge on the molecule once diluted in acid solution. This is clear from the proportion of free amino groups in the chitosan biopolymer [16, 17]. Chitosan-derived biomaterials have received considerable attention as an antimicrobial, functional, renewable, nontoxic, biocompatible, bioabsorbable, and biodegradable biopolymer agent [18–20].

The study aims to design new transgenic onion lines resistant to a fungal infection caused by *Aspergillus niger*. Thionin (Thio-60) is the target gene with antimicrobial activity expressed into antimicrobial thionin peptides. This gene was transformed via chitosan nanoparticles.

### Materials and methods

#### Plant material

The thionin gene is genetically modelled in the *Arabidopsis thaliana* plant according to previous study of Abdel-Razik et al. [19]. Onion cultivar was used as a transgenic model (Giza red), which is susceptible for fungal infection. They were purchased from Agricultural Research Center and regenerated on MS media [21].

### Obtaining of thionin gene and cloning

Edward’s protocol was used to extract total genomic DNA from *Arabidopsis thaliana* [22]. For each 25 µl PCR reaction, 50 ng of template DNA was used. Also, contained 12.5 µl of 2X master mix (Biolene), 1 µl of each forward and reverse primer (50nmole/base), and sterile d.dH 2O to fill the remaining 25 µl. The primers were designed with the snap gene® (2.3.3) and had the following sequences: Thio-60F: 5’ GCTGAAATTCCATGGAGGACAAAAAGA 3’, Thio-60R: 5’ GCTAAGCTTTCATAGACTAAAAATCAAT 3’. The PCR reaction was run for 40 cycles: 1 min at 95 °C, 1 min at 64 °C, and 1 min at 72 °C. On a 1.2% (w/v) agarose gel, thionin insert were run. GeneJETTM PCR Puriﬁcation Kit was used to purify the ampliﬁed PCR product (Thermo K0701). pMiniT Vector (NEB® PCR Cloning Kit, #E1202S-10-beta Competent E. coli) was ligated to the Thio-60 product. Following the manual’s instructions.

### Confirmation of bacterial transformation

Colony PCR was used to differentiate non-recombinant and recombinant colonies, and colony PCR was used to apply colony PCR. On LB agar plates supplemented with 100 µg/l ampicillin, bacteria containing modiﬁed plasmid were grown. Single colonies were used as templates for PCR reactions using the same thionin primers, followed by electrophoresis on a 1.2% gel.

### Chitosan nanoparticle transformation

#### Degree of deacetylation

Titration method was used to determine the degree of deacetylation for chitosan nanoparticles according to Czechowska-Biskup et al. [23] as follow: “Dried chitosan (0.2 g) was dissolved in 20 ml of 0.1 M hydrochloric acid and 25 cm³. After 30 min continuous stirring 25 ml of dH2O was added with continuous stirring for another 30 min. After complete dissolve of chitosan, titration with a 0.1 mol·dm⁻³ sodium hydroxide solution was performed. The degree of deacetylation (DA or DD) of chitosan was calculated as:

\[
DA\% = 2.03 \left( \frac{V_2 - V_1}{m + 0.0042(V_2 - V_1)} \right)
\]

where: \(m\): weight of sample, \(V_1, V_2\): consumed volumes of 0.1 mol·dm⁻³ sodium hydroxide solution, 2.03: coefficient of the molecular weight of chitin monomer unit, 0.0042: coefficient of the difference between molecular weights of chitin and chitosan monomer units”.

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UV–Visible spectra measurements and zeta potential and size

Another characterization method for chitosan nanoparticles is using UV–visible spectroscopy “JASCO V-630 UV–visible spectrophotometer (serial: C285061148) with Spectra Measurement software”. The measured zeta potential and size was estimated using Malvern (7.2) software.

Transmission electron microscopy

The examined CS nanoparticles were prepared as follow: “copper grids mesh coated with carbon 400 and handled on 1 drop of the prepared complex (CS/pDNA) and left for 1.5 min. The grid was stained with 1 drop of filtered solution of 2% uranyl acetate for 1.5 min, with removal of excess uranyl acetate [24]. The grids were dried for 10 min then photographed with transmission electron microscope (TEM) in the Regional Center for Mycology and Biotechnology, Al-Azhar University”.

Preparation of chitosan-DNA nanoparticles complex (CS/pDNA)

Mansouri et al. [25] designed the protocol of CS/DNA formation as follow: “chitosan nanoparticles (CS) were dissolved in 25 mM acetic acid and adjusted to pH 5.5 at a concentration of 0.08%. Both CS and recombinant pMiniT were incubated in water bath at 55 °C for 15 min and then added equally to each other with stirring on a vortex for 1 min”.

Transformation of chitosan/pDNA into Allium cepa

The method of chitosan nanoparticle transformation into plant tissue was developed by [20]: “Seedlings of A. cepa at age of five days were inoculated by syringe containing CS/pDNA complex at the plumule region. After that the explants (injected seedlings) were transferred on to MS medium supplemented with (2 mg/L BAP and 1 mg/L kin) hormones and 100 µg/L ampicillin then incubated at 25 ± 1 °C for 4 weeks to regenerate plants”.

Molecular analysis of transgenic Allium cepa lines

DNA from both leaves of non-transgenic and transgenic Allium cepa lines was isolated. DNA fragments of thio-60 transgenes were amplified by PCR with the same thionin primers and the same PCR program conditions.

Pathogenicity bioassay

A fungal-resistance bioassay, of the transgenic A. cepa lines, was applied to estimate the phytopathogen infection resistance. The thionin genes in transgenic plants were tested for antimicrobial activity against A. niger. Pathogenicity bioassay was performed via three methods: (1) Spore suspension infecting whole Allium cepa shoot; (2) infection of whole in vitro plant and (3) spore suspension infecting plants out-jars (after 2 weeks of growth).

Spore suspension infecting whole Allium cepa seedling

In another experiment, spore suspension was prepared by immersing fungal discs in 5 ml of sterile distilled water to release the spores. The spores were collected with a sterile Pasteur pipette, and their concentration was adjusted to $2 \times 10^5$ spores/ml using sterile water. This assay was applied for the whole shoot with some modifications.

The whole seedlings from mature transgenic and non-transgenic A. cepa, grown in vitro for 4 weeks, were placed in a Petri dish with wet filter paper and inoculated with the spore suspension (100 µl each). After inoculation, the shoots were incubated at room temperature under 16 h light/8 h dark conditions and high humidity for a week. Pictures were taken 5 days after inoculation [26].

Disease resistance assay

For fungal infection: this follows [26], 27 with some modification. “Fungi grew on potato dextrose agar till the surface covered with the fungal mycelia. Then, a block of the agar with mycelia was placed near to the base of in vitro transgenic and non-transgenic control plants (3 weeks old) grown on 100 ml MS medium and incubated at 25 °C with 16 h light/8 h dark. Photographing for the results was recorded 2 weeks after inoculation”.

Spore suspension with infected plant

The spore suspension of A. niger was prepared by submerging fungal discs in 5 ml of sterile dH₂O to release the spores (adjust concentration to $2 \times 10^5$ spores/ml using sterile dH₂O). This assay was applied for whole of plants from mature transgenic and non-transformed A. cepa, grown in vitro for 4 weeks, put in a Petri plate with wet filter paper, and inoculated with the spore suspension (100 µl each). After inoculation, these infected leaves were incubated at room temperature and high humidity for 7 days then photographed to record the infection symptoms [26].

Inhibitory protein bioassay

The antifungal activity of the transgenic Allium's product thionin proteins was tested against A. niger spores. Bradford’s method [28] of protein extraction was used to extract the proteins. Then, with some modifications, the inhibitory
effect of protein extracts on spore germination bioassay was used, as described by Maji et al. [29]. “The pathogens' spore suspension was made aseptically from a 7-day-old pure culture. On separate sterile eppendorf tubes, 100 µl of spore suspension and 100 µl of crude protein extracts were taken. One tube was kept as a control, with no extract added. Triplicates of each treatment were kept. The tubes were incubated at 25 ± 2 °C for 24 h. After the incubation period, observations were made under microscope to calculate the percentage inhibition” (using 100 × magnification power under JENLAB microscope).

Statistical analysis

The data in this study were analyzed using one-way ANOVA in SPSS 21 software for calculating means and the significance for 10 individuals for each sample. The data analysis of gel was performed using BioRAD Quantity One software (4.6.2). In SPSS 21 (IPM, USA), the data was subjected to an analysis of variance test. Standard deviations, and mean averages were calculated.

Results

The thio-60 gene was transformed into Allium cepa using chitosan nanoparticles. The transgenic plants exhibited resistance against Aspergillus niger infection.

Thio-60 was isolated from Arabidopsis thaliana and then obtained via PCR using specific primers. Following that, the PCR product of each gene was ligated into the pMini-T vector separately. The recombinant plasmids were then transformed into competent 10-beta (Fig. 1). A colony PCR was used to confirm thionin transformation into 10-beta for amplification. Thio-60 was found at approximately 640 bp.

Chitosan nanoparticle characterization

DD, morphology TEM measurements, UV–Visible spectroscopy, and zeta potential were used to characterise chitosan nanoparticles (Table 1). TEM images (Fig. 2a) separately depicted the complex of chitosan nanoparticles with modified plasmids carrying thionin genes. The absorbance of chitosan nanoparticles was determined to be 302.8 nm using UV–visible measurements (Fig. 2b). The distribution of zeta potential of these chitosan nanoparticles was 28 (Fig. 2c).

Transformation of chitosan/pDNA into Allium cepa

After four weeks of incubation at 25 °C, the regenerated transgenic and non-transgenic lines of A. cepa were fully grown (Fig. 3). Confirmation of thionin gene transformation into A. cepa was applied using PCR for both transgenic and non-transgenic lines. The band was detected at 640 bp for thio-60. The non-transgenic lines were applied to confirm that the thionin gene was not found previously in this species.

Pathogenicity test

The spore suspension bioassay, Aspergillus niger was applied to infect the entire plant out-jars. Fungal discs were used to infect the whole plant jar, and spore suspension was applied to infect A. cepa transgenic lines comparing to non-transgenic one. The inhibition percentage in the growth after and before infection was calculated and recorded in Table 2. The percentage in growth in non-transgenic lines (69.746%) was higher than transgenic ones (27.393%), and this is a high significant difference (Fig. 4).

In the inhibitory protein bioassay, the expressed total proteins were used to inhibit spores’ germination. The results obtained from infected onion jars were precise and made a difference between transgenic and non-transgenic lines (Fig. 4a), where the bioassay of spore suspension infecting shoot was shown in (Fig. 4b). The reverse effect of transgenic thionin protein on spore germination was explained in Fig. 5 and Table 3. The in vitro culture of transgenic A. cepa showed significant growth and resistance to fungal infection comparing to non-transgenic one, which showed high inhibition in growth. The results showed a highly significant
difference of transgenic *Allium* expressing *thio*-60 comparing to non-transgenic *Allium*. The inhibition percentage in weight of non-transgenic *Allium* was 36%, wherein the transgenic one with *thio*-60 was 13%.

### Discussion

This work based on transformation of antimicrobial gene (*thio*-60) into tissues of *A. cepa*. The thionin protein will be expressed and allow *A. cepa* to be resistant to phytopathogenic fungi (*A. niger*). Transformation process was performed by loading the modified plasmid carrying the target gene on nano-chitosan particles. Many antifungal genes were isolated from several plants, like Liu et al. [30] who isolated Pa-AMP-1 from *Phytolacca americana*. Also, Plattner et al. [31] isolated the antimicrobial BTH6 thionin.
from barley. Almaghrabi et al. [32] isolated a category of thionins from *Arabidopsis* and proved anti-nematodes.

Chitosan nanoparticle is a modern technique used for transformation because it has many advantages: non-toxic, biocompatible, and biodegradable compared with the *Agrobacterium* technique. The CS/pDNA complex was efficiently prepared and applied as an effective gene delivery system under controlled conditions. This agreed with Hussein [33], who applied chitosan nanoparticles as a carrier of pMiniT carrying thionin gene via transformation to *Paulownia* sp. Czechowska-Biskup et al. [23] used a simple titration method to determine the degree of deacetylation (DD), which evaluates the amine or acetyl amine groups on the glycoside unit of chitosan nanoparticle. The affinity between the nanoparticle and pDNA is determined by the positive charge of this group joining with the negative charge of pDNA. In addition, the degree of deacetylation determines the content of free amino groups in the polysaccharide, which have an essential role in binding between chitosan and DNA.

In our work, we used the ratio 1:1 (pDNA: CS) and confirm that DNA union with chitosan depend on both molecular weight and the DD of chitosan nanoparticles and these results were the same manner with Kiang et al. [24] who indicated that the ratio between the DNA and chitosan concentration must be the same ratio. Also, in agreement with Bivas-Benita et al. [34], who demonstrated that particle size and shape play an important role in gene transfer into cells.

According to Kiang et al. [24], who photographed chitosan nanoparticles in colon cancer cells using a plasmid carrying the HEK293 gene (human embryonic kidney cells); and Hallaj-Nezhadietel et al. [35], who photographed the pUMVC3-hIL12 complex on chitosan nanoparticles in colon cancer cells using a plasmid carrying the HEK293 gene (human embryonic kidney cells). Till now, only a few research have focused on the use of chitosan in plants, and the work in this field has been too limited, according to [20], who photographed chitosan nanoparticle binding with the pEGAD, and Hussien [33] who described chitosan nanoparticle using SEM.

Chitosan nanoparticles were used for the transformation of various genes in different sources like animal and human cell lines, according to Kiang et al. [24] and Hallaj-Nezhadietel et al. [35]. However, till limited application in gene transformation into plant tissues, until [20] used chitosan nanoparticles for gene transformation to potato lines.

The pathogenicity bioassay results agreed with Khan et al. [26], who produce transgenic potato cultivars (three commercial potato cultivars) resistant to *Alternaria solani* fungus to a high extent. It also agreed with Khan et al. [27], who partially produce a free disease-resistant potato cultivar to resist *Alternaria solani*, *Botrytis cinerea*, and *Fusarium oxysporum*.

The activity of protein extracts against the spore germination of phytopathogenic *A. niger* (Table 2 and Fig. 5) showed inhibition in spores germination treated with transgenic thionin protein. It was noticed that spores treated with H2O (as negative control) cause aggregation in spores, but still high number in growth and number of spores. In the case of spores treated with non-transgenic *Allium* protein, the number and germination of protein are less than negative control but still high. In the case of spores treated with protein extracted from transgenic *Allium*, expressing thionin protein, the spore germination was significantly inhibited.
Cammue et al. [36] found that thionin proteins inhibit about 20 phyto-pathogens, including *Botrytis cinerea*, *Fusarium* spp., *Phytophthora infestans*, and *Rhizoctonia solani*. Epple et al. [37] found that constitutive overexpression of an endogenous thionin in transgenic *Arabidopsis* increased resistance to *Fusarium oxysporum* f. sp. *matthiolae*, implying that thionins are defence proteins. Asano et al. [12] used the secreted antifungal thionin protein isolated from *A. thaliana* to suppress the toxicity of *Fusarium graminearum*, with results ranging from 20 to 50% inhibition using the spore suspension method.

**Conclusion**

This work introduced the transformation of thionin gene (*thio-60*) isolated from *Arabidopsis thaliana*. This gene was transformed into *Allium cepa* cultivar (Giza red) by chitosan nanoparticle transformation. The transgenic *A. cepa* tissues were regenerated by seed culture technique. Resistance of transgenic *A. cepa* cultivar lines were assessed against phytopathogenic fungi (*Aspergillus niger*). The successful transformation was confirmed by using conventional PCR. The whole plant culture was subjected to *A. niger* and showed resistance to fungal infection. Protein isolated from transgenic *A. cepa* generally causes inhibition for the growth of spore germination of *A. niger*. All these bioassays were applied to both transgenic *A. cepa* lines comparing to non-transgenic ones. The further studies will involve the impact on the onion’s fleshy and scale leaves beside the onion seedlings.

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**Data availability** All data materials are available in manuscript.

**Code availability** Not applicable.

**Declarations**

**Conflict of interest** All authors have nothing to disclose.

**Consent to participate** All authors agree to the content of manuscript.

**Consent for publication** All authors agree for publication.

**Ethical approval** Not applicable.

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