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Article

Engineered dsRNA–protein nanoparticles for effective systemic gene silencing in plants

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Abstract

Long-distance transport or systemic silencing effects of exogenous biologically active RNA molecules in higher plants have not been reported. Here, we report that cationized bovine serum albumin (cBSA) avidly binds double-stranded beta-glucuronidase RNA (dsGUS RNA) to form nucleic acid–protein nanocomplexes. In our experiments with tobacco and poplar plants, we have successfully demonstrated systemic gene silencing effects of cBSA/dsGUS RNA nanocomplexes when we locally applied the nanocomplexes from the basal ends of leaf petioles or shoots. We have further demonstrated that the cBSA/dsGUS RNA nanocomplexes are highly effective in silencing both the conditionally inducible *DR5-GUS* gene and the constitutively active *35S-GUS* gene in leaf, shoot, and shoot meristem tissues. This cBSA/dsRNA delivery technology may provide a convenient, fast, and inexpensive tool for characterizing gene functions in plants and potentially for *in planta* gene editing.

Introduction

Exogenous RNA application to plants may provide a powerful tool for characterizing gene functions and improving agricultural crop productivity [7]. While exogenously applied naked RNAs were reported to be effective in gene silencing in plants [11, 20, 25], a number of more recent studies have shown that naked RNAs are not effective in gene silencing in plants [5, 6, 10, 38, 40–42]. It is not surprising that naked RNA molecules do not effectively silence gene expression when introduced into plant tissues because RNA molecules carry negative charges due to their phosphate groups. Negatively charged molecules face difficulty crossing the plasma membrane, which also possesses negatively charged surfaces. On the other hand, nanoparticle–RNA complexes with neutralized surfaces have been proven capable of gene silencing, likely owing to their ability to traverse the plasma membrane. While nanoparticle–RNA complexes have been shown to be highly effective in silencing gene expression in living plant cells [3, 9, 10, 12, 14, 17, 21, 25, 26, 34, 38, 40–42], they are relatively inefficient for long-distance transport or systemic gene silencing within the plant. Zhang *et al.* [40, 42] and Demirer *et al.* [8] reported that nanoparticle complexes diffused only about 60 μm in the z direction and 3 cm in the x–y direction in the leaves from the infiltrated sites. While there have been reports on the long-distance transport of nanoparticle–RNA in plants, these studies document the silencing effects on genes in fungal pathogens, insect pests, or viruses that infect plants rather than on endogenous plant genes [20, 24, 29]. Hence, the attainment of long-distance transport or systemic gene silencing effects for nanoparticle–RNA complexes is highly desirable. This would facilitate the achievement of systemic gene silencing effects even when nanoparticle-RNA complexes are administered locally. In this study, we present the development of cationized bovine serum albumin (cBSA) and dsRNA nanocomplexes, along with their systemic gene silencing effects in higher plants.

Results

Chemical modification of cationized BSA and dsRNA nanocomplexes and their transport within a plant

To employ BSA for RNA delivery, we systematically adjusted the its net charge for capturing anionic dsRNA by conjugating the COOH groups of BSA with the amine groups of ethylenediamine (EDA) via carbodiimide chemistry (Fig. 1F). The degree of conjugation and net charge on the protein were optimized by adjusting the

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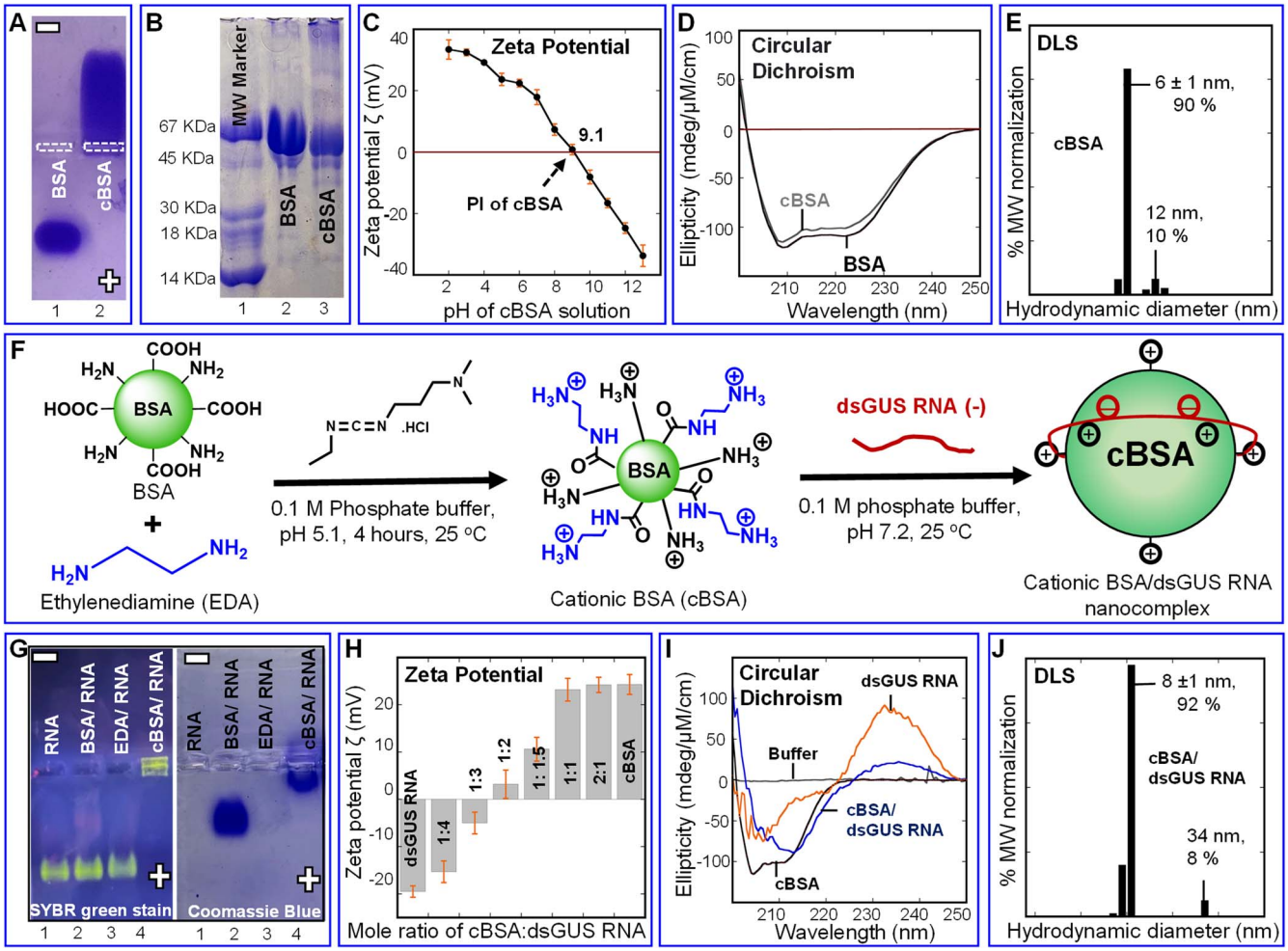

Figure 1. Synthesis and characterization of cBSA and cBSA/dsGUS RNA nanocomplexes. (**A**) Agarose gel (0.5%) of pure BSA (Lane 1) and cBSA (Lane 2) protein nanoparticles stained with Coomassie blue. (**B**) The SDS-PAGE of the molecular weight marker (Lane 1), unmodified BSA (Lane 2), and cBSA (Lane 3). (**C**) Zeta potential of cBSA as a function of pH (2~14). The arrow indicates the isoelectric point (PI) at pH 9.1. (**D**) CD spectra of BSA and cBSA. (**E**) The average hydrodynamic diameter of the cBSA was around 6 nm at pH 7. (**F**) Schematic illustration of the synthesis of cBSA followed by cBSA/dsGUS RNA nanocomplexes (middle row). Cationization of BSA by amidation of its carboxylic groups with EDA using carbodiimide EDC chemistry (left hand). The cBSA binds to the dsGUS RNA together via intermolecular H bonding and strong electrostatic interactions (right hand). (**G**) Titrations of cBSA/dsGUS RNA nanocomplexes were performed on agarose gels. The gels were stained with SYBR green dye to visualize the dsRNA bands (left) and with Coomassie blue to visualize the protein bands (right). Lane 1: dsGUS RNA, Lane 2: BSA/dsGUS RNA, Lane 3: EDA/dsGUS RNA, and Lane 4: cBSA/dsGUS RNA. Lanes 2 and 3 indicate that neither native BSA nor EDA interact with dsGUS RNA; however, Lane 4, cBSA/dsGUS RNA (1:1 mole ratio) shows stronger binding affinity forms ionic nanocomplex. The gels ran at pH 8.2 in 40 mM Tris-acetate buffer. (**H**) Zeta potential of cBSA/dsGUS RNA nanocomplexes with their respective mole ratios. (**I**) Far-UV CD spectra of phosphate buffer (10 mM, pH 7.0), dsGUS RNA (5 μM), cBSA (5 μM), and cBSA/dsGUS RNA nanocomplex (5 μM; 1:1 mole ratio). (**J**) Average diameter (nm) of the cBSA/dsGUS RNA nanocomplex was around 8 nm at pH 7, measured through DLS.

mole ratios of protein, EDA, and the carbodiimide condensing agent (EDC, Fig. S1). For example, the modified protein indicated a high positive charge moved toward the negative electrode in agarose gel (Fig. 1A, Lane 2) while there was little or no crosslinking [sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)] (Fig. 1B, Lane 3; Fig. S2). The reaction conditions were optimized for 100% conversion without any residual unmodified BSA or protein crosslinking. The cBSA with a high positive charge (Fig. 1A, Lane 2) was purified by dialysis and examined by Zeta potential titrations, which indicated a net charge of +23 mV and an isoelectric point of 9.1 (Fig. 1C). The UV circular dichroism (CD) spectra showed that its secondary structure is similar to that of the native protein (Fig. 1D), while dynamic light scattering (DLS) data (Fig. 1E) indicated a molecular size of 6 nm, further confirming that there has been no protein crosslinking.

The fully characterized cBSA (20 μM, pH 7.2) was subsequently evaluated for its ability to form nanocomplexes with double-stranded beta-glucuronidase RNA (dsGUS RNA) (20 μM, pH 7.2) (Fig. 1F) through a band shift assay conducted in agarose gel electrophoresis (Fig. 1G, Fig. S3). A mixture of a 1:1 mole ratio of cBSA and dsGUS RNA (RNA band stained with SYBR Safe dye) showed a significant shift in the band position (Fig. 1G, Lane 4), compared to the control and other lanes (Fig. 1G, Lanes 1–3). The absence of free protein or free dsRNA in this mixture is also evident when stained with Coomassie blue (Fig. 1G).

The nanocomplex formation was confirmed by Zeta potential titrations (Fig. 1H), CD spectra (Fig. 1I), and DLS (Fig. 1J). CD indicated considerable distortion of both the protein secondary structure (peaks at 210 and 222 nm) and the dsGUS RNA (270-nm band) (Fig. 1I), while DLS indicated a small increase in size from 6 to 8 nm (major fraction) and had a minor fraction of 34 nm. Moreover, cBSA was found to bind dsGUS RNA at a 2:1 to 5:1 mole ratio (Fig. S3), but these complexes had much larger particle sizes (40 to 600 nm) and thus were not further examined. Therefore, the

smaller 1:1 complex of cBSA/dsGUS RNA was selected to explore gene silencing in plants.

Systemic silencing effects of cBSA/dsGUS RNA nanocomplexes on auxin-induced expression of the *DR5-GUS* gene

An auxin-inducible synthetic promoter, *DR5* [35], derived from a soybean *GH3* promoter [13, 22], was used to drive the expression of the GUS reporter gene in tobacco (*Nicotiana tabacum*). The *DR5-GUS* transgenic tobacco leaf disks were incubated with cBSA/dsGUS RNA at concentrations of 0.01, 0.1, 1, or 10×10^{-6} M for 24 hours. Subsequently, they were treated with auxin (3×10^{-5} M Naphthaleneacetic acid (NAA)) for 18 hours before histochemical staining of GUS activity. The results of histochemical staining showed that a concentration of 1×10^{-6} M cBSA/dsGUS RNA was sufficient to achieve effective gene silencing. This concentration aligns with the physiological concentrations of plant hormones or growth regulators applied externally, indicating its effectiveness. Figure 2A illustrates the successful silencing of GUS enzyme activity in representative *DR5-GUS* transgenic tobacco leaf disks treated with 1×10^{-6} M cBSA/dsGUS RNA nanocomplexes. Gene silencing effects were observed in all treated leaf disks (8 out of 8). Additionally, we administered cBSA/dsGUS RNA complexes into leaf tissues through petioles (Fig. 2B). Initially, we used intact leaves with petioles for administration and achieved excellent gene silencing effects. However, due to variations in leaf surface areas among individual leaves, we encountered some discrepancies in gene silencing effects. This disparity might be partially attributed to variations in the concentrations of cBSA/dsGUS RNA complexes within leaf tissues due to differences in leaf surface areas. To reduce these variations, we standardized the surface area of tobacco leaves by trimming them (Fig. 2B) to ensure more comparable concentrations of the nanocomplexes across all experimental leaves. Following 72 hours of administration, no evident changes in the appearance or health of the experimental leaves were observed. About 0.4 ml of cBSA/dsGUS RNA nanocomplex solution (1×10^{-6} M), which was about 6.4 μg of dsGUS RNA in total, was fed into each square centimeter of the experimental leaves during the 72 hours of feeding. We then treated the leaf tissues with NAA (3×10^{-5} M) for 18 hours before staining for GUS activity. More than 90% of experimental leaves (11 out of 12) fed with the cBSA/dsGUS RNA nanocomplexes displayed reductions in the auxin-induced GUS activities, while cBSA nanoparticles or naked dsGUS RNA alone did not result in any gene silencing effects (Fig. 2C). Additionally, qPCR analysis conducted after 24 hours of feeding further demonstrated that the mRNA level of the *DR5-GUS* gene in leaves (10 out of 10) was drastically reduced by cBSA/dsGUS RNA nanocomplexes but not by cBSA nanoparticles or naked dsGUS RNA alone (Fig. 2D). Also, as shown in Fig. 2E and F, auxin-induced expression of *GH3.1* (GenBank: AF123503.1) [31] and *ANN12* (GenBank: AY965682.1) [4] genes in all leaves was not affected by cBSA/dsGUS RNA nanocomplexes. These results support that the silence effect of the cBSA/dsGUS RNA nanocomplexes is gene specific. These results also demonstrate that the cBSA may facilitate effective systemic gene silencing effects.

We also fed the cBSA/dsGUS RNA nanocomplexes to the basal ends of detached tobacco shoots (Fig. 3A) and observed silencing effects on auxin-induced expression of the *DR5-GUS* gene in shoot apical meristem tissues (marked a in Fig. 3A) and apical leaves (marked b in Fig. 3A). Tobacco shoots, along with their leaves, were fed with a solution containing 1×10^{-6} M of cBSA/dsGUS RNA for 72 hours. At the end of feeding, the leaves of the shoots were removed, and the bare shoots were longitudinally split into two halves to ensure uniform auxin inducibility during the subsequent auxin treatment. Figure 3B shows that the auxin-induced *DR5-GUS* expression in 10 out of 12 shoot and shoot apex tissues (83%) was drastically reduced by the cBSA/dsGUS RNA complexes but not by the cBSA or dsGUS RNA alone. Figure 3C shows qPCR results that cBSA/dsGUS RNA effectively silenced the auxin-induced expression of the *DR5-GUS* gene at the mRNA level, with 85% and 65% reduction in the leaf and shoot meristem tissues, respectively, after 24 hours of feeding. Again, as shown in Fig. 3D, the auxin-induced expression of the *GH3.1* and *ANN12* genes were not affected by the cBSA/dsGUS RNA nanocomplexes in all shoot tissues, including shoot apex tissues.

Systemic silencing effects of the cBSA/dsGUS RNA nanocomplexes on constitutively active genes

To explore efficiencies of the cBSA/RNA nanocomplexes on silencing of constitutively active genes, leaves of *35S-GUS* transgenic tobacco and poplar plants were detached and fed with the cBSA/dsGUS RNA complexes (1×10^{-6} M) through petioles (Fig. 4A and B). As shown in Fig. 4A, we observed 60% and 70% of the constitutively active *35S-GUS* gene in all experimental leaves (6 out of 6) of poplar and tobacco, respectively. The cBSA nanoparticles or naked dsGUS RNA had no silencing effects on expression of the *35S-GUS* gene in either plant species. We also administered the cBSA/dsGUS RNA nanocomplexes to the basal ends of *35S-GUS* transgenic poplar shoots (Fig. 4C). Consequently, using a qPCR technique, we detected approximately 60% reduction in the *35S-GUS* mRNA levels in shoot apical/meristem tissues in all experimental poplar shoots. Throughout the feeding period of 72 hours, about 6 ml of cBSA/dsGUS RNA nanocomplexes (about 96 μg of dsGUS RNA in total) was absorbed by each poplar shoot, including its leaves. These findings demonstrate the effectiveness of cBSA/dsGUS RNA nanocomplexes when applied to the basal ends of stem cuttings, as they can systematically silence constitutively active genes in shoot apex tissues.

Further, we have also shown that cBSA/dsPAP2 RNA nanocomplexes are effective to silence expression of the *Production of Anthocyanin Pigment 2* (*PAP2*) gene. Feeding the cBSA/dsPAP2 RNA nanocomplexes through petioles of *35S-PAP2* transgenic tobacco, we observed a 35% reduction in the *35S-PAP2* expression in all of the feeding leaves (6 out of 6) (Fig. S4). We used oxidative stress-inducible *RBOHB* gene (accession number: Ntab0768440 in http://www.tobaccodb.org/) [8, 39, 41] to monitor whether cBSA/dsGUS RNA nanocomplexes may cause unintentional stresses to the plant tissues and therefore alter expression of some genes non-specifically. Figure 4A shows that cBSA/dsGUS RNA nanocomplexes do not alter *RBOHB* gene expression, indicating that the silencing effects on the *35S-GUS* gene should be gene-specific.

Discussion

RNA interference (RNAi) technologies have significant potential for enhancing the productivity of agricultural crops. However, the production of RNAi transgenic plants often involves expensive and time-consuming processes. Early studies demonstrated gene silencing in plants using naked RNA, but more recent studies have shown that the application of naked RNA molecules does not result in gene silencing in plants [5, 6, 10, 40–42]. We believe that naked dsRNA molecules are unlikely to enter plant cells due to their negatively charged nature, which hinders their ability to cross the plasma membrane.

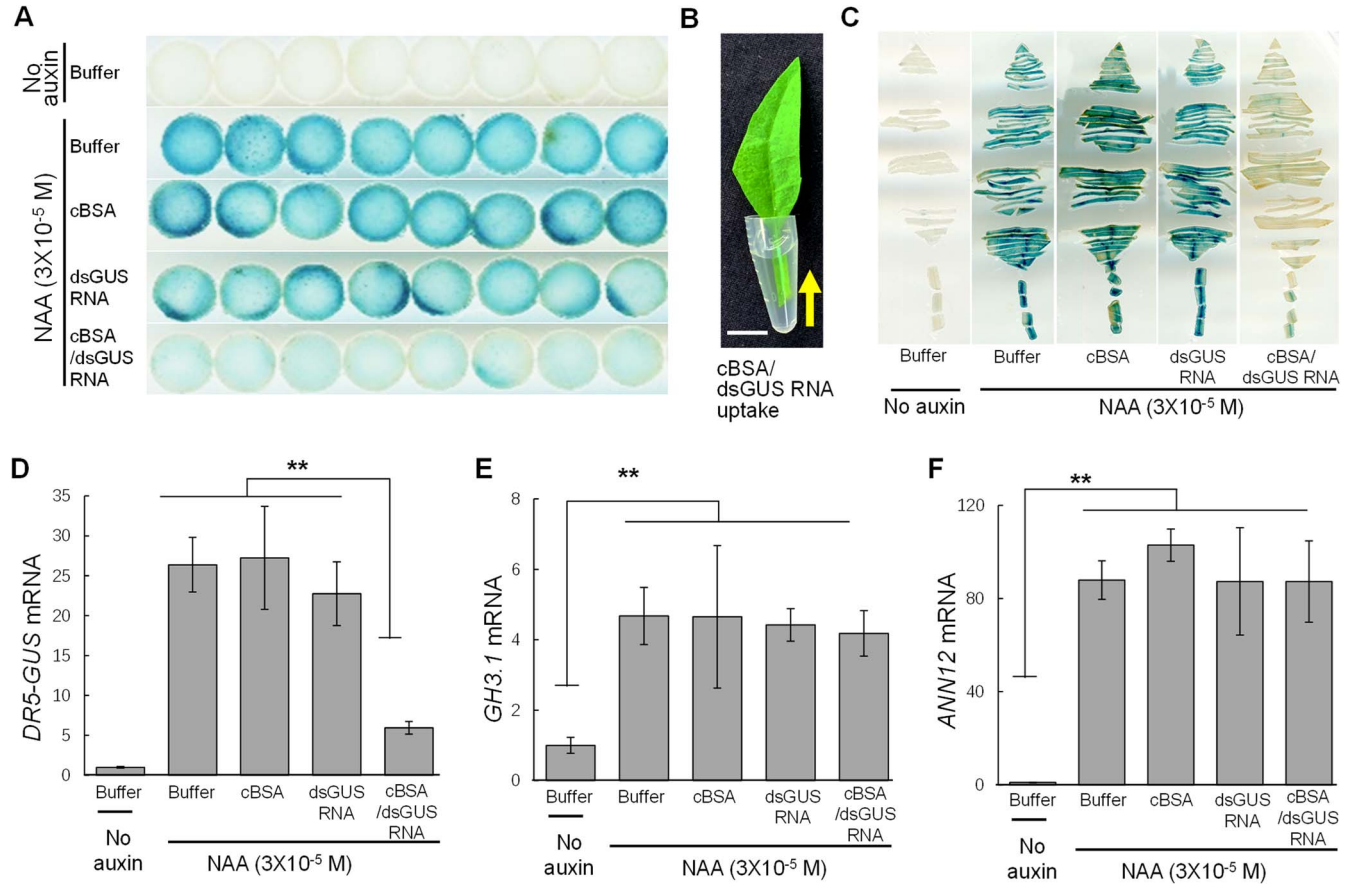

Figure 2. Systemic silencing effects of cBSA/dsGUS RNA nanocomplexes on expression of auxin-inducible *DR5-GUS* gene in tobacco leaves. (**A**) Histochemical staining of GUS activity shows that cBSA/dsGUS RNA nanocomplexes effectively reduced auxin-inducible GUS activity in the *DR5-GUS* transgenic tobacco leaf disks. The leaf disks were incubated in cBSA/dsGUS RNA solution (1×10^{-6} M) for 24 hours and then treated with auxin (3×10^{-5} M NAA) for 18 hours prior to histochemical staining of GUS activity. (**B**) cBSA/dsGUS RNA nanocomplexes were fed into leaf tissues from basal ends of leaf petioles. The feeding lasted for 72 hours and each square centimeter of leaf tissues received about 0.4 ml solution of cBSA/dsGUS RNA nanocomplexes (1×10^{-6} M). Scale bar, 0.5 cm. (**C**) Histochemical staining of GUS activity of *DR5-GUS* transgenic tobacco leaves shows BSA/dsGUS RNA nanocomplexes effectively reduced GUS activity. (**D–F**) qPCR analysis of expression of the *DR5-GUS* gene (**D**), two auxin-inducible genes *GH3.1* (**E**), and *NtANN12* (**F**) in the treated leaves shows the GUS transcript was drastically reduced by cBSA/dsGUS RNA nanocomplexes while expression of the *GH3.1* and *NtANN12* was not affected. *Elongation Factor 1α* gene (*NtEF1α*), a housekeeping gene, was used for normalizing the expression levels of all genes. ** indicates significant differences at $P \leq 0.01$ by ANOVA. The data presented are means ± AVEDEV, which have been calculated from a minimum of six biological replicates.

Traditional genetic engineering is a time-consuming process and often genotype dependent. Nanoparticle-mediated gene transfer or gene silencing does not rely on plant tissue culture, thus eliminating the need for this time-consuming process of optimizing various culture parameters [28]. Nanoparticles have been successfully utilized to facilitate DNA and RNA delivery into plant cells [10, 17, 40–42]. For instance, nano-RNA complexes have been utilized to facilitate the delivery of RNA molecules across the plant cell plasma membrane, leading to the silencing of target genes. However, the silencing effects of these complexes are somewhat limited to the local areas of application [10, 17, 40–42]. Also, some nanoparticles such as layered double hydroxide (LDH) nanosheets have been shown to facilitate the long-distance transport or systemic silencing effects of dsRNA molecules [20, 24, 29]. However, it is unknown whether these long-distance transported dsRNA nanocomplexes can penetrate plant cell membrane and silence plant genes, as these studies have primarily focused on investigating their impact on gene expression in insects, fungi, or viruses that infected the experimental plants [20, 24, 29]. As shown here, our cBSA/dsRNA nanocomplexes may present a highly useful and more convenient tool that not only enables the delivery of RNA molecules into plant cells but also facilitates their long-distance transport or systemic gene silencing effects within a plant.

Developing nano-RNA delivery technologies that can be applied to plants without the need for external forces is highly desirable. In previous studies, the delivery of nano-RNA or -DNA complexes into leaf tissues often required the use of external forces, such as needle-less syringe-mediated infiltration [8, 10, 17, 40–42]. Nevertheless, our cBSA/dsRNA nanocomplexes can be easily administered to plants by feeding them through the basal ends of leaf petioles or stems/shoots without relying on external forces such as high-pressure-mediated infiltration.

The small size of the cBSA/dsRNA nanocomplexes, their strong association, and their neutral charge provide possible reasons for their efficient long-distance transport or systemic effects in plant tissues. BSA is a globular monomeric protein of molecular mass of 65.4 kDa with 6-nm diameter [2]. To minimize the nanocomplex size for effective transport in the plant and across cell membranes, we adjusted the charge on cBSA to obtain a soluble RNA complex at 1:1 mole ratio. The cBSA is nontoxic to the plants and highly water soluble, enabling dsRNA transport over long-distances or triggering systemic gene silencing within the plant.

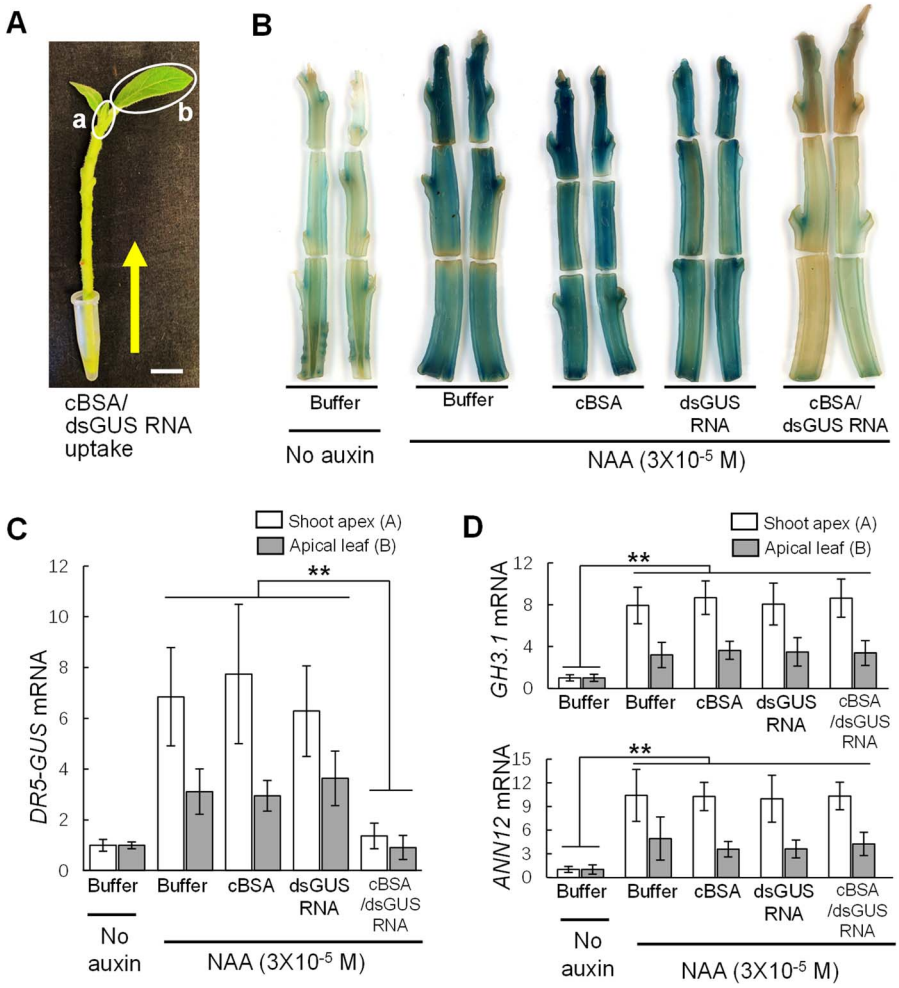

Figure 3. Systemic silencing effects of cBSA/dsGUS RNA nanocomplexes on auxin-induced expression of the *DR5-GUS* gene in tobacco shoots. (**A**) cBSA/dsGUS RNA nanocomplexes were fed into about 10-cm-long shoots from their basal ends. The feeding lasted for 72 hours, and a total of about 10-ml solution of cBSA/dsGUS RNA (1×10^{-6} M) was fed into each shoot. Scale bar, 1.0 cm. (**B**) Histochemical staining of GUS activities in *DR5-GUS* transgenic tobacco shoots fed with buffer, cBSA nanoparticles, naked dsGUS RNA, and cBSA/dsGUS RNA nanocomplexes. The treated shoots were split longitudinally into two halves after 72 hours of feeding and treated with auxin (3×10^{-5} M NAA) for 18 hours before histochemical staining of GUS activity, showing that cBSA/dsGUS RNA complexes effectively silenced the *DR5-GUS* gene expression. (**C–D**) qPCR analysis of expression of the *DR5-GUS* (**C**) and auxin-inducible *GH3.1* and *ANN12* (**D**) genes in shoot apical tissues (marked a in Fig. 3**A**) and leaves (marked b in Fig. 3**A**), demonstrating that cBSA/dsGUS RNA nanocomplexes silenced *DR5-GUS* gene at the mRNA level and also the effects are specific. Expression levels of *NtEF1α* were used for normalizing the expression levels of all genes assayed. The white and gray filled bar graphs represent apical meristem tissues (marked a in Fig. 3A) and leaf tissues (marked b in Fig. 3A), respectively. ** indicates significant differences at $P \leq 0.01$ by ANOVA. The data presented are means ± AVEDEV, which have been calculated from a minimum of three biological replicates.

Therefore, the cBSA-based gene delivery platform is facile, bioactive, environment-friendly, highly water soluble, scalable, and inexpensive to be multiplexed for silencing multiple gene targets.

The efficient long-distance transport or systemic silencing characteristics of the cBSA/dsRNA nanocomplexes may make the technology versatile for other applications. In the case of long-distance transport of the RNA molecules, one potential application is that our cBSA/dsRNA delivery technology may facilitate delivery of gRNA molecules to shoot meristem cells of *Cas9* expressing plants, so that *"in planta"* gene editing may be achieved. For this, gRNA nanocomplexes can be applied to the basal ends of shoots/stems or directly injected into the stem tissues of a *Cas9* expressing platform plant. The cBSA nanoparticles may expedite the transport of gRNAs to the shoot meristem cells, where gene editing may occur. Consequently, emerging shoots originating from these meristem cells can be gene edited. This method holds promise due to its simplicity and efficiency, eliminating the need for tissue culture, plant regeneration, or repeated genetic transformations for every gRNA introduced. Although these characteristics are highly desirable [28, 30], it needs to be experimentally demonstrated in the future how effectively BSA nanoparticles can facilitate the delivery of gRNA into meristem tissue for the purpose of *in planta* gene editing.

Another potential application is that the cBSA/dsRNA nanocomplexes might be used to transiently silence target genes in large-scale, field-grown plants, particularly orchard trees [16, 23], through trunk injection [1] for enhancing productivity. For example, silencing of the farnesyltransferase, *RACK1*, *OsGRXS17*, and *BrDST71* genes can enhance plant's drought tolerance [15, 19, 27, 37]. By suppressing the expression of the *AGAMOUS-like* gene in fruit can generate coreless fruits [16]. Similarly, inhibiting the expression of *MdSUMO2* gene can promote plants better growth potential under nitrogen deficiency stress [23]. Transient silencing of these genes using the cBSA/dsRNA technology may offer a safe, low-cost, nontransgenic approach for enhancing productivities of field-grown crops. Currently, the cost for dsRNA production is less

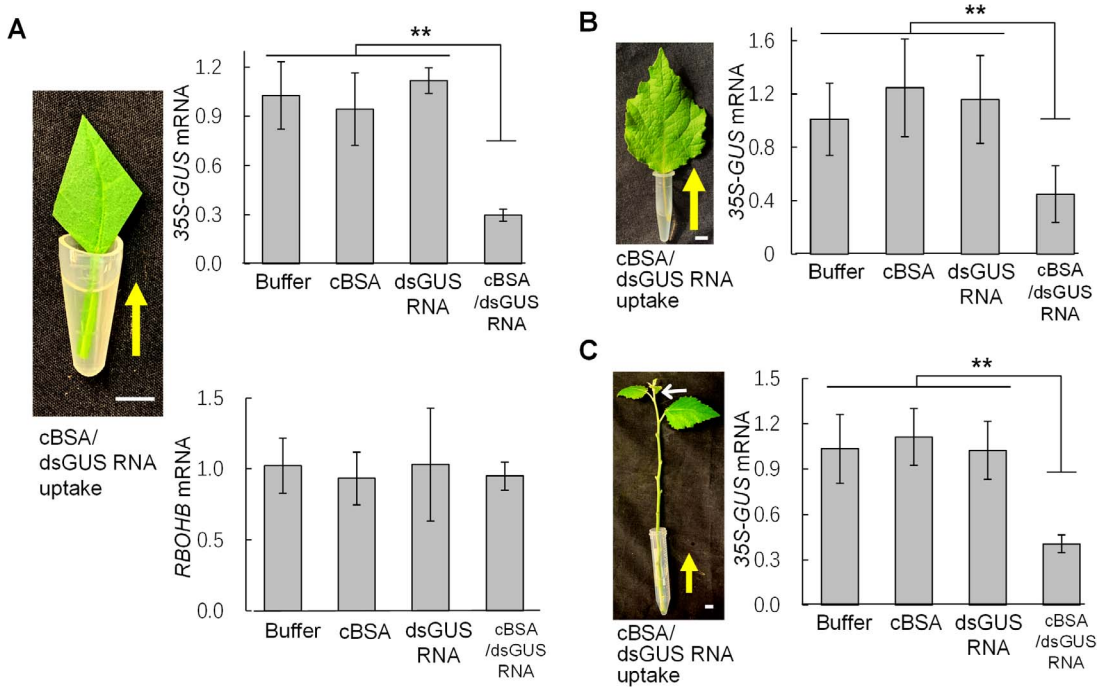

Figure 4. Systemic silencing effects of cBSA/dsGUS RNA nanocomplexes on expression of constitutively active 35S-*GUS* gene in tobacco and poplar. (**A**) Constitutively active 35S-*GUS* gene in tobacco leaves was specifically silenced at the transcript level by cBSA/dsGUS RNA nanocomplexes while expression of an oxidative stress-inducible *RBOHB* gene was not affected. cBSA/dsGUS RNA nanocomplexes were fed from basal ends of leaf petioles for 5 days, and approximately 0.6-ml cBSA/dsGUS RNA nanocomplex solution (1×10^{-6} M) was fed into each square centimeter of the leaves. Scale bars, 1.0 cm. (**B**) Constitutively active 35S-*GUS* gene in poplar leaves was specifically silenced at the transcript level by cBSA/dsGUS RNA nanocomplexes fed from the basal ends of petioles. The feeding lasted for 5 days, and approximately 0.5-ml nano-dsGUS RNA nanocomplexes solution (1×10^{-6} M) was fed into each square centimeter of the leaves. Scale bars, 1.0 cm. (**C**) Constitutively expressed 35S-*GUS* gene in poplar shoot meristem tissues (white arrow pointed) was specifically silenced at the transcript level by cBSA/dsGUS RNA nanocomplexes fed from the basal ends of 15-cm-long shoots. The feeding lasted for 6 days, and a total of about 6-ml cBSA/dsGUS RNA nanocomplexes solution (1×10^{-6} M) was fed into each shoot. Scale bars, 1.0 cm. For all qPCR analyses, expression levels of *NtEF1α* or *PtEF1α* were used as internal controls for normalizing expression levels of the 35S-*GUS* gene. ** indicates significant differences at $P \leq 0.01$ by ANOVA. The data presented are means ± AVEDEV, which have been calculated from a minimum of three biological replicates.

than $0.5/g ($500/kg) with no length limitation, and the cost of the short dsRNA production will be further reduced in future [33], which can make large-scale field applications of nano-dsRNA complexes economically feasible.

Conclusions

In summary, we have developed and used environmentally friendly and low-cost cBSA/dsRNA nanocomplexes for systemic gene silencing in higher plants effects. We have successfully shown that the cBSA/dsRNA nanocomplexes are highly efficient in systematically silencing both conditionally inducible and constitutively active genes in tobacco and poplar. The cBSA/dsRNA delivery technology described here may provide a convenient, fast, and nontransgenic tool to manipulate gene expression in higher plants that may be used for functionally characterizing novel genes, for *in planta* gene editing, and for enhancing crop productivity under field conditions.

Materials and methods

Material preparation

Bovine serum albumin (BSA) was purchased from Equitech-Bio Inc. (Kerrville, TX). EDA, *N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide hydrochloride, and 98% EDC [1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride] were purchased from Sigma Aldrich. SYBR Safe nucleic acid gel staining dye was obtained from Invitrogen (Fisher Scientific, MA). All chemicals were used directly without any further purification. Dialysis membrane (25 kDa molecular weight cutoff (MWCO)) was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Biology grade agarose was purchased from Hoefer Inc. (Holliston, MA). Two pieces of dsGUS RNA (GUS1 and GUS4), which were fully complementary to the GUS mRNA, were synthesized using the cell-free bioprocessing platform of Genolution (Seoul, South Korea). The GUS1 and GUS4 dsRNA sequences, which have lengths of 126 and 127 bp, respectively, were combined and then subjected to sonication to produce shorter fragments ranging from 20 to 30 bp in length. These fragmented sequences were then used for the further experiments (see Table S1 for the sequences of dsGUS RNA).

Synthesis of the cationized BSA

cBSA protein nanoparticles were prepared based on a modified version of previously reported procedure [18], which is described here. A stock solution of EDA (0.5 M, 50 ml, pH 5.1) was prepared with deionized water. Next, BSA solution (0.1 g in 8 ml of 0.1 M phosphate buffer, pH 5.1) was added to 1.5 ml of the EDA stock solution. The mixture was stirred for 15 minutes at room temperature, and then a fresh solution of 0.5-ml EDC (0.1 M, 0.5 ml in deionized water) was added to the mixture, which was stirred continuously. The final molar ratios of BSA/EDA/EDC were 1:200:200 (total of 10 ml of reaction volume), respectively. The mixture was stirred for 4 hours at room temperature and the

condensation reaction was stopped by adding 1 ml of acetate buffer (4 M, pH 4.75) to each reaction mixture. The excess EDA, EDC, and byproducts were removed by dialysis against a 10 mM sodium phosphate buffer at pH 7.2 using a 25 000 Da cutoff dialysis membrane for 2 days. After dialysis, the product yield cBSA was 80% (~8 mg/ml) as estimated by BSA absorbance at 280 nm and the known extinction coefficient. The net charge on cBSA was confirmed by Zeta potential measurements described below. The degree of chemical modification was carefully controlled by adjusting the EDA and EDC concentrations such that modified BSA charge varied gradually from a net negative to a net positive value.

Synthesis of the cBSA/dsGUS RNA nanoparticle soluble complexes

cBSA/dsGUS RNA complex nanoparticles were prepared by adding dsGUS RNA (20 μM, 5 ml) to a cBSA solution (20 μM, 5 ml) at various N/P (nucleic acid and protein) molar ratios followed by immediate gentle mixing. When conducting the bulk and plant feeding experiments, we prepared a dilute cBSA (75 μM, 5 ml, pH 7) solution mixed with dsGUS RNA (75 μM, 5 ml) to make cBSA/dsGUS RNA nanocomplexes with the required molar ratios, and the resulting solutions were incubated for 1 day. During this step, the resulting complex solutions were precipitated, then diluted to <1 μM concentration to solubilize the protein–nucleic acid complexes. The unbound protein or dsGUS RNA was removed by precipitation of the complexes (high concentration >10 μM) followed by centrifugation and redispersal of the nanocomplexes in buffer solution. When performing bulk preparation, cBSA/dsGUS RNA complexes were mixed in a 1:1 molar ratio (1 μM, 10 ml), and the nanocomplexes were equilibrated for 1 day; these complexed nanoparticles were further used in the plant feeding experiments, unless otherwise noted.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed using a method reported earlier, using a horizontal gel electrophoresis apparatus (Gibco model 200; Life Technologies Inc., MD) and agarose (0.5% w/w) in Tris acetate (40 mM, pH 8.2) buffer. Modified cBSA conjugates were loaded with 50% loading buffer (50% v/v glycerol and 0.01% w/w bromophenol blue). Detection of the dsGUS RNA and cBSA/dsGUS RNA complex samples was accomplished through additional staining with SYBR Safe DNA gel staining dye. Samples were dispensed into wells placed in the middle of the gel to allow the protein to migrate toward the negative or positive electrode, based on net surface charge. The formation of cBSA/dsGUS RNA complexes was confirmed by a gel shift assay in native agarose gel electrophoresis. A potential of 100 V was applied for an appropriate duration, and gels were stained overnight with 10% v/v acetic acid and 0.02% w/w Coomassie blue, followed by destaining in 10% v/v acetic acid overnight.

SDS-PAGE gel electrophoresis

Prepared polyacrylamide separating gels (12.5% w/w) and stacking gels (5% w/w) were directly used for SDS-PAGE gel to run the samples. The separating gel was poured into a 1.25-mm mold and allowed to polymerize for 30 minutes (6.5 cm long), after which the stacking gel was poured and similarly allowed to polymerize (1.5 cm long). Samples were prepared by combining 10 μL of 100 μM BSA-EDA conjugates with 30 μL SDS-PAGE loading buffer (2% w/v SDS, 10% w/w 2-mercaptoethanol), after which the solutions were heated at 90°C for 2 minutes and 20 μL of each sample solution was loaded per well. Gels were run in a vertical Bio-Rad Mini electrophoresis apparatus at 200 V until the dye front reached the bottom of the gel (~45 minutes). Eight hundred milliliters of SDS-PAGE running gel buffer [25 mM Tris, 192 mM glycine, 3.47 mM (0.1%) SDS, pH 8.3] was used for single gel run experiment. The gel was stained with Stain A solution (10% v/v acetic acid, 10% v/v isopropanol, and 0.02% Brilliant blue R250) for 4 hours, followed by Stain B solution (20% v/v acetic acid and 0.03% Brilliant blue R250) for 4 hours. Gels were destained with 10% acetic acid overnight prior to imaging.

Dynamic light scattering

The hydrodynamic radii of dsGUS RNA, cBSA, and cBSA/dsGUS RNA complexes were measured using a CoolBatch DLS apparatus as reported earlier, using Precision Detectors (Varian Inc., NJ) with a 0.5×0.5 cm^2 cuvette and a 658-nm excitation laser source at 90° geometry. The samples of the dsGUS RNA, cBSA, and the nanocomplex (20 μM) were diluted with 1 ml of phosphate buffer (100 mM, pH 7.2) and equilibrated for 300 seconds at 25°C with 5 repetitions and 100 accumulations. Precision Elucidate (v1.1.0.9) was used to run the experiment, and Deconvolve (v5.5) was used to process the data.

Zeta potential studies

The surface charge of cBSA and dsGUS RNA as well as complexed nanoparticles was characterized using Zeta potential analyzer. The samples were prepared by mixing 15 μM with 1 mM KCl electrolyte solution. The Zeta potential was from the Zeta Potential Analyzer (Brookhaven Zeta Plus, Holtsville, NY). The sample volume of 1.6 ml was taken in a polystyrene cuvette, then the electrode for the Zeta Potential Analyzer was immersed in the solution and connected to the instrument. The sample's Zeta potential was calculated using laser Doppler velocimetry and Smoluchowski fit of the instrument. Each measurement is the average of three runs with triplicates of different samples, and the standard deviation is calculated.

Circular dichroism study

CD spectroscopy was used to monitor the retention of secondary structure or any conformational changes in dsGUS RNA, cationic BSA (cBSA), and cBSA/dsGUS RNA nanocomplexes. The change in the ellipticity was recorded using a Jasco 710 spectropolarimeter. Each sample concentration was around 5 μM dissolved in 10 mM phosphate buffer (pH 7.2) to record the ellipticity values. Spectra were obtained using a 1 cm path length quartz cuvette from 300 to 200 nm. While collecting the data, the sensitivity of samples was set as 100 mdeg and the data pitch as 1 nm, with continuous scanning mode, 50 nm min^{-1} scanning speed, 1-second response, and 1.0 nm bandwidth. The baseline was measured with 10 mM phosphate buffer (pH 7.2) and subtracted from each spectrum. Three scans were used to get the average data, which were then normalized to a millimolar concentration of the RNA or protein per unit path length.

Plant growth

Transgenic *DR5-GUS* (*N. tabacum*), *35S-GUS* (*N. tabacum* and *Populus alba* × *P. berolinensis* hybrid clone), and *35S-PAP2* (*N. tabacum*) seeds were germinated, and seedlings were grown in a substrate consisting of a mixture of soil and vermiculite (3:1). The air relative humidity was approximately 80%. The tobacco seedlings were grown for 3–4 weeks and the poplar seedlings were grown for 5–6 months under a light intensity of 400 $mol \cdot m^{-2} \cdot s^{-1}$ before being used for experimentation.

Nanocomplexes and auxin treatment of plant tissues

The *DR5-GUS* and *35S-GUS* transgenic tobacco leaves were cut into a diamond shape with the leaf petiole in the middle. Leaves of similar size were used for the feeding experiment; the cBSA/ds-GUS RNA complexes were fed through the basal ends of leaf petioles. For poplar leaves, intact young leaves were used, and the nanocomplex feeding experiment was conducted through the basal ends of leaf petioles. To maintain plant growth, 1× Hoagland medium (Sigma-Aldrich) was added to the feeding solution every 2 days. Before auxin induction, the leaf tissues were cut into slices. Additionally, leaf disks of 0.8 cm in diameter were cut out from *DR5-GUS* transgenic tobacco leaves using a hole puncher. Subsequently, these leaf disks were immersed in the cBSA/dsGUS RNA (1×10^{-6} M) nanocomplex solution with gentle agitation for 24 hours before auxin induction with a light intensity of 100 $mol{\cdot}m^{-2}{\cdot}s^{-1}$.

The *DR5-GUS* tobacco shoots, which were approximately 10 cm long, were detached with two apical young leaves left. The detached shoots were then fed with cBSA/dsGUS RNA complexes from their basal ends. The *35S-GUS* poplar leaves, with the petiole left intact, were directly used in the feeding experiment by inserting the basal ends of leaf petioles into the feeding solution with a light intensity of 100 $mol{\cdot}m^{-2}{\cdot}s^{-1}$. The *35S-GUS* poplar shoots (~15 cm long) were detached with two apical young leaves left, and then were fed with nanocomplexes from their basal ends. To maintain plant growth, 1× Hoagland medium was added to the feeding solution every 2 days. The shoot tissues were split longitudinally into two halves before auxin induction.

For the auxin treatment, all leaf and shoot tissues were incubated in an NAA solution (3×10^{-5} M) with gentle agitation for 18 hours with a light intensity of 100 $mol{\cdot}m^{-2}{\cdot}s^{-1}$ before histochemical staining of GUS activity and qPCR.

Histochemical staining of GUS activity

Histochemical assays for GUS activity were conducted using the method described by Li *et al.* [22]. The tissues were immersed and incubated in the GUS dye solution at 37°C for 12–20 hours. Pigments in the tissues were removed through incubation in an increasing ethanol gradient, which progressed as follows: 20, 40, 60, 80, and 95%. After the pigments were fully removed, the tissues were rehydrated serially with 80, 60, 40, 20, and 0% ethanol before being analyzed photographically.

qPCR analysis

The qPCR was performed to quantify the expression of target genes in plants using the following commercially available kits: NucleoSpin RNA (MN, Germany) for total RNA extraction, iScript cDNA synthesis kit (Bio-Rad, USA) for reverse transcription of total RNA into cDNA, and iTaq universal SYBR green supermix (Bio-Rad, USA) for qPCR. The target genes for qPCR were *GUS*, *NtANN12*, and *RBOHB*. The expression levels of *NtEF1α* in tobacco (GenBank: AF120093) [32] and *PtEF1β* in poplar (GenBank: eugene3.00091463) [36] were used to normalize the expression levels of each target gene. The sequences of each pair of primers are listed in Table S2. All steps followed the guidelines of MIQE (Minimum Information for Publication of Quantitative Real-time PCR Experiments).

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Author Contributions

Yi Li and C.K. conceived the study. Yi Li, C.K., H.S., A.K., X.Y., G.A.T., Z.D., F.G.G., and H.D. were involved in the experimental design. H.S., A.K., D.T., J.D., L.Z., X.G., Yanjun Li, H.Y., L.Z., and X.G. performed the experiments. Yi Li, C.K., H.S., and A.K. conducted the data analysis. Yi Li, C.K., H.S., A.K., X.Y., G.A.T., Z.D., and F.G.G. wrote and edited the manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding authors upon request, and the detailed information of primer and dsRNA sequences used in this study is provided in supplementary documents.

Conflict of Interests

The authors declare no conflict of interest.

Supplementary data

Supplementary data is available at *Horticulture Research* online.

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