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# Systemic Gene Silencing in Plant Triggered by Fluorescent Nanoparticle Delivered Double-Stranded RNA

Li Jiang,[#a] Lian Ding,[#a] Bicheng He,[a] Jie Shen,[a] Zejun Xu,[b] Meizhen Yin*[b] and Xiaolan Zhang*[a]

A cationic fluorescence nanoparticle efficiently enters into plant with high transfection efficacy. Applying the mixture of G2/dsRNA to the model plant *Arabidopsis* root leads to significant knock-down the expression of important developmental genes and results in apparent phenotypes. This work reports a non-viral gene nanocarrier which triggers gene silencing in plant and leads to systemic phenotypes.

RNA interference (RNAi) is a process of post transcriptional gene silencing (PTGS) that triggered by introduction of double-stranded RNA (dsRNA) in the cells resulting in specific degradation of target mRNA.[1] dsRNA mediated RNAi has been reported to occur conservatively in different kingdoms including fungi, plant and animal system, and has emerged as a powerful tool for viral defence, gene function studies, crop improvement, transposon taming, and gene therapy.[2] Despite dsRNA can be easily delivered into animals by oral feeding or microinjection,[3] it is much more challenging to introduce dsRNA into plants, partly because of the obstruction from the rigid plant cell wall. There are generally two methods to introduce dsRNA-RNAi in plants, one is Agrobacteria T-DNA mediated stable genetic transformation,[4] and the other is transient virus-derived vector mediated gene silencing. [4, 5] However, both the transgenic technique and virus derived vectors are time-consuming, have species-dependent efficiency and potential harm to human health.[6] Therefore, it is necessary to explore more simple and efficient methods to utilize RNAi in plant.

Recently, non-viral carriers such as functionalized cationic polymers,[7] dendrimers[8] and nanoparticles[9] can rapidly enter into live animal cells and exhibit low cytotoxicity as well as high gene delivery efficiency. The cationic fluorescence nanoparticles G1-G3 (**Scheme 1** and Scheme S1) have been demonstrated as promising non-viral vectors for delivering DNA into live animal cells.[8a] Up to now, however, there is no report of non-viral gene-vectors worked in plant. To explore the possibility of delivering nuclei acid into plant cells by using non-viral carrier, we preliminary screened some different types of gene carriers (G1, G2, and G3), which possesses a central perylene-3,4,9,10-tetracarboxdiimide (PDI) chromophore and

precise cationic groups at the periphery. The fluorescence nanoparticles have good water solubility (>10 mg/mL) and high photostability. The maximum emissions of nanoparticles in water are around 617 nm which can be traced in red channel of a fluorescence microscope.[8a]
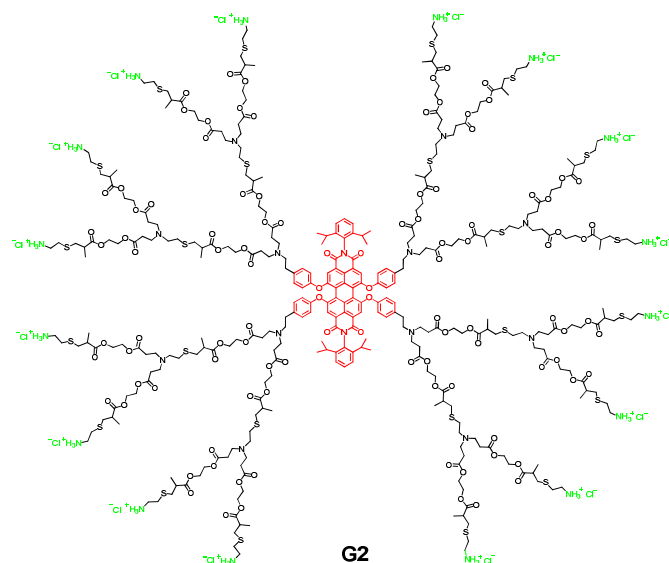
Scheme 1. Chemical structure of G2. The central PDI chromophore (red) can emit red fluorescence. The peripheral cationic groups (green) provide water-solubility and can electrostatic bind DNA and dsRNA for cell uptake.

The dynamic light scattering (DLS) data of G1, G2, and G3 in water and the complex sizes of nanoparticle and DNA are shown in Table 1. The appropriate sizes of complexes below 200 nm imply that the nanoparticles are in suitable size for *in vitro* and *in vivo* applications.[10] We found that the nanoparticles can efficiently enter into plant cells and act as an excellent non-viral carrier for efficient delivery of DNA and dsRNA. Further, the nanoparticles delivered dsRNA efficiently knocked down the expression of two important

developmental genes, demonstrating systemic RNA interference in the plant system.

Table 1. Dynamic light scattering (DLS) data of G1, G2, and G3, and the complex sizes of nanocarrier and DNA at N/P=8:1 in water.

| No. ⁻	Diameter (nm)
G1	2.2±0.5
G2	3.6±0.3
G3	6.4±0.2
G1/DNA	129.9±1.1
G2/DNA	136.5±3.4
G3/DNA	136.6±2.1

The ability of delivering DNA into animal cells *in vitro* by cationic fluorescence nanoparticles (G1, G2, and G3, Scheme S1) has been demonstrated previously.[8a] To explore whether they can deliver DNA through the rigid plant cell wall, we mixed these nanoparticles (red) with CXR reference dye-labeled DNA (blue) at N/P ratio 2:1 (charges ratio of nanoparticle/DNA), and applied the mixture to the root tip of 10-day-old wild-type *Arabidopsis* seedling. As shown in Fig. S1, all the three nanoparticles can deliver DNA into *Arabidopsis* root. The delivery efficiency was different, with G1 the lowest (Fig. S1 A-D) and G3 the highest (Fig. S1 I-L). G2 has middle delivery efficiency but is very close to the effect of G3 (Fig. S1 E-H). The lower delivery efficacy of G1 may due to its smaller outer branches and less positive charges. All the treated seedlings developed normally indicating that these nanoparticles have no any impact on plant development.

Considering that G2 has satisfactory delivery efficiency, reasonable synthesis cost as well as relatively simple production steps for widely use in future as a general tool for gene delivery *in vivo*, we chose G2 as the gene carrier for further application. Next, the ability of G2 to penetrate different parts of the *Arabidopsis* root was investigated (Fig. 1). The root tip is an actively dividing tissue covered by a protective, thimble-shaped root cap, while the middle part of the root is generally covered by tubular outgrowth of root hair cells. The complex of G2/DNA was pipetted to the root tip (arrow in Fig. 1A) or the middle part of root (arrow in Fig. 1F). After 24 h, we observed the fluorescent images by fluorescence microscopy. Both G2 (red) and DNA (blue) were detected in the *Arabidopsis* root away from the site of application, moving upward (Fig. 1B-1E) as well as downward (Fig. 1G-1J), indicating that G2 can pass through the cell wall of root cap and root hair, and deliver DNA efficiently throughout the root tissues. While treatment with water control (Fig. 1K-1L) or DNA alone (Fig. 1M-1N) showed no detectable fluorescence signal in the root, suggesting that DNA alone cannot penetrate into plant cells by itself.
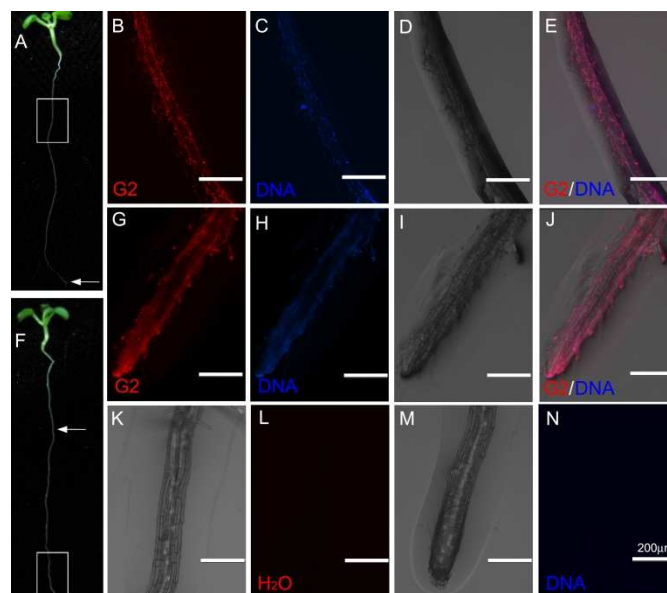
Fig. 1 Fluorescence images of G2 and DNA in *Arabidopsis* root.
Dendrimer G2 can deliver DNA into plant system through both root tip (A-E) and root hair (F-J), in which A and F are the schematic images showing the G2/DNA application sites (arrows) and the corresponding observation areas (boxes) after 24 h. Fluorescence images of the boxed areas in A and F are shown in (B-E) and (G-J), respectively. G2 is shown in red (B, G), DNA is labeled by CXR Reference Dye in blue (C, H). D and I represent the bright field; E and J show the corresponding merged field. Seedlings treated with H₂O (K-L) or DNA (M-N) alone are used as negative controls and show no fluorescence signal. Bars=200μm.
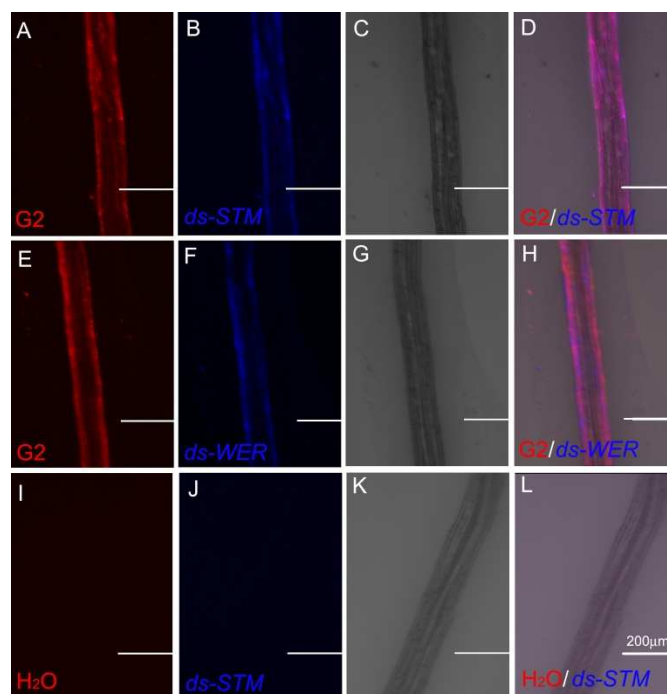

Fig. 2 Fluorescence images of G2 and dsRNA in *Arabidopsis* root.
(A-D) Fluorescence images of G2/ds*STM* after 24 h treatment in *Arabidopsis* root (red: G2, blue: ds*STM* labeled with CXR Reference Dye). (E-H) Fluorescence images of G2 and ds*WER* after 24 h treatment in *Arabidopsis* root (red: G2, blue: ds*WER* labeled with

CXR Reference Dye). (I-L) *Arabidopsis* root treated with H_2O/dsSTM serves as negative control. Bars=200µm.

Since double-stranded RNA (dsRNA) is the key initiator of post-transcriptional gene silencing,[11] and is of great importance for gene function studies, we next explore whether G2 can deliver dsRNA into plant cell. Two genes were chosen to make dsRNA: *SHOOT MERISTEMLESS* (*STM*) and *WEREWOLF (WER)*. *STM*, Class I knotted-like homeodomain protein, is specifically expressed in the shoot apical meristem (SAM) and required for SAM formation and maintenance throughout the plant lifecycle.[12, 13] *WER,* a R2R3 type MyB-related transcription factor, is specifically expressed in the root epidermal cells and is essential for specifying the non-hair identity in the root epidermis.[14] *dsSTM* and *dsWER* were synthesized and labeled with the blue-emitting CXR reference Dye, and then the mixture of G2/*dsRNA* was applied to the root tip of *Arabidopsis*. After 24 h, the blue signal was observed throughout the root system (Fig. 2A-2H), whereas the *dsSTM* (Fig. 2I-2L) or *dsWER* (data not shown) alone showed no signal, suggesting that G2 can efficiently deliver dsRNA into plant cells. Thus, G2 can act as a non-viral gene carrier that efficiently delivers both DNA and dsRNA into plant cells.

Subsequently, we test the possibility of whether G2-delivered dsRNA can silence gene expression in plant cells. After 3 days continuous treatment with G2/dsRNA complexes at N/P ratio 2:1 (1µg dsRNA, once per 24 h) on the root tip of *Arabidopsis*, quantitative real time RT-PCR analysis was used to examine the transcription levels of *STM* and *WER* in the treated seedlings versus H_2O/dsRNA control. As shown in Fig. 3, the expression of both *STM* and *WER* were significantly suppressed. The transcription levels of *STM* and *WER* were decreased to 16% and 13% of the corresponding control treatment, respectively (Fig. 3A and 3B). Semi-quantitative RT-PCR verified such repression (Fig. 3C and 3D), suggesting that G2-delivered dsRNA can interfere the endogenous gene expression in *Arabidopsis*. This is the first report of utilizing a non-viral gene delivery system that triggers gene silencing in plant.
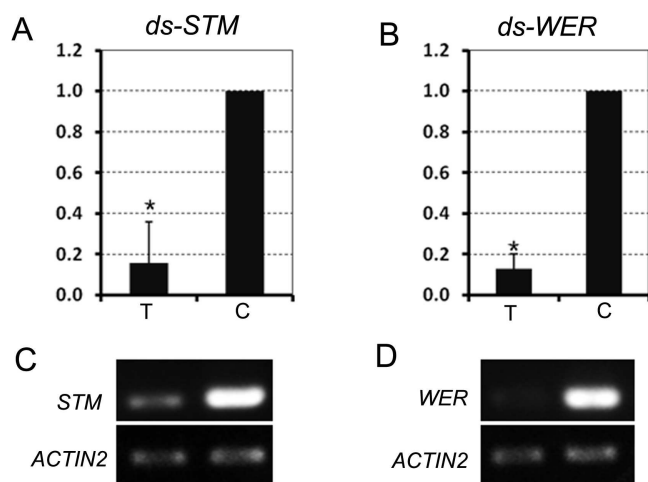
Fig. 3 RNA silencing by the mixture of G2/dsRNA in *Arabidopsis*.
(A-B) Quantitative real time RT-PCR analysis of the transcription levels of *STM* (A) and *WER* (B) in the G2/dsRNA treated seedlings (T) versus H_2O/dsRNA control (C). Asterisk shows the significant difference between treatment and control as detected by t-test at

$p<0.01$. (C-D) Semi-quantitative PCR analysis of *STM* (C) or *WER* (D) expression in the G2/dsRNA treated seedlings (T) versus H_2O/dsRNA control (C). *Arabidopsis ACTIN2* is used as an internal control and data normalization.
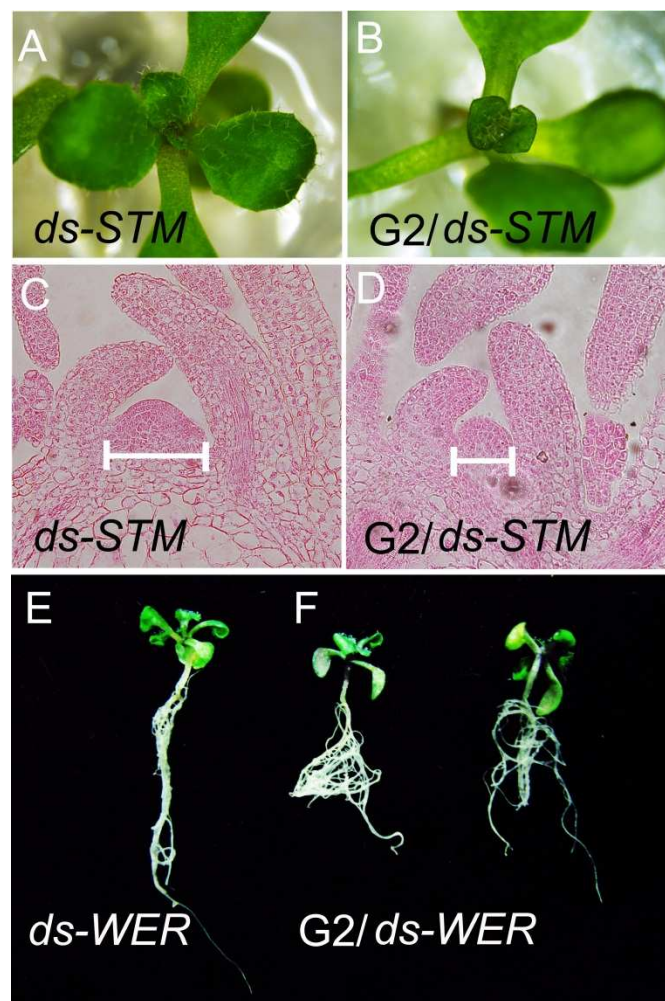
Fig. 4 Phenotypic characterization of the G2-delivered dsRNA silencing in *Arabidopsis* seedlings. (A-B) Shoot morphologic images of the H_2O/*dsSTM* control (A) and the G2/*dsSTM* treated plant (B). (C-D) Histological sections show the shoot apical meristem (SAM) of a H_2O/*dsSTM* control plant (C) and a G2/*dsSTM* treated seedling (D). The white brackets indicate the relative size of the SAM. (E-F) Representative root morphology of the H_2O/*dsWER* control (E) and the G2/*dsWER* treated roots (F).

To further characterize the efficiency of gene silencing in *Arabidopsis*, we treated 10-day-old seedling with G2/dsRNA complexes at N/P ratio 2:1 (1µg dsRNA, once per 24 h) for continuous 5 days to record any phenotypic changes. Unlike the animal system, plant continuously produces new organs even during postembryonic stage. Plant organogenesis occurred in the shoot tip called shoot apical meristem (SAM), which produces lateral organ primordia in a repetitive manner, and meantime maintains a pool of stem cells relatively constant.[15] *STM* has been shown to be required for SAM maintenance, loss-of-function of *STM* leads to abolished SAM in mature embryos or prematurely terminated SAM during postembryonic stage.[13] In this study, we found that G2/*dsSTM*-treated plants showed retarded growth (Fig. 4A, 4B) and reduced

meristem size (Fig. 4C, 4D) compared with the mock-treated plants. This is consistent with the reduced *STM* expression in the treated seedling (Fig. 3A, 3C) and the proposed role of *STM* in SAM function,[13] indicating that G2 can efficiently deliver *dsSTM* into the root and silence *STM* in the *Arabidopsis* shoot. Next, we compared the phenotypic changes in the G2/*dsWER*-treated plants with mock-treated (H$_2$O/*dsWER*) plants. *WER* is a regulator of root hair patterning, loss-of-function of *WER* results in hairy root phenotype with ectopic root hair cell formation.[14] However, there was no apparent difference in the root hair production between G2/*dsWER*-treated and mock-treated plants, despite the expression of *WER* was shown to decrease more than seven-fold (Fig. 3B, 3D). This may due to the fact that five-day treatment maybe too short to cause substantial changes in root hair formation. Surprisingly, G2/*dsWER*-treated plants produce more lateral roots (32.33±5.04) compared with the control (23.33±0.58). Given that *WER* has additional role in flower time control,[16] lateral root formation may be a new function of *WER* through such transient RNA interference analyses. Further research is required to verify the putative role of *WER* during later root production, probably combined with genetic, developmental as well as biochemical tools. Therefore, the non-viral carrier G2-delivered dsRNA can cause gene silencing in plant with observable phenotypic defects.

Conclusions

In summary, we reported water-soluble cationic fluorescence nanoparticles that contain a fluorescent PDI chromophore in the center and precise peripheral positive charges. The central PDI chromophore allows the tracing in the plant via fluorescence microscopy. The cationic fluorescence nanoparticle can efficiently enter into plant cells and act as an excellent non-viral carrier for DNA and dsRNA. Moreover, in the model plant *Arabidopsis*, applying the mixture of G2/ds*STM* or G2/*dsWER* to the root lead to dramatic knock-down the expression of *STM* and *WER* in the whole seedling. The specific RNA interference of *STM* and *WER* mediated by G2-delivered dsRNA resulted in reduced SAM size and increased lateral root, respectively. To the best of our knowledge, up to date, there is no report on systemic gene silencing of important developmental genes in plant through a non-viral gene delivery system. Our work provides a new way for gene transfection in plant, without the difficulties of genetic transformation and virus-related concerns to human health.

Acknowledgements

Notes and references

[a] College of Agronomy and Biotechnology, Beijing Key Laboratory of Growth and Developmental Regulation for Protected Vegetable Crops, China Agricultural University, Beijing 100193, China, E-mail: zhxiaolan@cau.edu.cn
[b] State Key Laboratory of Chemical Resource Engineering, Key Laboratory of Carbon Fiber and Functional Polymers, Ministry of Education, Beijing University of Chemical Technology, Beijing 100029, China, E-mail: yinmz@mail.buct.edu.cn
# These authors contributed equally to this work.

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