A lentinan-loaded hydrogel with a core-shell structure in-duces broad-spectrum resistance against plant virus by acti-vating the expression of CML19

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Abstract

Control of plant virus disease largely depends on the induced plant defense achieved by the external application of synthetic chemical inducers with the ability to modify defense-signaling pathways. However, most of the molecular mechanisms underlying these chemical inducers remain unknown. Here, we developed a lentinan-loaded hy-drogel with a core-shell structure and discovered how it protects plants from different virus infections. The hydrogel was synthesized by adding a chitosan shell on the sur-face of the polyanion sodium alginate-calcium ion-lentinan (LNT) hydrogel (SL-gel) to form CSL-gel. CSL-gel exhibits the capacity to prolong the stable release of lenti-nan and promote calcium ions release. Application of CSL-gel on the root of plants induces broad-spectrum resistance against TMV, TuMV, PVX and TRV. Further-more, RNA-seq analysis identified that the calmodulin-like protein 19 gene (CML19) is upregulated by the sustained release of calcium ions from the CSL-gel, and silenc-ing and overexpression of CML19 alter the susceptibility and resistance of tobacco to TMV. Our findings provide evidence that the novel and synthetic CSL-gel with the sustainable release of LNT and calcium ion strongly inhibits the plant virus infection. This study uncovers a novel mode of action by which CSL-gel with the stable release of calcium ion triggers CML19 expression.

Introduction

Inducing and activating plant immune response is one of the most effective ways to combat plant virus diseases (Hammerschmidt et al., 2001). Plant virus that has a variety of infection modes, a wide range of host and a long infection cycle causes a common systemic infection (Scholthof, 2005, M. et al., 1982). Control of plant virus disease largely depends on the application of the virus passivation agents. However, these agents are not able to provide complete control of the plant viral diseases owing to their ability to partially reduce the virus infection and inhibit the replication and transfer of the virus in the plant(Yang et al., 2016, Yamakawa and H., 1998). Inducers with the ability to activate plant resistance are becoming an increasingly important way to control plant virus disease. Previous studies showed that they can significantly induce the expression of resistance-related genes and enhance the immune response of plants to biotic stresses(Yamakawa and H., 1998). However, the application of the existing inducers in the field is limited because their lasting time is shorter than the existing period of virus infection. In addition, most of the inducers consist of biological polysaccharides with a structure that is easily degraded at the complicated environment in the field, rustling in the reduced efficacy on the control of virus disease (Xiang et al., 2013, Lin et al., 2006, Xiu et al., 2006,

Tang et al., 2012). Therefore, there is an urgent need to develop a novel inducer agent with the ability to last the release time of loaded drugs for the field application.

Calcium ions (Ca^{2+}) as an essential nutrient element, can promote plant growth, improve photosynthesis, and increase the synthesis and accumulation of organic matter (Tang et al., 2012, Kwon et al., 2009). Ca^{2+} as a second messenger is also involved in the signal transduction of plant to stress responses (Batistic and Kudla, 2012, Defalco et al., 2010, Dodd et al.). Previous studies showed that Ca^{2+} signals are perceived by four types of calcium signaling sensor proteins, including calmodulin (*CaM*), calmodulin-like protein (*CML*), calcium-dependent protein kinase (CDPK), and calcium-like protein Calcineurin B-like (CBL) (Braam and Davis, 1990, Mccormack et al., 2005, Boonburapong and Buaboocha, 2007). However, CML, CDPK, CBL are found only in plants and some protists. *CMLs* , as one of the plant-specific Ca^{2+} receptors, participate in plant biological stress, abiotic stress as well as many developmental processes(Scrase-Field and Knight, 2003, Bender and Snedden, 2013, Nakahara et al., 2012). It has been documented that plant defense response, plant growth and development as well as cytokine regulation, induce changes in intracellular calcium ion concentration, which results in the induced expression of *CMLs* gene (Roberts and Harmon, 1992, Mcainsh and Pittman, 2009). Accumulated evidence showed that the up-regulation of *CML* gene expression is able to significantly improve the plant resistance to biotic stress (Ma et al., 2008, Bo et al., 2017). For example, *CML41* was found to reduce *P. syringae* infection by regulating plasmodesmal closure through mediating Ca^{2+} signaling in response to bacterial pathogens(Bo et al., 2017). Leba *et al.* showed that *CML9* is involved in plant resistance to pathogenic bacteria through a flagellin-dependent pathway in plants(Leba et al., 2012). Tobacco calmodulin-like protein, rgs-CaM, binds to the RNA silencing (RNAi) suppressor of the tobacco etch virus to inhibit RNAi and increase tobacco resistance to the virus (Nakahara et al., 2012). *CML19* , also known as arabidopsis centrin 2, was found to be regulated by abiotic stress (UV resistance) and play diverse roles in DNA damage repair (Liang et al., 2006). Therefore, CML can be regarded as an important target site to stimulate plant defense response.

Recent studies showed that sustained-release hydrogel as a drug carrier has attracted more interest in delivering therapeutic agents to control human diseases(Jeong et al., 1997, Asamura et al., 2010). Advantages of hydrogel include a prolonged drug release and action time, increased stability of loaded drugs, and enhanced control efficiency of disease(Wasikiewicz et al., 2005, Builders et al., 2008, Ali et al., 2004). In addition, many studies showed that sustained-release hydrogel exhibits the ability to carry multiple drugs and provide various control of multiple diseases. For example, porphyrin photosensitizer sinoporphyrin sodium (DVDMS) and Polylactic acid-copolymerized glycolic acid (PLGA) are loaded into the sodium alginate-chitosan hydrogel that provides the dual action of antibacterial and skin regeneration (Mai et al., 2020). Therefore, the development of dual functions of hydrogel that could deliver a range of drugs and provide broad-spectrum resistance to multiple diseases is required.

Our previous studies found that coating the surface of hydrogel with amino oligosaccharides can prolong the release time of drugs loaded in the hydrogel (ALA-gel) (Xiang et al., 2019). However, amino oligosaccharides are highly soluble in water, resulting in the slow dissolve of the ALA-gel surface and the reduced drug release time from the ALA-gel. To overcome the drawbacks of ALA-gel and improve the sustained release of drugs loaded in the hydrogel, a multiple-functional sodium alginate-lentinan hydrogel (SL-gel) with a dense chitosan shell (CSL-gel) was developed aiming at improving the control efficacy against plant virus diseases. As expected, CSL-gel exhibited stable and sustained LNT release and broad-spectrum anti-virus activities. According to the RNA-seq, the sustainable and controlled release of calcium ions activated the expression of *CML19* that enhances the resistance of tobacco against TMV with a lasting time up to 30 days after CSL-gel treatment.

Materials and Methods

Chemical reagents

The following chemical materials and reagents were used and purchased from commercial suppliers without further purification: SA and chitosan (viscosity of 200 ± 20 mPas, Shanghai Aladdin Biochemical Technology

Co., Ltd., Shanghai, China); $CaCl_2$ (Kaixiang fine chemical co. LTD, Shanghai, China); LNT (purity:98%, Watson International Ltd).

Synthesis of CSL-gel

The SA, $CaCl_2$, lentinan (LNT) and chitosan were used to synthesis the CSL-ge following the method as described previously (Xiang et al., 2019, Gao C, 2009) : 1 g SA and 1 g LNT were dissolved in 125 mL of distilled water and stirred until completely dissolved. Subsequently, the SA-LNT mixed solution was slowly dropped into 1000 mL of aqueous $CaCl_2$ (30 g/L) solution (stirred at 400 revolutions/min for 30 min) to form a hydrogel precursor (SL-ge) immediately (loading efficiency (Lin Y H, 2008) of 15.2% and encapsulation percentage (Lin Y H, 2008) of 89.4%). Next, 10 g/L aqueous chitosan solution was added to the surface of the SL-gel and stirred for 2 h (400 revolutions/min) to form the CSL-gel. The CSL-gel was washed three times with deionized water.

Characterization of the chitosan core-shell of CSL-gel

Scanning Electron Microscopy (SEM), Zeta potential analysis, Fourier Transform infrared spectrometry (FTIR) and elemental analysis were was performed by Beijing Zhongke Baitei Technical Service Co., LTD. Briefly, SEM (SU-8020, Hitachi, Japan) was used for observation of the surface composition of the hydrogel according to the manufacturer's instructions. Zetasizer Nano ZS90 (Malvern Panalytical, Almelo, Netherlands) was used to measure the surface charge of the hydrogel. FTIR spectrometer Nicolet 6700 (Thermo Scientific, USA) was used to collect infrared spectra in the range 4000-400 cm^{-1} by 32 scans following the manufacturer's instructions. Elemental analyzer (Vario EL cube, Elementar Analysensysteme Madison, WI, U.S.A, Frankfurt, Germany) were used to analyze the elemental composition on the formation of the chitosan shell in the surface of the hydrogel.

Measurement of LNT and calcium ion release from CSL-gel

The standard curve of LNT was determined by UV-vis spectrophotometry (UV-vis, PGEN-ERAL) according to the method as described previously (Xiang et al., 2019). The sustained-release ability and rate of CSL-gel and SL-gel were tested according to the previous methods (Xiang et al., 2019): Briefly, 50 pieces of CSL-gel or SL-gel were added into 10 mL of deionized water (pH 5) at 20 degC and an UV-vis spectrophotometer was used to measure the absorbance of LNT in solution at 24-336 h, respectively. The same method was used to measure the cumulative release of calcium ions and investigate the effects of different temperatures (Temperature: 10, 20, 30, 40, 50, and 60 degC, pH=5), different pH values (pH: 3.0, 5.0, 7.0, and 9.0, adjusted by Tris-HCl, at 20 degC), and different concentrations of cations (The concentrations of NaCl solution were 0.1, 0.2, 0.4, and 0.8 mol/L, respectively, pH=5, at 20 degC) on the sustained-release ability and rate of CSL-gel as well. The cumulative release rate (CRR) of LNT was calculated according to formula 3. The release kinetics of LNT from CSL-hydrogel and SL-hydrogel were analyzed using Higuchi (Formula 4) and Korsmeyer-Peppas models (Formula 5) (Korsmeyer R W, 1983).

$$\text{CRR } (\%) = \frac{\sum_0^t C_t V_{\text{total}}}{Q_1} \times 100\% \quad (3)$$

C_t represents the release LNT concentration at time t, V_{total} represents solution volume, and Q_1 represents the total quality of loaded LNT.

Higuchi model: $Q_t = Kt^{1/2}$ (4)

Korsmeyer-Peppas model: $Q_t = Kt^n$ (5)

Q_t represents the cumulative release rate of LNT until time t, K represents the kinetic constant, and n is an index that reflects the following release mechanisms: Fickian diffusion ($n < 0.43$), non-Fickian or anomalous diffusion ($0.43 < n < 0.85$), and case II transport ($n > 0.85$).

Plant treatment with CSL-gel

50 pieces of CSL-gels or SL-gels were evenly embedded near the root of each plant at the approximately four-leaf stage. Each treatment group has twelve repeats. All plants were grown in the same condition with

12 h of light and 12 h of darkness at 25 and were given 10 mL of water every 24 h.

Virus inoculation and visualization of disease symptoms

All viruses were inoculated 14 days after plants were treated with different hydrogels. The rubbing method was used to inoculate TMV labeled with a green fluorescent protein (GFP) in hydrogel-treated plants or *CML19* silenced plants. The agroinfiltration was used to inoculate TRV-GFP, TuMV-GFP and PVX in hydrogel treated plants. The infection of virus-GFP in *N. benthamiana* was observed under UV lamp (Blak-Ray B-100AP, Upland, CA, U.S.A.) at the appropriate time (TMV-GFP: 2 and 7 dpi, TRV-GFP: 5 and 9 dpi, TuMV-GFP: 12 and 16 dpi). The infection of PVX in *N. benthamiana* was observed under a fluorescent lamp at 18 and 22 dpi. At the same time, the inoculated leaves and young leaves of each group were collected in liquid nitrogen and stored at -80 degC for a subsequent experiment.

Quantitative real-time PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA)

Total RNA was extracted from inoculated leaves and young leaves using RNA extraction kit (Promega Biotechnology Co., Ltd., Shanghai, China). Next, the reverse transcription kit (Shanghai Titan Technology Co., Ltd., Shanghai, China) was used to synthesize cDNA. Quantitative PCR (qPCR) was performed according to the previous method using a CFX Touch Real-time PCR machine (Bio-Rad, USA) and Quantinova SYBR Green PCR Kit (Chongqing Shuguang Biotechnology, China) [66].*Actin* was used as an internal reference. Quantification of the relative changes in gene transcript levels was performed using the $2^{-\Delta\Delta^{\sim}T}$ method. Each sample has three biological replicates. All primers of qPCR were listed in **Table 4** . The crude extracts prepared from leaf samples at different inoculation times were used to perform ELISA assay. TMV-GFP antibodies and horseradish substrate coloration were used to detect the protein accumulation of TMV-GFP (Ma L S, 2018, Peng H R, 2019).

Construction of *CML19* silencing vector

CML19 cDNA was amplified from leaf cDNA using primers*CML* -TRV-F and *CML* -TRV-R (**Table S1**). The fragment was then inserted into VIGS vector Pash18 at the EcoR I and Xba I site. Finally, the recombinant plasmids were introduced into*Agrobacterium tumefaciens* GV3101. The empty vector, TRV-GFP, was used for the control treatment.

Construction of *CML19* overexpression vector

The full-length *CML19* was cloned into the pgR107 vector digested with the same restriction sites. Empty vector PVX:00 was used as a negative control. The resulting plasmid was transformed into Agrobacterium tumefaciens GV3101 for Agrobacterium infiltration. The corresponding primers refer to **Table S1** .

Western blot

Total plant leaf protein was extracted using RIPA lysis buffer (ThermoFisher, MI, USA) and the protein concentration of the extracted samples was measured using the Pierce BCA Protein Assay Kit. Cell lysates were loaded on SDS-polyacrylamide gels and electrophoretically separated. Proteins were transferred to PVDF membranes, blocked with 3% BSA for 0.5 h at room temperature, and incubated with primary antibodies overnight at 4 °C. After washing 3 times with TBST, the cells were incubated with the secondary antibody for 1 h at room temperature. Signal was detected with the aid of a chemiluminescence instrument using standard ECL kits.

RNA-seq and analysis

The CSL-gel and SL-gel were embedded into soil planted with *N. benthamiana* and 10 mL of water was given to the soil every 24 h. Water-treated plants severed as the negative control. After 7 days, the leaves at the same position of the plant were picked and sent to Kedio Biotechnology Co., Ltd. for transcriptome sequencing (NCBI Bioproject Number: PRJNA823004). RSEM software (v 1.3.1) was used to map the clean reads of each sample to the *N. benthamiana* reference genome (solgenomics Niben.genome.v1.0.1), and FPKM conversion was performed to analyze the expression level of each Unigene. NOIseq software (v 1.20.0)

was used to analyze the DEGs of different hydrogel-treated samples and control samples, and the default recognition criteria for DEGs were |log2(fold change)|[?]2 and FDR[?]0.05.

Subcellular localization of CML19

The agroinfiltrated *N. benthamiana* leaves were selected for microscopy visualization at 48 hours after infiltration. The fusion green fluorescent signals were observed under a Zeiss laser scanning confocal microscope (Zeiss LSM780, Germany).

Measurement of plant physiological index after CSL-gel treatment

A total of 50 pieces of CS-gel or S-gel were buried in the soil, and each plant was given 10 mL of water every day for consecutive 14 days and placed in a constant temperature growth room at 25 degC (12 h of light and 12 h of darkness). After 14 days, the plant height, plant width, leaf width, fresh weight, and dry weight of *N. benthamiana* were measured. Each treatment group has twelve replicates.

Biosafety evaluation of CSL-gel

10 crucian carp with a length of 3-4 cm were placed into 1 L of pure water (pH=5.5) with different amounts of CSL-gel (the number of CSL-gel pieces per 1 L of water was 50, 100, 200, and 400). Pure tap water was used as a blank control, and each group has three repeats. The life activity and death of crucian carp were observed at 24 h intervals. Each treatment group has four replicates.

Statistical analysis

All experimental data presented here involved at least three replicates. The data are presented as means and standard deviations. The statistical analysis was performed with SPSS software (version 17.0) and OriginPro 8.

Results

Synthesis of CSL-gel and characterization of chitosan core-shell

To obtain the sustained release of lentinan and calcium ions, we developed a novel and multiple-functional hydrogel by cross-linking the mixed solution of sodium alginate and lentinan with calcium ions to form a hydrogel precursor (SL-gel). A chitosan shell was built on the surface of SL-gel to form a lentinan-loaded hydrogel with a core-shell structure (CSL-gel) (**Figure 1a**), which is expected to provide the stable and sustained release of lentinan (LNT) and calcium ions (**Figure 1b**). To assess the formation of a chitosan shell on the surface of CSL-gel, scanning electron microscopy (SEM) and Zeta potential were employed. SEM images of SL-gel and CSL-gel were illustrated in**Figure 1c** . SEMs showed that the surface of the SL-gel with large pores was loose, but a dense chitosan shell covering the SL-gel was observed on the surface of CSL-gel (**Figure 1c**). Moreover, the elemental analysis showed that elemental nitrogen was detected on the surface of the CSL-hydrogel (**Table 1**), indicating that chitosan was added on the surface of the CSL-gel. To avoid the influence of LNT on the FTIR and zeta analysis, we performed the Fourier transform infrared spectrometry (FTIR) and zeta analysis using the hydrogel without LNT to characterize the chitosan shell. **Figure 1d**showed a shared peak located at approximately 3340 cm^{-1} was observed in the FTIR spectrum of the S-gel and CS-gel. However, the CS-gel features a fully developed peak on 3340 cm^{-1}, which is attributed to the overlap associated with the peaks of the -OH stretching vibration of SA and the -NH stretching vibration of the chitosan in comparison to the S-hydrogel. Besides, the CS-gel exhibited a peak at approximately 2927 cm^{-1}, which was formed by the C-H stretching vibration of chitosan, suggesting that the reaction of adding chitosan to the SL-gel was successful. Sodium alginate is composed of carbon, hydrogen, and oxygen, and the surface is negatively charged. The zeta potential analysis showed that the surface of CS-gel has a positive charge, but the S-gel with a negative charge (**Figure 1e**). Taken together, our findings strongly indicate that a chitosan shell is added to the SL-gel, which has the potential to stabilize the structure of CSL-gel and provide the sustained release of LNT and calcium ions.

Sustainable release of LNT and calcium ion of CSL-gel

To assess the release property of loaded drugs in CSL-gel, we measured the release rate of LNT at the corresponding time according to the regression equation of LNT (y = 0.0028x + 0.0266, R^2 = 0.9998, **Figure S1**). As shown in **Figures 2a** and**2b** , CSL-gel maintained a stable and cumulative release of LNT from the first day to the 14 days after soaking. However, a substantial amount of LNT was released from SL-gel on the first day and the LNT release slightly increased with the extension of soaking time and reached the maximum amount on the eighth day after soaking. At the ninth day, a limited amount of LNT was released following the extension of the soaking. Furthermore, we used Korsmeyer-Peppas and Higuchi models to determine the release of LNT. **Figure 2c** showed that a clear linear correlation (R^2=0.989) between the cumulative release rate and the logarithm of time from CSL-gel in the Korsmeyer-Peppas model fitting curve was observed. Similarly, a high linear correlation between the cumulative release rate and the square root of time during the release of LNT from the CSL-gel (R^2=0.9823) was also observed in the Higuchi model fitting curve (**Figure 2d**). However, no linear correlation between the cumulative release rate and the square root of time (R^2=0.8683) and between the cumulative release rate and the logarithm of time (R^2=0.933) of SL-gel was observed. The corresponding dispersion coefficient (n) of SL-hydrogel was 0.3089 ($n < 0.43$), indicating that the release of LNT from SL-gel under these conditions followed Fickian diffusion (**Table 2**). In contrast, the dispersion coefficient (n) of CSL-gel was 0.6305 ($0.43 < n < 0.85$), which indicates the CSL-gel followed non-Fick diffusion mechanism (**Table 2**). These findings demonstrated that the chitosan shell controls the release of LNT from the CSL-gel, resulting in the cumulative and sustainable release of LNT.

Soil is a complex system in which the properties, such as pH, temperature, and ion concentration, are dynamic due to the impact of external factors such as fertilization, irrigation, and rainfall (Reth et al., 2005). The drug release from hydrogel will be influenced by different soil environments. To assess the release rate of LNT at different soil properties, we measured the release of LNT from hydrogel at different pH, temperatures, and ion concentrations. As shown in**Figure 2e** , the cumulative LNT release rate of CSL-gel highly increased following the increased temperature, which is caused by the high swelling of the hydrogel at a high temperature. Similarly, the corresponding release rate of LNT from CSL-gel largely increased from 7.54% to 41.35% when the pH increased from 3 to 9 (**Figure 2f**). This observation follows the fact that at low pH most of the carboxyl groups on alginate are deionized and form strong hydrogen bonds with hydroxyl groups, resulting in a tight shrinkage of the calcium alginate pellet that prevents the LNT release from the CSL-gel. Interestingly, we found that ion concentration present in the soil positively regulated the release of LNT from CSL-gel. As shown in**Figure 2e** , the cumulative release rate of LNT highly increased from 33.7% to 99.5% following the increased Na^+concentration from 0 to 0.8 M. The ion exchange between Na^+ and Ca^{2+} strengthens the electrostatic repulsion between -COO^-, which ultimately increases the swelling of the hydrogel to stimulate the release of LNT (Xiang et al., 2017).

CSL-gel promotes plant growth and enhances the resistance against plant virus

The chitosan present on the surface of CSL-gel can form a robust electrostatic force with sodium alginate, resulting in the accelerated release of calcium ions from the gel. To test our hypothesis, we soaked the SL-gel or CSL-gel in water and measured the concentration of Ca^{2+} in the water solution at 2-hour intervals. As shown in **Figure 3a** , the amount of Ca^{2+}released from the CSL-gel was significantly higher than that of SL-gel. To assess whether the released Ca^{2+} can promote plant growth and development, we measured the plant height, leaf width, dry weight, and fresh weight of *N. benthamiana* at 14 days after SL-gel or CSL-gel treatment. **Figure 3b-g** showed CSL-gel-treated plants displayed a significant increase in plant growth, including height, plant width, leaf width, dry weight compared to that of the water-treated plant. In addition, the growth indexes of plants treated with CSL-gel were higher than those of the S-gel-treated and control plants (**Figure 3b-g**). Moreover, After 19 days of treatment, the growth trend of CSL-gel-treated plants was significantly higher than that of the water-treated and SL-gel-treated groups (**Figure S2**). These results demonstrated that CSL-gel promotes plant development.

To examine the anti-virus activity of the CSL-gel with the sustained release of LNT and calcium ion, we applied the CSL-gel into the soil surrounding the plant root. SL-gel serves as a control. We inoculated the

TMV-GFP by rubbing on the leaves of the plant at 7 days after CSL-gel application. As shown in **Figure 3h** , at 2 dpi the number of green fluorescent spots in CSL-gel-treated *N. benthamiana* was the same as that on the SL-gel-treated plants. At 7 dpi, the green fluorescent signals present in the young leaves of the CSL-gel-treated plant were significantly weaker than that of the water and SL-gel treatment groups (**Figure 3h**). qPCR analysis showed that the number of TMV-*CP* transcripts between the two treatment groups was no significant differences, but at 7 dpi the expression level of TMV-*CP* in the CSL-gel-treated plant was significantly lower than that in the SL-gel-treated and control plants (**Figure 3i-j**). Taken together, we can conclude that the CSL-gel with the sustainable release of LNT and calcium ion significantly enhances the tobacco resistance against TMV as well as promotes plant development.

CSL-gel induces *CML19* expression and silencing of*CML19* enhances the susceptibility to TMV

The increased Ca^{2+} concentration in plant cells promotes the expression of *CML* that plays diverse roles in plant development and resistance in response to biotic stresses[40]. To examine the impact of CSL-gel on the genome-wide expression of plant genes, we performed RNA-seq on the CSL-treated, SL-treated and water-treated plants at 10 days after treatment, respectively. Transcriptomic analysis showed that a total of 514 differentially expressed genes (DEGs) were upregulated in *N. benthamiana* treated with CSL-gel compared to SL-treated and water-treated plants (Figure S3). *CML* genes in the DEGs list were checked and we found that *CML19* was specifically upregulated after CSL-gel treatment. To confirm the RNA-Seq results, the relative expression of *CML19* in plants treated with CSL-gel was quantified by qPCR. SL-gel treated plant severs as a positive control.**Figure S4** showed that the *CML19* expression in the CSL-gel treated plants was significantly higher than that in the SL-gel treated plants at 7 days after treatment.

To further determine whether upregulated *CML19* expression by CSL-gel contributes to TMV resistance, we generated the *CML19*RNAi silencing construct to transiently knockdown the expression of*CML19* in tobacco by agroinfiltration. qPCR analysis showed that the *CML19* expression level in the silenced plant was 40% reduced compared with the wild-type plant at 8 days after agroinfiltration (**Figure 4a**), indicating that the silencing construct is effective in reducing *CML19* expression. Eight days after infiltration, all infiltrated leaves were inoculated with TMV-GFP.**Figure 4e** showed that at 6 dpi, the level of GFP signals present in silenced plants and WT plants were similar. At 8 dpi, strong GFP signals were observed in the inoculated leaves of silenced plants compared with that in WT plants. Similarly, in the young leaves of the silenced plant, increased GFP signals were visualized, while in the WT plant limited GFP signals were observed in the young leaves**(Figure 4e**). At 10 dpi, pronounced green fluorescence signals were visualized in the young leaves of the silenced plant, but slightly expanded GFP signals were observed in the young leave of the WT plant (**Figure 4e**). In addition, the number of TMV-*CP*transcripts was quantified by qPCR. **Figure 4b-c** showed that the expression of TMV-*CP* in the silenced plant was significantly higher than that in the WT plant at 6, 8 and 10 dpi. Based on our findings, we can conclude that silencing *CML19* promotes TMV infection in *N. Benthamian* , indicating that CSL-gel induces*CML19* expression that contributes to TMV resistance.

Overexpression of *CML19* enhances the antiviral function of *Nicotiana benthamiana*

To further confirm the antiviral function of *CML19* in *N. benthamiana* , we generated the *CML19* overexpression construct tagged with GFP and transiently overexpressed it in *N. benthamiana* using agroinfiltration. Western blot detection showed that the accumulation of GFP-CML19 protein was observed in the overexpression leaves at two days after infiltration (**Figure 5a**). Two days after infiltration, all infiltrated leaves were inoculated with TMV-GFP.**Figure 5c** showed that at 2 dpi, strong GFP green fluorescent signals were observed on the young leaves of the control plant compared to that *GFP:CML19-* overexpressed plant. Limited GFP signals were observed on the young leaves of the overexpression plant, while marked signals were visualized on the young leaves of the control plant (**Figure 5c**). In addition, qPCR analysis showed that the expression of *TMV-CP* in the GFP:00 plant was significantly higher than that in the overexpression plant at 2 and 4 dpi (**Figure 5 b**). These results indicated that overexpression of *CML19*inhibits TMV infection in *N. benthamiana* .

The anti-TMV activity of CSL-gel mainly depends on the chitosan shell and calcium ion

CSL-gel promotes the expression of *CML19* and the silencing of*CML19* enhances TMV infection. To further determine that*CML19* induced by calcium ion released from the CSL-gel contributes to the resistance against TMV infection, we generated the CS-gel and S-gel without LNT to eliminate the role of LNT on the anti-TMV activity. As shown in **Figure 6a** , at 2 dpi, the number of green fluorescent spots in the inoculated leaves of *N. benthamiana* treated with CS-gel was lower than that of the S-gel and water-treated plants. qPCR analysis showed that the expression levels of TMV-*CP* were significantly lower in the inoculated leaves of*N. benthamiana* treated with CS-gel than that in the water and S-gel-treated plants. At 7 dpi, the green fluorescence signals and the number of TMV-*CP* transcripts of the water control group and S-gel treatment group were significantly higher than that of CS-gel control. However, there was no significant difference between the water-treated and the S-gel-treated plans on TMV infection at 2 and 7 dpi. These findings indicated that *CML19* induced by CSL-gel also plays an important role in anti-TMV.

CSL-gel induces broad-spectrum resistance against different plant viruses

To assess whether CSL-gel induces broad-spectrum resistance against different viruses, *N. benthamiana* plants treated with CSL-gel were separately inoculated with the tobacco rattle virus (TRV), potato virus X (PVX) and turnip mosaic virus (TuMV) by rubbing. SL-gel and water treated plants were included as controls. At the early stage of infection (TRV: 5 dpi, TuMV: 12dpi), the number of green fluorescent spots in CSL-gel-treated*N. benthamiana* was similar to SL-gel-treated plants. However, in the late stage of infection (TRV: 9 dpi, TuMV: 16 dpi), the green fluorescent signals present in the young leaves of the CSL-gel-treated plant were significantly lower than that of the water and SL-gel treatment groups (**Figure 7a-b**). **Figure 7c** showed that 18 days after inoculation, symptoms caused by PVX were relatively mild in the young leaves of and CSL-gel-treated plants, while PVX caused severe symptoms in the young leaves of water-treated plants. At 22 dpi, PVX infection developed severely curly young leaves in the water-treated plants, and plants treated with SL-gel showed mild symptoms. However, no symptoms caused by PVX infection were observed in the CSL-gel-treated plants (**Figure 7c**). Furthermore, qPCR analysis showed that the number of virus-CP transcripts in the CSL-gel-treated plant was significantly lower than that in the SL-gel and water-treated plants (**Figure 7d-i**). Therefore, we can conclude that CSL-gel inhibits the infection caused by the majority of viruses in *N. benthamiana*plants.

Safety test of CSL-gel

The toxicity test of pesticides on aquatic organisms is an important measure strategy for the safety evaluation of pesticides (Tian et al., 2005). Our previous studies showed that the LC_{50} of LNT to crucian carp at 24, 48, 72, and 96 h was about 1.6, 0.8, 0.4, and 0.4 mg/mL, respectively. To evaluate the CSL-gel safety, the number of deaths of *crucian carp* was examined in the water immersed with a series of numbers of CSL-gel at 24 h, 48 h, 72 h and 96 h. Pure tap water was used as a negative control. As shown in **Table 3** , no dead *crucian carp* was observed in the CSL-treated water with LNT concentration lower than 336 mg as well as in the water control. However, dead *crucian carp* was observed at 48 h in the water treated with 400 pieces of CSL-gel with an LNT concentration at 336 mg, indicating that CSL-gel maintained a relatively high safety to environmental organisms.

Discussion

Plant viruses cause a common systemic disease named plant cancers that differ from other plant diseases (Bawden et al., 1936). They can entirely rely on the host plant to acquire nutrients for replication, movement, and other life activities, resulting in damage to plant chloroplasts and photosynthesis, reduced accumulation of plant carbohydrates, and inhibiting plant growth (Roossinck et al., 2015, Roossinck and Condit, 2013). Traditional methods depend on virus deactivators and plant immunity inducers to minimize the infection (Ryu et al., 2017). However, most virus deactivators and plant immunity inducers are not able to completely inhibit the proliferation and movement of the virus in the plant after the virus infection (Chen et al., 2009). In this study, a lentinan-loaded hydrogel with the core-shell structure was developed, which features the stable and sustainable release of lentinan and calcium ions. The prolonged release of LNT and calcium ions

significantly promotes plant growth and development and provides broad-spectrum resistance against TMV, TuMV, PVX and TRV. In addition, we found that the sustained release of calcium ions from the CSL-gel activates the expression of calmodulin-like protein 19 (*CML19*), and the silencing of *CML19* enhances the susceptibility of tobacco to TMV. We summarize the action of CSL-gel in **Figure 8** . Therefore, the low-cost and easily synthesized CSL-gel with a novel mode of action triggering *CML19* expression has the potential to be utilized in the field against severe virus disease and increase the yield of crop plants.

Our results showed that the chitosan shell prevents the rapid bursting of the hydrogel, resulting in the controlled and stable release of LNT and calcium ions and the extended-release time (**Figures 1 and 2**). Polysaccharide immunity inducer, such as amino oligosaccharides, chitosan, chitin, and lentinan, has been widely used in anti-plant virus diseases (Zhao et al., 2007). Lentinan, as a biological polysaccharide, has multiple disease-resistant functions, such as closing plant stomata, inducing the expression of plant resistance genes and improving plant disease-related enzyme activity (Yin et al., 2010). However, previously generated hydrogel loaded with LNT is confined in field application due to the limited induction time of lentinan and its instability in the complex field environment. The newly synthesized hydrogel with a core-shell structure that functions as a slow-release carrier prolongs the action time of lentinan on plants, promotes plant growth and induces the resistance of *N. benthamiana* to TMV (**Figure 3**). At present, plant immunity inducers as an alternative agent are more effective in controlling plant virus disease (Ryu et al., 2017). Many studies reported that plant immunity inducers can induce the expression of plant disease-related genes, and promote the activities of stress-related enzymes to comprehensively improve the resistance of plants to pathogens (Chen et al., 2009). For example, Lentinan has been used as a common inducer in the prevention and treatment of plant virus diseases in the field. It can significantly promote the activity of SOD, POD and CAT and stimulate the expression of *PR1* and *PR3* to improve plant resistance (Zhao et al., 2007). Previous studies showed that chitosan was able to increase the activity of plant defense-related enzymes (PAL, PPO, POD, CAT, SOD), and induce the production of secondary metabolites associated with disease resistance and induce phenolic metabolic pathways to improve the antagonism of plants to fungi, bacteria and viruses (Orzali et al., 2010, Hadwiger et al., 1981).

Calcium ion (Ca^{2+}), as one of the important nutrient elements, can increase the germination rate of plant seeds, promote the development of plant roots and leaves, and increase the absorption of nutrients to enhance plant growth. Previous studies showed that the application of calcium ion at a concentration in the range of 0-14mmol/L increases plant growth, root growth, and dry matter accumulation (Chowdhury and Choudhuri, 2010). $Ca^{2+,}$ as a universal second messenger, can regulate the plant response to biotic and abiotic stresses. For example, it can regulate plant cell membrane protective enzyme systems to alleviate the effects of drought, salt stress, and water stress on growth (Jones and Lunt, 1967, Wigdorowicz-Makowerowa, 1982). The activities of antioxidant enzymes, such as SOD, POD, and CAT, in soybeans and apples treated with calcium ions were significantly improved (Munir et al., 2016). Furthermore, the application of calcium fertilizer effectively prevents the occurrence of brown spot disease (Xiaomeng et al., 2017). Our results showed that the chitosan shell promotes the CSL-gel to stably and sustainably release Ca^{2+} owing to the strong electrostatic interaction between SA and chitosan (**Figure 2**). The plants treated with CS-gel exhibit significantly enhanced growth than those treated with the SL-gel (**Figures 3 and 6**). This evidence is consistent with previous reports, indicating that CSL-gel with the stable and sustainable release of calcium ions promotes plant growth and improves plant resistance against viruses.

Previous studies showed that Ca^{2+} is perceived by calmodulin (CaM) and calmodulin-like (*CML*) proteins to participate in physiological and biochemical reactions in plants (Batistic and Kudla, 2012, Defalco et al., 2010). As a type of plant-specific Ca^{2+} receptor, *CML* involves various physiological activities in the process of plant growth and development, such as regulating plant defense responses, enhancing plant anti-stress responses, and controlling plant hormone levels (Min et al., 2009). For example, overexpression of *Arabidopsis CML8* confers enhanced resistance to *Pseudomonas syringae* in an SA-dependent process (Xiaoyang et al.). *Arabidopsis CML9* is rapidly and strongly induced by *Pseudomonas syringae* and abiotic stress and abscisic acid (ABA), which acts as a positive regulator in response to*Pseudomonas syringae* and a negative regulator to salt stress (Leba et al., 2012). Tomato plants overexpressing *CML44* exhibit higher

antioxidant enzyme activity and greater tolerance to abiotic stresses (Munir et al., 2016). Our results showed that CSL-gel strongly induces the expression of *CML19* and the silencing of *CML19*enhances the infection of TMV (**Figure 4**). In addition, overexpression of *CML19* inhibits TMV infection, suggesting that *CML19* plays a positive role in the resistance to TMV. We further found that CML19 localizes in the plant cytosol and nucleus (**Figure S5**). The CSL-gel maintains a stable and cumulative release of calcium ions into the soil, which activates plant *CML19* to adapt to the increased calcium ions in the environment. In turn, the accelerated expression of *CML19* enhances the resistance against TMV. However, the mechanisms underlying this observation remain unknown. Because CML as a sensor of Ca^{2+} regulates diverse plant processes, the identification of CML19 interactors will aid us in understanding the anti-virus activity of CML19 in the subsequent studies.

Taken together, a hydrogel with a core-shell structure and sustainable release of lentinan and calcium ions based on the polycationic properties of chitosan was developed in this study. This hydrogel has multiple functions including the promotion of plant growth, continuous induction of plant resistance against the different virus, and enhancement of *CML19* expression to improve TMV resistance. These findings presented here form a solid basis for us to understand the resistance against TMV, highlight the importance of *CML* in plants against TMV infection and expand our tools to control plant virus diseases as well in the future.

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Tables:

Table 1. Elemental analysis of the SL-gel and CSL-gel.

Table 2. Kinetic parameters associated with LNT released from the SL-gel and CSL-gel.

Table 3. Toxicity of CSL-gel to Crucian Carp.

Figure Legends:

Figure 1. Synthesis of CSL-gel and characterization of chitosan shell . (a). schematic representation of the synthesis of CSL-gel. (b). Diagram of CSL-gel and SL-gel drug release simulation. (c). SEM images of surface composition of SL-gel and CSL-gel. (d). Zeta potential of the S-gel without LNT and chitosan shell and CS-gel without LNT. (e). FTIR spectra of the S-gel and CS-gel.

Figure 2. CSL-gel exhibits sustainable and cumulative release of LNT . (a). Comparison of the cumulative release of LNT from the SL-gel and CSL-gel. (b). The cumulative release rate of LNT from CSL-gel and SL-gel. (c). The fitting curves for the Korsmeyer-Peppas model. (d). The fitting curves for the Higuchi model. (e). The cumulative release rate of LNT from CSL-gel at different temperatures. (f), The cumulative release rate of LNT from CSL-gel at different pH. (g). The cumulative release rate of LNT from CSL-gel at different Na^+ concentrations. Double asterisks indicate separation among CSL-gel and SL-gel at the same amount by Duncan's multiple comparisons (**, $p < 0.01$). Vertical bars indicate standard deviations (n = 3).

Figure 3. CSL-gel with the sustainable release of calcium ions promotes plant growth and significantly enhances the resistance of *N. benthamiana* against TMV . (a). Comparison of calcium ions cumulative release between SL-gel and CSL-gel. (b-f).Comparison of plant height, plant width, leaf width, dry weight, and fresh weight between CSL-gel and SL-treated plants. (g). Representative pictures showing the plant growth between different treatments. (h). CSL-gel treated *N. benthamiana* plants display enhanced resistance against TMV. *N. benthamiana* plants were inoculated with TMV–GFP constructs by rubbing and representative pictures were photographed at 2 and 7 dpi. (i) qPCR analysis showing the expression level of *TMV-CP* in the inoculated leaves of *N. benthamiana* at 2 dpi (j). qPCR analysis showing the expression level of TMV-CP in the inoculated leaves of *N. benthamiana* at 7 dpi. The expression level was normalized to Actin. Mean values displayed in each bar followed by different letters are significantly different according to Duncan's multiple range test ($p < 0.05$). Vertical bars indicate standard deviations (n = 3).

Figure 4. CSL-gel triggers *CML19* expression and silencing *CML19* increases TMV infection in *N. benthamiana* .The leaves of *N. benthamiana* were inoculated with TMV-GFP by rubbing. The green GFP fluorescence signals were visualized at 6, 8, and 10 dpi under UV light. Representative pictures are presented. (a). qPCR analysis showing the relative expression of *CML19* in the silenced *N. benthamiana.* (b-d). qPCR analysis showed the expression level of TMV-CP in the inoculated leaves of *CML19* silenced *N. benthamiana* at 6, 8 and 10 dpi. (e). TMV-GFP was inoculated by rubbing after *CML19* silencing and representative pictures were taken at 2 and 7 dpi. The asterisk indicates a significant difference according to Duncan's multiple range test (*, $p < 0.05$; ***, p¡0.001). Vertical bars indicate standard deviations (n = 3).

Figure 5. Overexpression of *CML19* inhibits TMV infection. (a). Western blot analysis showed the accumulation of GFP-CML19 protein in the overexpressed *N. benthamiana* and empty vector GFP:00 control plants. (b). TMV-GFP infection was hindered in *CML19* -overexpressed plants compared to the control plant.TMV-GFP was inoculated by rubbing after *CML19* overexpression and representative pictures were taken at 2 and 4 dpi. (c). qPCR analysis showed the expression level of *TMV-CP* in the *CML19* overexpressed leaves of *N. benthamiana* at 2 and 4 dpi was significantly higher than that in the control plant (GFP:00). The asterisk indicates a significant difference according to Duncan's multiple range test (*, $p < 0.05$; ***, p¡0.001). Vertical bars indicate standard deviations (n = 3).

Figure 6. Comparison of the anti-TMV activity of S-gel and CS-gel. (a). *N. benthamiana* plants treated with CS-gel display increased resistance against TMV. The leaves of *N. benthamiana* were inoculated with TMV-GFP by rubbing. The green GFP fluorescence signals were visualized at 2 and 7 dpi under UV light. Representative pictures are presented. (b). qPCR analysis showing the relative expression of *TMV-CP* in the inoculated leaves of *N. benthamiana* at 2 dpi (f). qPCR analysis showed the expression level of *TMV-CP* in the young leaves of *N. benthamiana* at 7 dpi. Mean values displayed in each bar followed by different letters are significantly different according to Duncan's multiple range test ($p < 0.05$). Vertical bars indicate standard deviations (n = 3).

Figure 7. CSL-gel treated *N. benthamiana* plants exhibit significantly increased resistance against TRV, TuMV and PVX . The leaves of *N. benthamiana* were inoculated with TRV, TuMV and PVX by rubbing or agroinfiltration 14 days after CSL-gel treatment. (a). The green GFP fluorescence signals were visualized at 5 and 9 days after TRV inoculation under UV light. Representative pictures are presented. (b). The green GFP fluorescence signals were visualized at 12 and 16 days after TuMV inoculation under

UV light. Representative pictures are presented. (c). Disease symptoms caused by PVX were observed at 18 and 22 days after inoculation and representative pictures are presented. (d-e). qPCR analysis showing the relative expression of *TRV-CP* in the inoculated leaves of *N. benthamiana* at 5 dpi and in the young leaves at 9 dpi. (f-g). qPCR analysis showing the relative expression of T*uMVV-CP* in the inoculated leaves of *N. benthamiana* at 12 dpi and in the young leaves at 16 dpi. (h-i). qPCR analysis showing the relative expression of *PVX-CP* in the inoculated leaves of*N. benthamiana* at 18 dpi and in the young leaves at 22 dpi. Mean values displayed in each bar followed by different letters are significantly different according to Duncan's multiple range test ($p < 0.05$). Vertical bars indicate standard deviations ($n = 3$). All experiments were repeated three times and similar results were obtained.

Figure 8. Schematic representation of a proposed action model of CSL-gel on plants to improve broad-spectrum resistance to different viruses.

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