Early warning of *Diaporthe* infection in kiwifruit soft rot by plasmonic dimer-enhanced Raman spectroscopy

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**Highlights**

- Screening a pair of *Diaporthe* genus-level primers for high-sensitive Raman assays
- All-in-one sensing of 12 isolates of *Diaporthe* genus with a high accuracy of 100%
- Much higher sensitivity than PCR at an early stage of 24 h for *Diaporthe* infection
- Lighting up point-of-care tests for early warning of kiwifruit soft rot infection
Early warning of *Diaporthe* infection in kiwifruit soft rot by plasmonic dimer-enhanced Raman spectroscopy

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**SUMMARY**

*Diaporthe* genus is the dominant pathogens of kiwifruit soft rot with long incubation period and rapid onset and very hard to detect in advance. It is of great significance to develop point-of-care tests for disease prevention and field management. Here we screen a pair of genus-level primers for constructing plasmonic dimer Rayleigh/Raman spectroscopy, which enables rapid, specific, and sensitive detection of *Diaporthe* genus in real kiwifruits. Dark-filed Rayleigh scattering clearly visualizes the target-induced dimer assembly of nanoprobes. Plasmonic dimer-enhanced Raman spectroscopy has high specificity for 12 common isolates of *Diaporthe* genus in one batch samples with highly accurate results and much higher sensitivity than polymerase chain reaction (PCR). It realizes the recognition of *Diaporthe* infection at an early stage of 24 h when the kiwifruits do not have any noticeable symptoms. It demonstrates a bright prospect of point-of-care tests for early warning of kiwifruit soft rot infection and quality control.

**INTRODUCTION**

Kiwifruit is highly prized for its delightful taste, rich vitamin C content, balanced composition of dietary fiber, vitamin E, minerals, and health benefits like antioxidant properties and relief of constipation.1,2 However, kiwifruit is susceptible to various fungal pathogens which cause soft rot disease during growth stages and postharvest storage, resulting in severe economic losses.3–5 One of the typical pathogens is *Diaporthe* genus, which is responsible for diseases in a broad range of plants hosts and distributed all over the world.6–9 Unlike the other typical pathogen, *Botryosphaeria dothidea*, there are plenty of pathogenic species in the *Diaporthe* genus, accompanied by constant discovery of new species,10–13 which increase the difficulty of detection. Therefore, a genus-level detection method for *Diaporthe* at early stages of infection is crucial to prevent the fast spread of kiwifruit soft rot, especially in the first 24 h, which could help pick out the infected fruits, carry out management methods in time, and greatly reduce the rate of fruit decay. However, to date, there is relatively little research focusing on rapid detection of *Diaporthe* spp. in infected kiwifruits, let alone the detection of genus-level pathogen infection. It is imperative to develop a sensitive and fast diagnostic method for early warning.

Traditional detection methods are generally based on pathogens’ morphological and microscopic features, pathogenicity testing, and sequence analysis of the internal transcribed spacer (ITS), which are time consuming for the culture steps. Polymerase chain reaction (PCR) is a simpler and less time-intensive DNA-based method that is widely used to detect and identify plant pathogens.15,16 But to date, at least 72 h is needed on positive reports from PCR analysis on kiwifruit soft rot infection, i.e., its sensitivity falls short of what is needed, limiting its application, although it is the only feasible fast detection method at present. Hence, developing a convenient, sensitive and rapid detection method for early diagnosis of soft rot pathogens is of great significance.

In recent years, single particle detection (SPD) has been widely concerned because of its high sensitivity and low sample consumption.17–19 Accurate quantitative detection can be realized by measuring target-induced changes in spectral profiles of single nanoparticles.20,21 Analyzing single particles effectively avoids the interference of the average effect. Various excellent scattering nanoprobes have been proposed for SPD measurement by dark-field microscopy (DFM), such as silver nanoparticles, gold nanobiconical,
gold nanorods, gold nanoparticles (GNPs), etc. Among them, GNPs are especially suitable for sensing applications because of their good water solubility, high stability, and easy synthesis; its large optical absorption also makes it have good scattering intensity.22

In addition, surface-enhanced Raman spectroscopy (SERS) is an emerging analytical technology for field detection with the advantages of high sensitivity, rapidness, selectivity, and nondestructive detection.23–25 It has been widely applied as a rapid diagnostic assay in chemical and biological sensing.26–30 Highly sensitive pathogen detection is usually accomplished by the label-based SERS assay,31,32 where nanoparticles are functionalized with specific target recognition elements and Raman reporters (RRs). The hot spots are derived from the binding events between targets and specific ligands on nanoparticles, leading to enhanced Raman scattering of the adsorbed RRs within the gaps, which could reveal the existence or quantification of targets.

Here we screened a pair of genus-level primers, each of which was used to fabricate single-primer-tethered GNPs, developing an ultrasensitive SERS assay for early detection of pathogenic species of the Diaporthe genus in kiwifruit. The positive reports could be realized after 24 h infection of the kiwifruit. GNPs were co-functionalized with RRs and primers to enable the specific recognition capability and SERS readout. Helper DNA was introduced to adjust the density of primers modified on GNPs, promoting the formation of target-induced small clusters of GNPs rather than large clusters. DFM assisted with an RGBtoHSI algorithm was employed to optimize the concentration of helper DNA and primers. The optimal SERS assay exhibited an excellent detection specificity for Diaporthe genus and ultrasensitivity to detect pathogen infection at an early stage compared with the PCR assay. It promises a bright prospect for early warning of soft rot pathogen infection in kiwifruit for point-of-care applications.

RESULTS AND DISCUSSION

Screening the primer and the degenerate primer for the Diaporthe genus

Primer design was based on ITS gene sequences from different species of the Diaporthe and other genera. ITS sequences were obtained from the NCBI GenBank nucleotide sequence database and aligned to determine the conserved and variable regions of the Diaporthe genus for primer design. One of PCR detection primers (D.sp-29, black rectangle in Figure 1A) was designed to amplify 456 bp from the ITS gene based on the conserved regions. A degenerate primer (D. sp-JB, blue rectangle in Figure 1A) was developed according to the variable region adjacent to the conserved region of D.sp-29. The detection primer D.sp-29 (P1) and the degenerate primer D. sp-JB (P2) were used here to shorten the length of the target fragment and produce hot spots with a smaller gap (Figure S1). Primer sequences were checked by NCBI Primer-BLAST to confirm their specificity in the NCBI GenBank database.

The specificity and accuracy of the primers were verified by the PCR assay and 1.0% agarose gel electrophoresis (see details in the supplemental information). The designed specific primers can amplify a clear single band from 4 D. spp. strains, while 12 fungal species of other genera showed no amplification under the same conditions (Figures 1B and 1C), which confirms that the primers and PCR analysis could specifically detect D. spp. strains. Moreover, the PCR assay was compared with traditional pathogen identification method to investigate the accuracy of the primers in detecting pathogen-infected fruits. As shown in Figure 1C and Table S1, the result of PCR assay was consistent with that of the pathogen isolation and identification. The samples of 2, 3, 7, 8, 9, 10, 11, 13, 14, and 15 were identified as D. spp. by traditional methods, and amplified specific DNA bands were also consistently shown in gel electrophoresis image, indicating that the identification result of the specific primers was accurate.

Principle on plasmonic dimer-enhanced Rayleigh/Raman spectroscopy

The principle of the SERS assay was based on sequence-specific DNA hybridization and the formation of a Y-shaped DNA duplex structure with designed sequence orientation (Figures 2 and S1). Two DNA probes (marked as P1 and P2) and helper DNA were separately anchored onto RRs-modified GNPs to synthesize plasmonic nanoprobes. Both P1 and P2 contain two segments: one is the detection primer or degenerate primer designed before, which is complementary to a portion of the target; another segment of P1 and P2 could be complementary to each other (Table S2). In the presence of the target fragment, the target strand could hybridize with DNA probes to form a Y-shaped DNA duplex during annealing, resulting in the formation of hot spots with minimized gap distance between two nanoparticles and enhanced SERS signal.33,34 However, no DNA duplex was formed without target since
the Tm of a complementary segment in P1 and P2 was below room temperature. Besides, to avoid lower sensitivity caused by the formation of large target-induced aggregation of plasmonic nanoprobes, helper DNA was introduced during the fabrication of nanoprobes to adjust the density of DNA probes modified on GNPs.35,36

The conjugation of DNA on GNPs was achieved by a typical salt aging method,37 followed by characterization using UV-vis absorption spectroscopy and zeta potential analysis. According to the single particle scattering theory, the larger the particle size, the stronger the scattering light of GNPs, i.e., large-sized GNPs have stronger scattering light and should provide better SERS enhancement; however, their stability is insufficient especially when DNA is modified because large particles will consume more probes and the modification process becomes more unstable, which is likely to cause aggregation, interfere with the formation of dimers, and cause false-positive signals. Overall, we chose 40 nm GNPs for the SERS analysis.

The citrate-capped 40 nm GNPs exhibited a surface plasmon resonance (SPR) absorption peak at 528 nm. The SPR peak of RRs-modified GNPs had no apparent shift compared with the unmodified GNPs, while the further conjugation of DNA induced a slightly red shift of absorbance peak to 530 nm, indicating the successful immobilization of DNA on GNPs (Figure S2A). The gradual decrease in zeta potential stated the change of the surface environment of GNPs after modification of RRs and DNA, which also confirmed the successful attachment of RRs and DNA to GNPs (Figure S2B). Transmission electron microscopy

Figure 1. Screening the primers for Diaporthe genus

(A) Sequence alignment of target fragments in ITS genes from Diaporthe and other genera including (1–10) ten isolates of Diaporthe, (11–12) two isolates of Botryosphaeria, (13–14) two isolates of Altemaria, (15) Botrytis fabae isolate, (16–18) three isolates of Botrytis cinerea, (19) Penicillium expansum isolate, and (20) Pestalotiopsis trachicarpica isolate. The illustrated sequences of the primer D.sp-29 (P1) and the degenerate primer D.sp-JB (P2) are illustrated at the top which has 5 degenerate bases and leads to 32 combinations, and the letter K represents T and G bases, S represents G and C bases, W represents A and T bases, Y represents T and C bases, W represents A and T bases, Y represents T and C bases.

(B) Specificity examination of the D.sp-29 for Diaporthe genus, Lanes 1–16 are different fungal isolates, (1–2) Botryosphaeria, (3) Penicillium expansum, (4) Altemaria, (5) Botrytis cinerea, (6) Arthrinium saccharicola, (7) Cladosporium anthropophilum, (8) Cryptosporiopsis diversispora, (9) Diaporthe cotoneastri, (10) Diaporthe nobilis, (11) Epicoccum nigrum, (12) Neofusoccum parvum, (13) Diaporthe amygdali, (14) Diaporthe capsica, (15) Fusarium fujikuroi, (16) Mucor circinelloides, and (M) the DNA marker.

(C) Accuracy examination of the primers and PCR assay on the pathogen-infected soft rotted kiwifruits. Lanes 1–16 were different soft rotted kiwifruits infected by different pathogens. The lanes 2, 3, 7, 8, 9, 10, 11, 13, 14, and 15 were Diaporthe spp-infected fruit samples.
(TEM) has been used to observe the morphologies of the nanoprobe and evidence of the formation of dimer after the presence of the target DNA (Figure S2C).

To achieve better analytical performance, the concentration ratio of DNA probe and helper DNA used for modifying GNPs was optimized by DFM observations according to the maximum ratio of plasmonic dimers in total nanoprobes, since different aggregation states of nanoprobes show distinct plasmonic colors under DFM. As shown in Figure 3A, owing to the localized surface plasmon resonance (LSPR) coupling effect, there were mainly four types of spots in dark-field images: green, yellow, orange, and red spots. The scattering spectrum of green dots presented a peak at approximately 540 nm. In contrast, the scattering spectra of yellow, orange, and red dots showed peak wavelengths at 575, 594, and 622 nm, respectively (Figure 3B). According to previous work,38–41 these dots could represent three aggregation states based on the LSPR peak. Green dots and yellow dots represented single nanoprobe and GNP dimers, respectively, whereas orange and red dots were large clusters of GNPs.38,42,43 For the following analysis, orange and red dots were classified and counted together. DFM-assisted condition optimization was based on the assumption that target-induced aggregation tended to form GNP dimers rather than large clusters with an appropriate ratio of DNA probe to helper DNA. That is, there were more yellow dots and fewer red dots under DFM observations.

In order to improve the accuracy and efficiency of classification and quantitative analysis, the RGBtoHSI algorithm was used to distinguish cluster types of nanoprobes and count the number of corresponding dots in dark-field images. The algorithm contained three parts: converting a color model, categorizing and counting plasmonic signals, and redrawing image spots (Figure S3). Each image was processed by converting the color model from RGB (red, green, blue) to HSI (hue, saturation, intensity), more in line with human visual perception for color.44,45 Green, yellow, and red spots could be easily distinguished according to the hue value, where green spots had hue values ranging from 60 to 150 and yellow spots had hue values ranging from 40 to 60. The Hue value of red spots contained two ranges: from 0 to 40 and 340 to 360.46 The remaining spots represented interference signals such as dust and off-focus spots (Figure 3D).39 Then, the proportion of different color spots was counted to demonstrate target-induced aggregation states of plasmonic nanoprobes with different DNA probe densities. In addition, the spots were redrawn with small rectangles according to the results of cluster type classification.

Figure 2. The pair of genus-level primers, P1 and P2, assisted the target-programmed GNP dimerization, leading to enhanced SERS signal and distinct plasmonic signals in DFM imaging

P1 and P2 are the selected primer, and P2 has 5 degenerate bases with 32 combinations, and the letter K represents T and G bases, S represents G and C bases, W represents A and T bases, Y represents T and C bases, respectively.
We noted that the synthesized plasmonic nanoprobes also appeared yellow and red spots under DFM due to inevitable aggregation during the synthesis or washing procedure (Figure S4A). Therefore, target-induced aggregation and inherent aggregation were counted and compared. Here, a fragment of genomic DNA of *D. spp.* (named GZGC1-2, Table S2) was chosen as the target, and PBS buffer was used as a blank sample. As shown in Figure S4, there was approximately 22% aggregation in plasmonic nanoprobes including GNP dimers and large clusters, while target-induced aggregation significantly increased to ~40%. Actually, only ~18% of clusters were induced by target DNA. Therefore, the inherent aggregation in nanoprobes was subtracted for subsequent optimization of DNA probe density. The quantitative results of aggregation states under different experimental conditions were shown in Figure 3E, where the increase

(Figure 3C). The redrawn images were consistent with the original DFM images, indicating the feasibility of the algorithm (Figures 3A–3C).
of yellow and red dots and the corresponding decrease of green dots compared to the blank control indicated the aggregation induced by target DNA. The dark-field images and statistics of aggregation states for optimizing the ratio of DNA probe/helper DNA were detailed in the Datas S1–S6. DFM Imaging and Analysis. With the increased concentration of helper DNA, the proportion of yellow dots displayed a trend of decrease, increase, and decrease, while the proportion of red dots first increased then decreased. The decrease of green dots was relatively stable, but when the ratio of DNA probes to helper DNA reached 1:6, target-induced aggregation significantly reduced. It was obvious that with a ratio of 1:4, the proportion of GNP dimers reached a maximum and the proportion of large clusters was relatively low. Furthermore, A FAM dye labeled P1 (P1-FAM) was applied to estimate the conjugation number of effective DNA probes on each GNP, which might help explain the results of DFM.

Figure 3F indicates that the number of effective DNA probes first increases and then decreases with the increased concentration of helper DNA. We conjectured that without helper DNA, the steric hindrance resulting from high density influenced the hybridization ability of DNA probes, leading to only eight strands of effective DNA on each GNP. However, the increase of helper DNA reduced the influence of steric hindrance, so the amount of effective DNA increased, which resulted in the formation of more large clusters. The number of effective DNA and the proportion of target-induced large clusters reached the maximum at the ratio of 1:1 along with the lowest GNP dimer proportion. As the concentration of helper DNA continued to increase, DNA probe density on GNP decreased with a reduction of effective DNA probes, contributing to an increased proportion of GNP dimers and decreased proportion of large clusters. Nevertheless, when the concentration ratio of DNA probe/helper DNA reached 1:6, there were only two strands of effective DNA on each GNP, and the number of target-induced aggregations significantly reduced. Here, the ratio of 1:4 was the optimal condition with the most GNP dimer and the least large clusters and was used for subsequent SERS detection; neither too much nor too little effective DNA was conducive to the formation of GNP dimers.

Analytical performance of Rayleigh/Raman spectroscopy

DFM has better sensitivity than SERS from the perspective of single particle imaging, and DFM has better accurate detection of hybridization process. The data obtained by DFM are based on the analysis of the scattering signal response of a single particle, excluding the interference of the average effect of the system. Nevertheless, DFM requires complex equipment and setups and is not suitable for field detection. Nevertheless, the SERS technique is possible to develop into a portable tool for field detection by the use of portable Raman spectrometer. As for the test cost, the commercial production of DNA probes has been achieved and its cost is affordable.

To evaluate the analytical performance of our strategy, SERS spectra of targets with different concentrations were collected under the optimal experimental conditions. Target fragments of GZGC1-2, probe 1 (100 pM), and probe 2 (100 pM) were heated to 95°C and annealed to 37°C before SERS analysis. As shown in Figure S5A, the SERS signals of 4-mercapto benzoic acid (4-MBA) at 1071 cm⁻¹ and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at 1326 cm⁻¹ were significantly enhanced compared with the control group, and the intensities gradually increased along with the increase of target concentration from 1 pM to 100 pM, indicating the increasing formation of GNP dimers with the increasing target concentration. However, SERS signal no longer increased when the target concentration was higher than 100 pM (Figures S5B and S5C). We speculated that the double-stranded target was the reason for this phenomenon. When the concentration of targets exceeded that of the nanoprobes, the excess complementary strands competitively hybridized with the target strands during annealing, thus influencing the hybridization between DNA probes and target strands, leading to the appearance of comparable SERS signal.

DFM was used to reveal the aggregation state of nanoprobes induced by different concentrations of the target. As shown in Figure 4A, target-induced aggregation of nanoprobes increased along with the raising of target concentration from 0 pM to 100 pM, indicating the increasing formation of Y-shaped DNA duplex-linked nanoprobe aggregation and thus contributing to a gradual increase in the SERS signal. Nevertheless, there was no significant difference in aggregation induced by 1 nM or 10 nM targets where the complementary strands were excessive, which might be the reason for no difference in the SERS signal. The dark-field images and statistics of aggregation states for detecting different concentrations of targets were detailed in the Datas S7–S12. DFM Imaging and Analysis. Note that there was still a linear relationship between SERS intensity of DTNB at 1326 cm⁻¹ (4-MBA at 1071 cm⁻¹) and target concentration logarithm within
A range of 1–100 pM (Figures 4Ba and 4S5D). In addition, 30 random SERS spectra of 10 pM target fragment were collected to evaluate the reproducibility. A statistical analysis of the peak strength of DTNB at 1326 cm\(^{-1}\)/\(C_0\) had a relative standard deviation (RSD) of 10.32% (Figure 4C), indicating a good repeatability of the SERS assay.

To examine the specificity of the SERS assay, target fragments in genomic DNA of \(D.\) spp. and other genus were detected at 10 pM, the sequences of which were shown in Table S2. Inspiringly, no significant signal change was observed for DNA fragments of other genus compared with the blank control. In contrast, the SERS intensity dramatically increased with the presence of DNA fragments of \(D.\) spp., implying that only the target fragments of \(Diaporthe\) could induce the formation of Y-shaped DNA duplex and GNP dimers, which attributed to the excellent selectivity of the assay (Figures 4E, 4F, and 4S6). Yet we noticed that the SERS intensity of different species of \(D.\) spp. varied, which was the consequence of the single or two base differences in sequences. Despite this fact, the assay could still be applied for specific detection of \(D.\) spp. in practical use.

Figure 4. Nanoprobe optimization and its reproducibility and specificity
(A) Statistical results of nanoprobe aggregation induced by different concentrations of target fragment. Detailed DFM images and statistical data were listed in Datas S7–S12. DFM Imaging and Analysis.
(B) A linear relationship between the intensity of characteristic SERS peak at 1326 cm\(^{-1}\) and the logarithm of concentration of target fragment.
(C) The statistical deviation of SERS intensities at 1326 cm\(^{-1}\).
(D) Comparison of nanoprobe aggregation induced by double-stranded target DNA and single-stranded target DNA, respectively, in DFM observations.
(E and F) Histogram of the SERS intensity at 1071 cm\(^{-1}\) and 1326 cm\(^{-1}\), respectively, produced by the DNA samples from different Fungal isolates. SXJY2-1, CDHY1-1, GZHY3-1 belong to \(Diaporthe\) genus, while GZHY3-1 belong to \(Alternaria\) sp., WZBLN2-2 belong to \(Botryosphaeria\) sp., HBR2-5 belong to \(Penicillium\) sp. The error bars represent the SD of repeated experiments.

a range of 1–100 pM (Figures 4B and 5S). In addition, 30 random SERS spectra of 10 pM target fragment were collected to evaluate the reproducibility. A statistical analysis of the peak strength of DTNB at 1326 cm\(^{-1}\) had a relative standard deviation (RSD) of 10.32% (Figure 4C), indicating a good repeatability of the SERS assay.

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In addition, based on the above inferences of the competition between the complementary strands and nanoprobes, UV-vis spectroscopy, DFM, and agarose gel electrophoresis were used to further explore the differences between the aggregation of nanoprobes induced by single-stranded target and double-stranded target. As shown in Figure S7, single-stranded target-induced nanoprobes aggregation appeared a red shift of UV-vis spectra, while nanoprobes aggregation induced by double-stranded target showed no significant distinction. This result indicated that the existence of excess complementary strands (>1 nM) would affect the hybridization of target DNA and nanoprobes (100 pM). Thus, nanoprobes aggregation was not detectable by UV-vis spectroscopy, which verified our speculation mentioned above. Similarly, DFM analysis revealed that single-stranded target induced about 40% of nanoprobes aggregation, whereas only 18% of aggregation was induced by double-stranded target (Figure 4D). Furthermore, agarose gel electrophoresis was performed to test the formation of Y-shaped duplexes by the hybridization of DNA probes and target strands. As shown in Figure S8, a clear retardance was observed in lane 6 which demonstrated the formation of Y-shaped duplexes. However, it is obvious that Y-shaped duplexes were in the minority and most targets were left. The results indicated that the complementary strands in the double-stranded target had an effect on the hybridization of the nanoprobes and the target strands. Despite the influence, the SERS assay still shows great potentials in the application of D. spp. detection.

**Double-blind test of genomic DNA of plant pathogens**

In order to identify the practicability and reliability of the SERS assay, a double-blind test was performed. Genomic DNA from 18 known pathogens of kiwifruit soft rot was extracted and fragmented with ultrasonic treatment, followed by randomly labeling the samples from 1 to 18 (Table S3). The gel electrophoresis results showed the size of ultrasonic DNA fragments (Figure S9). The 18 samples were detected by another researcher with the proposed SERS assay. The ultrasonic DNA fragments were diluted to 5 ng/μL for SERS detection. In Figure 5, sample no. 4, 6, 9, 14, 16, and 17 generated the SERS signals similar to the blank control, whereas the signals of the rest samples at 1071 cm$^{-1}$ and 1326 cm$^{-1}$ are significantly enhanced. The detection results indicated that sample no. 4, 6, 9, 14, 16, and 17 were not *Diaporthe* species, while the rest 12 isolates belonged to *Diaporthe* genus. The SERS detection results are consistent with the labeling results of the researcher who was preparing pathogen DNA (Table S3), implying the specificity and feasibility of the SERS assay for analysis of unamplified genomic DNA in practical use. It is worth noting that the SERS intensities of *Diaporthe* sp. differed similarly on account of base mismatches between primer or degenerate primer and target fragment (Figure S10). It should be noted that there are other species of
D. spp., but they were not analyzed in this work due to the infeasibility in isolating these pathogens from the kiwifruit rot. The above tested 12 species of D. spp. were prevalent in China.

**Early warning of infected kiwifruits**

To clarify the applicability and sensitivity of our SERS method for detecting pathogens-infected kiwifruits, healthy kiwifruits were inoculated with *Diaporthe nobilis* to simulate the pathogen infection, and sterile water was inoculated as control. The symptoms of kiwifruits at different times after pathogen infection were observed by peeling. For fruits without peeling, no symptoms can be observed from the appearance after inoculation (Figure S11). It is hard to judge whether the disease occurs from the appearance of the kiwifruit, which is also the main reason for the difficulty in preventing and controlling kiwifruit soft rot. However, an initial lesion with a small pale yellow or light brown spot appeared on the peeled flesh after 48 h of inoculation, and the lesion area further expanded with the duration of the infection. Eventually, the lesion formed typical symptoms with dark brown lesion and water-soaked appearance, as shown in Figure 6A after 96 h of infection. An indistinct water-soaked appearance was observed 96 h after inoculation (Figure 6A). In contrast, the control group did not show the symptom or tendency for rot. Therefore, the

![Figure 6](image)
detection of presence of a pathogen before a lesion emerges is of extreme significance for the early warning and timely control measures of kiwifruit soft rot. The infected fruit tissues at different time points were collected to extract genomic DNA and then used to detect the sensitivity of the SERS pathogen detection method.

As showed in Figures 6B–6E, the SERS signals generated from infected kiwifruit for 0 and 12 h were similar. Meanwhile, there was no difference between the SERS signals of the control group and its corresponding infection group at these two time points. The results indicated that the SERS assay we developed could not detect the pathogen-infected fruit within 12 h. However, the kiwifruit infected for 24 h generated significantly higher SERS signal than those of the control group and 0 h infected kiwifruit, and the SERS intensity gradually increased with the duration of infected time. Meanwhile, the PCR assay was performed to compare the detection sensitivity with the SERS assay. As shown in Figure 6F, the DNA amplification bands were observed at 60 h after infection, yet not in earlier time. These results suggested that the SERS assay was more sensitive than the PCR or gel electrophoresis and could diagnose diseased kiwifruit without any noticeable symptoms, making it better suited for early warning of D. spp. infection.

The appropriate analysis strategy for spot detection needs to be fast, simple, and portable. Based on this, we chose a portable Raman spectrometer for spot detection. As for the test cost, the price of DNA probe is not as expensive as expected. Moreover, SERS requires fewer probes; this enables our synthesized probes to complete many tests, which reduces the cost.

Conclusion
In summary, we have developed the plasmonic dimer rayleigh/Raman spectroscopy assay that enabled detecting Diaporthe infection in kiwifruit at an early stage of 24 h, of which the specificity was realized by a pair of genus-level primers. DFM and RGBtoHSI algorithms were successfully applied to optimize the ratio of DNA probes and helper DNA, promoting the sensitivity of the SERS assay. The sequence-specific DNA hybridization and the formation of Y-shaped DNA duplex structure between target and nanoprobe ensured the specific and sensitive detection of the Diaporthe genus. A double-blind experiment confirmed the reliability of our SERS assay. Compared with the PCR assay, the proposed SERS assay displayed better sensitivity to diagnose Diaporthe infection before any noticeable symptoms. The early warning of pathogen infection at the first 24 h has great prospects in controlling disease spread and helping informed decision-making for field management. We envision that our SERS assay holds excellent potential for fast, early diagnosis of pathogen and quality control of kiwifruit in future.

Limitations of the study
Some limitations to the findings of this study must be acknowledged. First, Diaporthe has been identified as the main pathogens of kiwifruit in recent years. Although we have tried our best to detect the pathogens we own, there are still other pathogens belonging to the Diaporthe genus are not included due to insufficient experimental conditions, so the comprehensiveness of the detection results needs to be further improved. Second, we have proved that our method has a good application prospect in practical production, but the nanoprobe still needs to be prepared and stored in advance. Therefore, there are still some challenges in using this method for real samples.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105650.

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AUTHOR CONTRIBUTIONS

Ting Yu, Baomei Zhou, Hao Jiang, Min Xu, and Mengke Su collected the data and performed the analysis; Ting Yu wrote the paper; Huanhuan Li, Kui Liu, and Yongsheng Liu provided actual samples; Min Miao and Honglin Liu conceived and designed the analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| Chloroauric acid | Nanjing Chemical Reagent Co., Ltd | Cat#50780 |
| Hydroxylamine hydrochloride | Nanjing Chemical Reagent Co., Ltd | Cat#C02150123 |
| 4-mercaptobenzoic acid | Aladdin Industrial Inc. (Shanghai, China) | Cat#M100780 |
| Tris (2-carboxyethyl) phosphine hydrochloride | Aladdin Industrial Inc. (Shanghai, China) | Cat#T1656 |
| Trisodium citrate | Sinopharm Chemical Reagent Co., Ltd | Cat# C49-11863 |
| Chloride | Sinopharm Chemical Reagent Co., Ltd | N/A |
| Sodium dodecyl sulfate | Sinopharm Chemical Reagent Co., Ltd | Cat#20106ES76 |
| Disodium Hydrogen Phosphate Dodecahydrate | Sinopharm Chemical Reagent Co., Ltd | Cat# D16-1013064 |
| Sodium dihydrogen phosphate dihydrate | Sinopharm Chemical Reagent Co., Ltd | Cat# S817970 |
| Sodium hydroxide | Sinopharm Chemical Reagent Co., Ltd | Cat# S817979 |
| 5,5'-dithiobis-(2-nitrobenzoic acid) | Sangon Biotechnology Co., Ltd. (Shanghai, China) | Cat#D0944 |
| Oligonucleotides in Table S2 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| Ultrapure water (18.2 MΩ-cm) | Millipore water purification system | N/A |

### Oligonucleotides

| Oligonucleotide | Source | Identifier |
|-----------------|--------|------------|
| D. sp-29 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| D. sp-485 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| D. sp-JB | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| ITS1 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| ITS4 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| P1 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| P2 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| Helper DNA | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| D.sp-29-FAM | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| G2GC1-2 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| G2GC1-2' | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| SXJY2-1 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| SXJY2-1’ | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| SDZB3-2 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| SDZB3-2' | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| CDHY1-1 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| CDHY1-1’ | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| WZBLN2-2 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| WZBLN2-2’ | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| HBR22-5 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| HBR22-5’ | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| G2HY3-1 | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |
| G2HY3-1’ | Sangon Biotechnology Co., Ltd. (Shanghai, China) | N/A |

### Bacterial and virus strains

| Strain | Source | Identifier |
|--------|--------|------------|
| D. anhuiensis | isolation from rotted fruits | MT877015 |
| D. sojae | isolation from rotted fruits | MT877050 |
| D. biguttulata | isolation from rotted fruits | MT877049 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Honglin Liu (liuhonglin@mail.ustc.edu.cn).

Materials availability
This study did not generate any new unique reagents.

Data and code availability
All data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Pathogen isolation and identification
The pathogen isolation and identification were performed as described by Li. The margins between symptomatic and healthy tissues were cut from rotted fruits, surface disinfested in 1% NaClO and 70% ethanol solution, washed, dried, plated on potato dextrose agar (PDA) containing 50 mg/liter of streptomycin sulfate, and incubated at 25°C for 3 days. Hyphal tips were transferred to PDA to obtain pure cultures and DNA extraction was mentioned as above. The internal transcribed spacer (ITS) was amplified using specific primer pairs of ITS1/ITS4 and sequenced. The sequences were compared with those deposited in the National Center for Biotechnology Information (NCBI) library using the BLAST algorithm online at https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Pathogen culture and infection
All the pathogens were cultured in PDA plates at 25°C. As for in vitro infection, ripe healthy fruits cv. Xuxiang (Actinidia deliciosa) were surface-disinfected with 1% NaClO solution for 10 min, rinsed three times in sterile distilled water and dried. Four holes (2 mm diameter and approximately 3 mm deep, same interval) were

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Botrytis fabae      | isolation from rotted fruits | N/A        |
| D. podocarpi-macrophyll | isolation from rotted fruits | MT877048   |
| Alternaria alternata | isolation from rotted fruits | N/A        |
| D. passiflorae      | isolation from rotted fruits | MT877046   |
| D. incompleta       | isolation from rotted fruits | MT877061   |
| Penicillium expansum| isolation from rotted fruits | MT877045   |
| D. phaseolorum      | isolation from rotted fruits | MT877041   |
| D. novem            | isolation from rotted fruits | MT877036   |
| D. lithocarpus      | isolation from rotted fruits | MT877024   |
| D. longicolla       | isolation from rotted fruits | N/A        |
| Botrytis cinerea    | isolation from rotted fruits | MT877017   |
| Fusarium graminearum| isolation from rotted fruits | N/A        |
| Fusarium avenaceum  | isolation from rotted fruits | N/A        |
| D. nobilis          | isolation from rotted fruits | MT877025   |

Software and algorithms
Imagej Image processing software https://imagej.net/
wounded with sterile nails, followed by inoculation of 10μL of D. nobilis conidial suspension adjusted to 1 × 10⁵ conidia/mL. Afterwards, treated kiwifruit were sealed in polyethylene-lined plastic boxes and kept in the thermostatic cultivation room at 25°C with 80–90% relative humidity. After 12 h of inoculation, the samples were collected from 5 fruits interval 12 h. The frozen fruit samples were ground to powder using liquid nitrogen for DNA extraction.

METHOD DETAILS

Chemicals
Chloroauric acid (HAuCl₄·4H₂O) and hydroxylamine hydrochloride (NH₂OH·HCl) were supplied by Nanjing Chemical Reagent Co., Ltd. 4-mercaptobenzoic acid (4-MBA), Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Aladdin Industrial Inc. (Shanghai, China). Trisodium citrate (C₆H₅Na₃O₇·2H₂O), chloride (NaCl), sodium dodecyl sulfate (SDS), Disodium Hydrogen Phosphate Dodecahydrate (Na₂HPO₄·12H₂O), Sodium dihydrogen phosphate dihydrate (NaH₂PO₄·2H₂O) and Sodium hydroxide (NaOH) were all bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) and oligonucleotides in Table S2 were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). Ultrapure water (18.2 MΩ·cm) was produced by Millipore water purification system.

Synthesis procedures of 40 nm GNPs
40 nm GNPs were prepared by a seed-growth method for nanoprobe synthesis. First, 15 nm GNPs were prepared as seeds. 98.9 mL of water and 1 mL of trisodium citrate solution (30 mg/mL) were added to a clean flask on a hotplate and heated to boiling under gentle stirring, keeping for 7 minutes. Then, 0.1 mL HAuCl₄·4H₂O (98.5 mg/mL) was added swiftly to the flask and reacted for another 7 min under vigorous stirring. The solution was cooled on ice and stored at 4°C. Seed-growth method was repeated three times to synthesize 40 nm GNPs. Each procedure is as follows: 9.4 mL GNPs, 10 mL water, 0.2 mL NH₂OH·HCl (250 mM), 0.2 mL trisodium citrate (10 mg/mL) were mixed together in a flask and stirred for 5 min at 650 rpm. Then, 0.4 mL HAuCl₄·4H₂O (1% w/v) was added to the mixture and reacted for 1 h.

Preparation of gold nanoprobes
First, GNPs were modified with Raman reporters (DTNB, 4-MBA) by adding Raman reporters (RR) in ethanol to the GNP solution with a final concentration of 250 nM and incubating for 2 h. The solution was centrifuged and washed three times to remove excessive reagents and the precipitate was redispersed in water for DNA modification. Thiol-modified DNA probes and helper DNA (100 μM) were reduced by 1m MTC Ep or 1h fIr s t .T r e a t e d D N Ap r ob e( 6 m L, 100 m M) and helper DNA (24 mL, 100 μM) were added to 0.5 mL RR-modified GNPs and shake at 37°C for 12 h. Then, 0.1M phosphate buffer (pH 7.4) and 0.1% SDS were added to the solution with final concentrations of 0.01M and 0.01%. For the next 10 h, 2 M NaCl were added to the solution at 2-h intervals with a final of 0.2 M, followed by shaking overnight. The resulting solution was washed and redispersed in assay buffer (0.01M phosphate buffer, 0.01% SDS, 0.2M NaCl) for further use.

DNA extraction
The DNA extraction of kiwifruit was performed using a commercial Plant Genomic DNA Extraction Kit (Biofit, Chengdu, China) according to the manufacturer’s protocol. The DNA extraction of pathogen was performed with cetyltrimethylammonium bromide (CTAB) method.5 The DNA concentration and quality was determined by measuring the absorbance using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). For dark field and SERS measurement, the DNA solution was sonicated in ice bath with consecutive 3-s-on, 6-s-off cycles for 40 min using a handheld ultrasonic cell crushing apparatus (Biosafer250up).

PCR protocols
The PCR assay was performed in a total volume of 25 μL using 2× Rapid Taq Master Mix (Vazyme Shanghai, China). The thermal cycling conditions were as follows: Initial denaturation 95°C – 3 min, 35 cycles: 95°C – 30 s, 50°C – 30 s for the primer (D. sp-29 and D.sp-485), 65°C – 30 s for the primer (D. sp-JB and D.sp-485), and 55°C – 30 s for the primer (ITS1 and ITS4), 72°C – 30 s, final extension 72°C – 10 min and hold at 4°C. PCR...
products were separated in 1% (w/v) agarose gels stained with Gel-red nucleic acid dyes and exposed to ChemiDoc XRS (BIO-RAD, USA) to visualize DNA fragments.

**Dark field imaging and SERS measurement**

50 μL of extracted nucleic acid was added to 100μL of nanoprobe mixture (probe 1 and probe 2 were mixed at a 1:1 ratio with the concentration of 100 pM). The solution was heated to 95°C for 3 min, and slowly cooled to 37°C in a mini dry bath (MIULAB, Hangzhou, China). After the hybridization process, SERS spectra were collected by a portable Raman microscope (BWTek, Delaware, USA) under excitation at 785 nm with 10% power, and the exposure time is 10s for three accumulation. For dark field imaging, microscope slides were cleaned by the piranha solution (H2SO4:H2O2 = 7:3, v/v), and the hydrophilic property of slides was enhanced at the same time. The DFM images and scattering light spectrum were acquired with a 60X, 0.7 NA dry objective and a triple grating spectrometer (Zolix, Beijing, China). The related results are shown in the Data S1–S12. DFM Imaging and Analysis.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Estimating hybridization efficiency of DNA probes**

To quantify the number of effective DNA probes on each GNP, the fluorescence spectrum of complementary chain labeled with FAM (D.sp-29'-FAM) was detected for the calibration curve approach. D.sp-29'-FAM and the probe 1 were incubated together for hybridization, followed with centrifugation and fluorescence measurement of the supernatant. The concentration of the unhybridized D.sp-29'-FAM was calculated according to the standard curve, which was converted to the concentration of D.sp-29'-FAM participating in the hybridization. Finally, the number of effective DNA probes loaded on each GNP was calculated by the following equation:

\[
N_{DNA} = \frac{N_{total-DNA}}{N_{total-GNP}} = \frac{C_{DNA}}{C_{GNP}}
\]