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AGROBACTERIUM-MEDIATED TRANSFORMATION OF *VERTICILLIUM DAHLIAE* WITH GFP GENE TO STUDY COTTON-PATHOGEN INTERACTION USING A NOVEL INOCULATION METHOD

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Abstract

Verticillium dahliae is a soil-born fungal pathogen which causes *Verticillium* wilt in economically important crops including cotton. We conducted a study to monitor the interaction between the fungus and cotton. *V. dahliae* was transformed with the gene encoding green fluorescent protein. The gene can be constitutively expressed and fluorescence was clearly visible in both hyphae and spores. Due to heterogeneous gene insertion, the growth rate, colony morphology and pathogenicity of fungus transformants showed differences compared with corresponding wild type. Similarly, quantitative real-time PCR analysis also indicated significant differences in the gene expression among different *V. dahliae* transformants. To study cotton-pathogen interaction, we devised a novel inoculation method and developed a successful infection by keeping GFP-expressed mycelial plug alongwith aseptic cotton seedlings. After 6-day inoculation, the LSM microscopic image showed that the fungus rapidly formed a mycelial network on the surface of the stems and colonized into plant tissue, displayed an intercellular infection pattern. The early events during cotton colonization by *V. dahliae* can be successfully observed in 10 days including the plant growth period. Besides, pathological changes of seedlings like tissue discoloration, wilting, stem dehiscence and necrosis can be clearly observed without the influences of soil and other microbes. This inoculation method provides a rapid, effective and environmental friendly technique for the study of cotton-pathogen interaction and identification of resistant plant cultivars.

Key words: *Agrobacterium*, Transformation, *Verticillium dahliae*, GFP, Cotton.

Introduction

Cotton (*Gossypium* spp.), one of the most important economic crops in the world, has suffered great fiber quality reduction and significant yield losses due to a vascular disease called *Verticillium* wilt (Khaskheli *et al*., 2014). The representative symptoms showed in the susceptible cotton include leaf chlorosis, defoliation and brown discoloration of the vascular tissue (Sink & Grey, 1999). *Verticillium dahliae* Kleb is a soil-born fungus which can cause the serious disease. It is widely distributed all over the world and can produce persistent resting structures known as microsclerotia (Tjamos *et al*., 2005). Until now, there are few resistant resources in upland cotton with resistance to *Verticillium* wilt, and no cultural practices can be always effective to control this disease (Miao *et al*., 2010; Xu *et al*., 2011; Bibi *et al*., 2013).

During long term process of co-evolution, plants have evolved a complicated defense mechanism against pathogens. It is of great importance for agricultural production to understand and recognize the interaction between plants and pathogens. The green fluorescent protein (GFP) derived from the jellyfish *Aequorea Victoria* is a 27 kDa protein and can generate striking green fluorescence upon excitation by UV or blue light (Shimomura *et al*., 1962; Bogs *et al*., 2004). Since the gene encoding it was cloned and successfully expressed in heterologous systems, GFP has proved to be an ideal marker to study fungal-plant interactions (Chalfie *et al*., 1994; Freitag *et al*., 2004; Skadsen *et al*., 2004; Zhang *et al*., 2013). For GFP expression, no exogenous substrates or cofactors are needed except of light and oxygen (Spellig *et al*., 1996). It facilitates the analysis of living tissues without cell lysis and tissue distortion (Chen *et al*., 2003). By fluorescence microscopy, fungus infection and pattern of colonization during the entire disease cycle can be observed and the role of race-specific resistance can also be examined (Lorang *et al*., 2001; Eynck *et al*., 2007; Vallad & Subbarao, 2008; Li *et al*., 2011).

There are a number of plant inoculation methods devised by different researchers to monitor fungal colonization and assess the host cell's resistance (Koga *et al*., 2004; Ruz *et al*., 2009; Tefera & Vidal, 2009). As reported, the type of infection method would have a significant effect on fungal biomass in inoculated tissue using the same inoculum (Berruyer *et al*., 2006). Some studies also indicated that the rate of fungus colonization may depend on inoculation method and plant growth condition (Tefera & Vidal, 2009). For soil-born fungus, methods such as spraying of plants, root-dip inoculation, stem injection and soil-drench inoculation are most widely used (Siddiqui & Shaukat, 2002; Infantino *et al*., 2006; Nel *et al*., 2007; Gao *et al*., 2011), and effective ways have made to study plant-microbe interaction. However, poor experimental conditions control such as untreated polluted soil and unreasonable use of spore suspensions would cause serious fungus spread, which deserves concern.

The present study was planned to figure out the characteristics of GFP-transgenic *V. dahliae* isolate Anyang in terms of growth rate and colony morphology, mitotic stability and pathogenicity. Besides, a novel inoculation method is developed to monitor plant host-pathogen interactions. Due to economical importance of cotton crop we used cotton seedlings as host for developing infection with GFP-transformed *V. dahliae* and successful infection was developed as visualized from morphology of seedlings. The advantage of this inoculation method is discussed.

Materials and methods

Fungal strain and culture conditions: *V. dahliae* isolate Anyang (ACCC no. 36207), a popular *V. dahliae* strain (Vd), was isolated from Anyang City, Henan Province, China. It was supplied by the Cotton Research Institute of the Chinese Academy of Agricultural Science and was preserved at the Agricultural Culture Collection, Beijing, China. The isolate was cultured on potato dextrose agar (PDA) medium and incubated at 25°C in the dark. For conidial production, Vd was sub-cultured from PDA plates into Czapek's medium on a shaker at 150 rpm at 25°C for 3 to 5 days. Fungal cultures were filtered through four layers of sterile gauze to retain the mycelia. The concentration of the spore suspension was counted with a haemocytometer and adjusted to a concentration of 10^6 spores mL^{-1} with sterile distilled water for immediate use in transformation experiment.

Plasmid vector, bacterial strain and transformation method: Transformation vector Sk1044 was kindly provided by Prof. Baolong Zhang (Jiangsu Academy of Agriculture Sciences, China) (Chen *et al*., 2011). The binary plasmid contains a hygromycin B phosphotransferase gene (hptB) as a selection marker and enhanced green fluorescent protein gene (eGFP) regulated by the gpdA promoter and B-tubulin terminator. Both *Escherichia coli* strain DH5α used during maintenance of the plasmid and *Agrobacterium tumefaciens* strain LBA4404 used for ATMT are preserved in our lab.

V. dahliae strain Anyang was transformed with the GFP report gene by *Agrobacterium tumefaciens*-mediated transformation (ATMT) as described by Eckert *et al*. (2005). Discrete colonies generated on PDA supplemented with 50 $\mu g \cdot mL^{-1}$ hygromycin B were checked under the fluorescence microscope.

Growth and Mitotic stability analysis of *V. dahliae* transformants: A 10 µL drop of the spore suspension (10^6 spores per mL) was placed on the surface of PDA medium and maintained in the dark at 25 °C during the growth study. Colony diameter was measured with a pachymeter along two axes, and the mean values were recorded. A Sony DSCF707 Cyber-shot 5MP Digital Still Camera (Sony, Japan) was used to capture growth images showing distinct colony morphologies.

For mitotic stability analysis, single-spore transformants were transferred to PDA medium containing 50 $\mu g \cdot mL^{-1}$ hygromycin B for subcultivation, and the test was determined as described previously (Flowers & Vaillancourt, 2005; Dobrowolska & Staczek, 2009).

PCR and quantitative RT-PCR (qRT-PCR) analysis: Genomic DNA from fungal mycelia was isolated using the method described by Al-Samarrai & Schmid, (2000). PCR analysis of transformants was performed using eGFP specific primers (5'-ACGGCAAGCTGACCCTGAAG-3' and 5'-CTCGTCCATGCCGAGAGTGA-3') and amplified a 590-bp fragment of eGFP gene during 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s (Chen *et al*., 2011). *V. dahliae* Specific ITS1-F primer (5'-CATCAGTCTCTCTGTTTATACCAACG-3') and ITS1-R primer (5'-CGATGCGAGCTGTAACTACTACGCAA-3'), which can amplify the internal transcribed spacers (ITS) of the ribosomal gene were used to confirm fungus, and a 324-bp product was amplified during 40 cycles of 94°C for 60 s, 51°C for 30 s and 74°C for 60 s (Zhu *et al*., 1998).

Total RNA from fungus mycelium of individual transformant was extracted with Tiangen RNAprep pure Plant Kit (Tiangen® Biotechnology, Beijing, China), and the first-strand cDNA was synthesized using the SYBR® RT reagent Kit with gDNA Eraser (TaKaRa® Biotechnology, Dalian, China) according to the manufacturer's instruction. EGFP and actin gene sequences were amplified from the cDNA and genomic DNA as described above, using an annealing temperature of 57°C. Reactions contained both eGFP gene-specific primers mentioned above and actin gene-specific primers (5'-GCCCTCTTCCAGCCCTCCGTTCTC-3' and 5' -TCGGCGTGGTTTTGTGGTGAG-3') (Dobinson *et al*., 2004). The qRT-PCR machine was set with 40 cycles and an annealing temperature of 60 °C. All reactions were performed in triplicate on a Bio-Rad DNA Engine thermo cycler (Bio-Rad Laboratories, Hercules, CA).

Pathogenicity analysis of transformants: Spore suspensions of both the wild type Vd and transformants strains were obtained as described above, and the concentration was adjusted to 10^6 spores per mL. Cotton seedlings cv CT1236 (susceptible) were infected by root-dip inoculation method as described by Qu *et al*. (2005). Disease severity of each plant was assessed 21 days post-inoculation (dpi) according to the method described by Yang *et al*. (2013).

Plant-pathogen interaction studies: Seeds of cotton (*Gossypium hirsutum* L) cv CT1236 (susceptible) were delinted with sulphuric acid and dried in the air, sterilized with 0.1% $HgCl_2$ for 10-15min and washed with sterile distilled water for 3-5 times. Plump, mature seeds were chosen and sown in the medium comprising half-strength MS macroelements, which had a pH of 5.8 and was supplemented with 2% sucrose and 0.7% agar at 28°C in dark treatment (Murashige & Skoog, 1962). For this experiment, we prepared 20 autoclaved bottles with the medium and each bottle contained 3-4 sterilized seeds. After 3 days of germination, bottles were transferred to a growth chamber under above mentioned conditions. The *V. dahliae* transformant which showed similar virulence with the wild-type strain and high level of fluorescence should be selected for cotton inoculation. On 4^{th} day, aseptic seedlings were inoculated with a mycelial agar plug (0.9 cm) taken from the growing edge of a 20-day-old fungus culture. The agar plug was placed onto MS medium against the stem base of cotton seedlings. For mock inoculation, the same size of agar plug without fungus mycelium was placed against seedlings.

Microscopic observations: Microscopic analyses were performed with a ZEISS LSM 780 laser scanning microscope, digital images of GFP tagged strains were acquired by scanning with 488 nm excitation and 493-556nm emission filters. A Leica DMIRB fluorescent microscope equipped with GFP fluorescence filter (450-490nm, LP515) and a Leica MZ95 stereomicroscope were also used to observe fungal strains and inoculated plant tissues. Both photo micrographs were taken by digital cameral (Leica DFC 300FX), operated with IM50 software (Leica DFC Twain, 6.2.1). All objects were directly placed on a glass slide in a water droplet, covered with a cover slip, and observed under microscope.

Statistical analysis: The analysis of variance was conducted between different treatments. The significant differences between different *V. dahliae* genotypes were worked out by One-way ANOVA ($p<0.05$). Mean separations were performed using the method of Tukey. Origin Pro 8.5 version (Origin lab corporation, Wellesley Hills, Wellesley, MA, USA) was used to prepare graphs.

Results

Growth analysis of *V. dahliae* transformants: The growth characteristics of four *V. dahliae* transformants (named Vdgfp-1, Vdgfp-2, Vdgfp-3 and Vdgfp-4) were analyzed and displayed in Fig. 1. The growth evolution represented by the colony diameter average up to 11 days' incubation time (Fig. 1a). Results revealed that the growth rate of all the transformants was faster than wild type, being maximum in Vdgfp-1. Defects in white mycelium presence and hyphae growth shape displayed representative phenotypes of *V. dahliae* transformants (Fig. 1b).

Mitotic stability of *V. dahliae* transformants: To determine mitotic stability, transformants were successively cultured on PDA medium for at least 7 generations in the absence of antibiotic selection. These subcultured transformants maintained the ability to grow on selection medium and the GFP expression was also observed. However, the repeated selection of these transformants up to 5 generation resulted in a gradual loss of the hygromycin resistance, but still kept the GFP fluorescence (Fig. 2e). Surprisingly, the fungal transformants recovered the growth after transferred onto PDA medium without adding the antibiotic (Fig. 2f). Microscopic observation of the mycelium morphology using bright-field microscopy exhibited that antibiotic affected the transformant growth significantly, and tumour-like structures were seen (Fig. 2c-d), showing hyperplasia as compared with the same transformant growing on the medium without hygromycin B (Fig. 2b), whose growth pattern was similar to the wild type (Fig. 2a).

Molecular analysis of transformants: *V. dahliae* fungus was identified from a PCR product of 324-bp as amplified with specific primers (Fig. 3a). Presence of eGFP gene in the genome of the transformant was confirmed by PCR with eGFP-F and eGFP-R specific primers, and amplified a band of expected size of 590 bp (Fig. 3b). A qRT-PCR analysis revealed that eGFP gene was all expressed in the *V. dahliae* transformants Vdgfp-1, Vdgfp-2, Vdgfp-3 and Vdgfp-4, the highest and the lowest expression level among these transformants were found in Vdgfp-3 and Vdgfp-1, respectively (Fig. 4).

GFP expression in *V. dahliae* transformants: Randomly selected transformants were investigated for GFP fluorescence by ZEISS LSM 780 microscope. As Fig. 5 showed, GFP fluorescence was detected during different stages of *V. dahliae* development, including free-living mycelium, spores and germinated ones (Fig. 5b-h) while untransformed recipient strains did not show any fluorescence at the applied excitation and detection conditions (Fig. 5a). Parts of hyphae were showing very weak or no GFP fluorescence (Fig. 5e-f). As reported, it may happen in senescing mycelium and collapsed cells. The cytoplasm was transferred from the older to younger hyphae and active growing hyphal tips always kept strong reporter gene expression (Eckert *et al*., 2005; Riedel *et al*., 2009). Fig. 5g-h showed dendritic structure of *V. dahliae* mycelium. The hyphal knots and vacuoles, which expressed reduced fluorescence can be clearly observed.

Pathogenicity analysis of transformants: The pathogenicity of fungus transformants showed a significant difference compared with wild-type strain (Fig. 6a). Among them, Vdgfp-1 transformant was significantly more virulent than the wild-type strain, while Vdgfp-3 and Vdgfp-4 showed significantly lower virulence. Vdgfp-2 transformant exhibited similar virulence relative to the wild-type strain. DI of cultivars CT1236 (susceptible) inoculated with wild type strain Vd and Vdgfp-2 transformant was 32.50 and 30.83, respectively. Therefore, Vdgfp-2 transformant was selected for further plant-pathogen interaction studies. Of the 4 transformants assayed, growth of plants infected with transformants was extremely inhibited. They all showed typical *Verticillium* symptoms, including wilting, chlorotic, and tissue necrosis comparable to the symptoms caused by the wild-type strain (Fig. 6b).

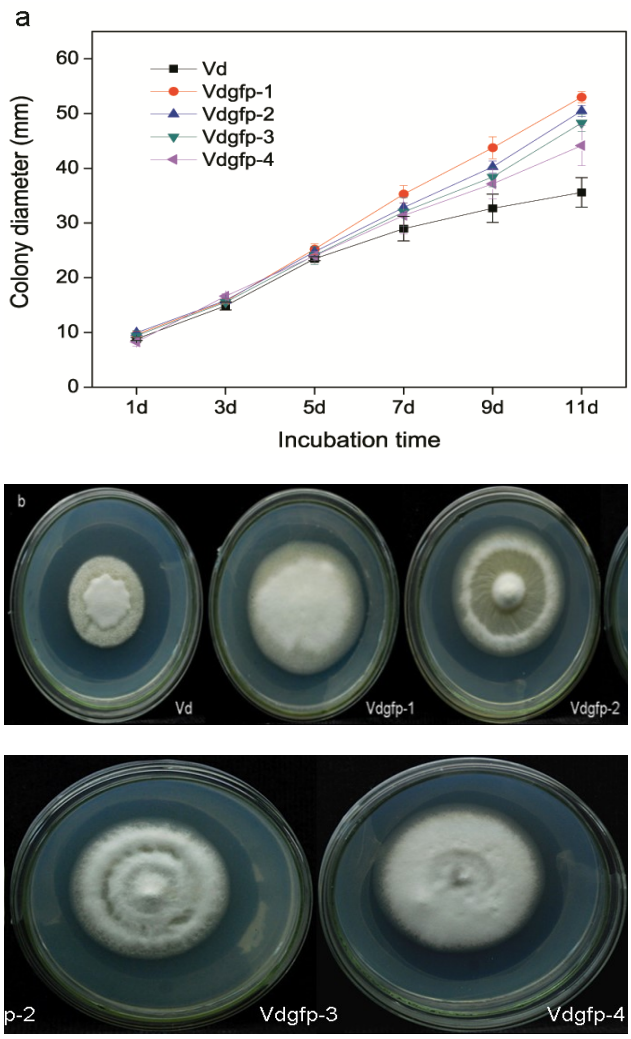

Fig. 1. Colony growth characteristics of *V. dahliae* transformants spores when cultivated on PDA. (a) Transformants were all showing increasing growth rate compared with wild type. (b) Representative colony morphology of *V. dahliae* transformants. Pictures were taken after 11 days of incubation at 25°C.

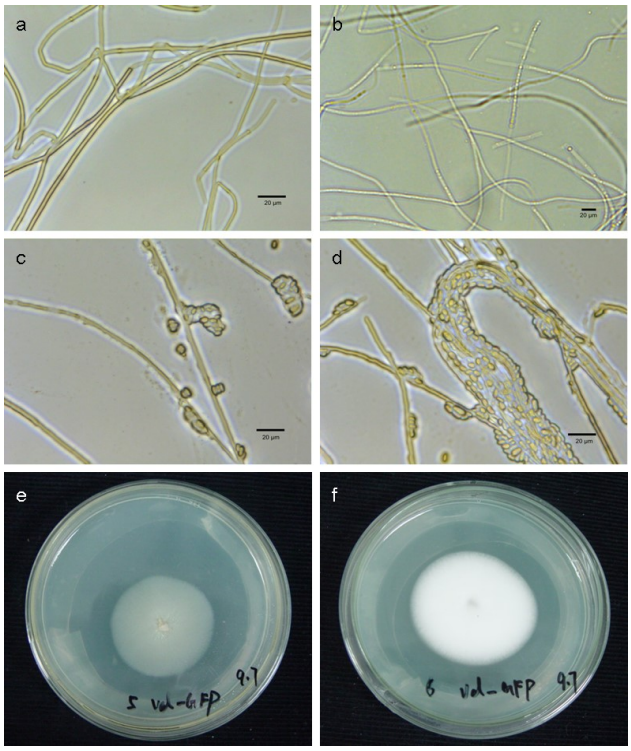

Fig. 2. The effect of hygromycin B on mycelium growth of *V. dahliae*. (a) Wild type (b) Transformants growing without hygromycin B. Mycelium morphology was showing no difference with wild type. (c-d) Transformants growing with hygromycin B were showing structural changes compared with wild type (Fig. 2a) and transformants growing without hygromycin B (Fig. 2b). (e) Transformants lost growing ability on PDA containing 50 $\mu g \cdot mL^{-1}$ hygromycin B. (f) Transformants recovered growth after transferred onto PDA without hygromycin B.

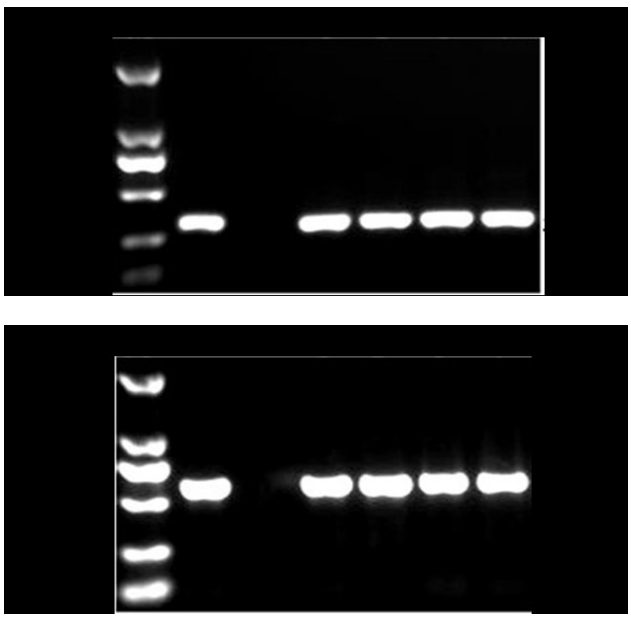

Fig. 3. PCR analysis of *V. dahliae* transformants from seventh generation. Genomic DNA was subjected to PCR with the specific primers to indentify *V. dahliae* fungus and eGFP gene. Lane M. Marker DL2000; Lane 1-4. Transformants of *V. dahliae*. (a) Lane +. Wild-type of *V. dahliae*; Lane –. The wild type of *Fusarium oxysporum*. (b) Lane +. The Sk1044 plasmid; Lane –. The wild-type of *V. dahliae*.

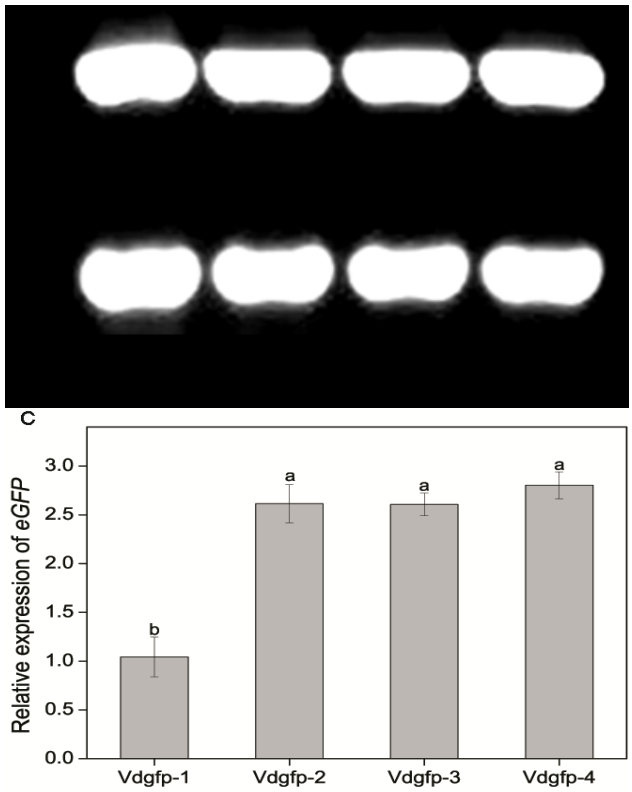

Fig. 4. Quantitative RT-PCR analysis of *V. dahliae* transformants. (a) The cDNA amplication of eGFP gene. (b) The cDNA amplication of actin gene. (c) Transcript levels of eGFP gene in fungus transformants Vdgfp-1, Vdgfp-2, Vdgfp-3 and Vdgfp-4. The GFP expression level of Vdgfp-1 was significantly low compared with other transformants. Error bars indicate standard deviations, based on three independent measurements of four independent RNA preparations. Different letters denote statistically significant differences at $p<0.01$.

Plant-pathogen interaction studies: Using the method of sterilized seedling inoculation, infection processes can be observed in 10 days including the period of seedlings growth. Growth condition of seedlings and inoculation method were showed in Fig. 7a-b. Mock inoculation of seedlings was displayed in Fig. 7c. During the incubation period, sterilized condition should be confirmed to make sure that seedlings would not be influenced by other microbes except *V. dahliae*. Fig. 7g-i showed visual colonization processes under microscope corresponding to pathological changes of the seedlings (Fig. 7d-e). Before inoculation, aseptic seedling was very healthy with green stem and white root. 2-day inoculation later, the stem closely attached to fungus plug became yellow and presented a low level of symptoms (Fig. 7d). Using the same plant to make sections, we observed a tight attachment of hyphae to the inoculation position of the stem under LSM microscope (Fig. 7g). After 4-day inoculation, the stem of seedlings appeared significant wilting, and the lateral root nearby became brown (Fig. 7e). The corresponding microscopic image displayed a dense mycelial network around the stem and root (Fig. 7h). Fig. 7f showed 6-day post inoculation, tissues became discolored yellow and dark brown with a distinctive rift produced on the stem. We also observed the fungus entered into the tissue and showed an intercellular infection along the stem under LSM microscope (Fig. 7i). Fig. 7j-o showed the morphologic characteristic of different positions in uninoculated and 6-dpi seedlings under the observation of stereomicroscope. Symptoms like stem dehiscence, necrosis and wilting that mentioned above can be clearly observed.

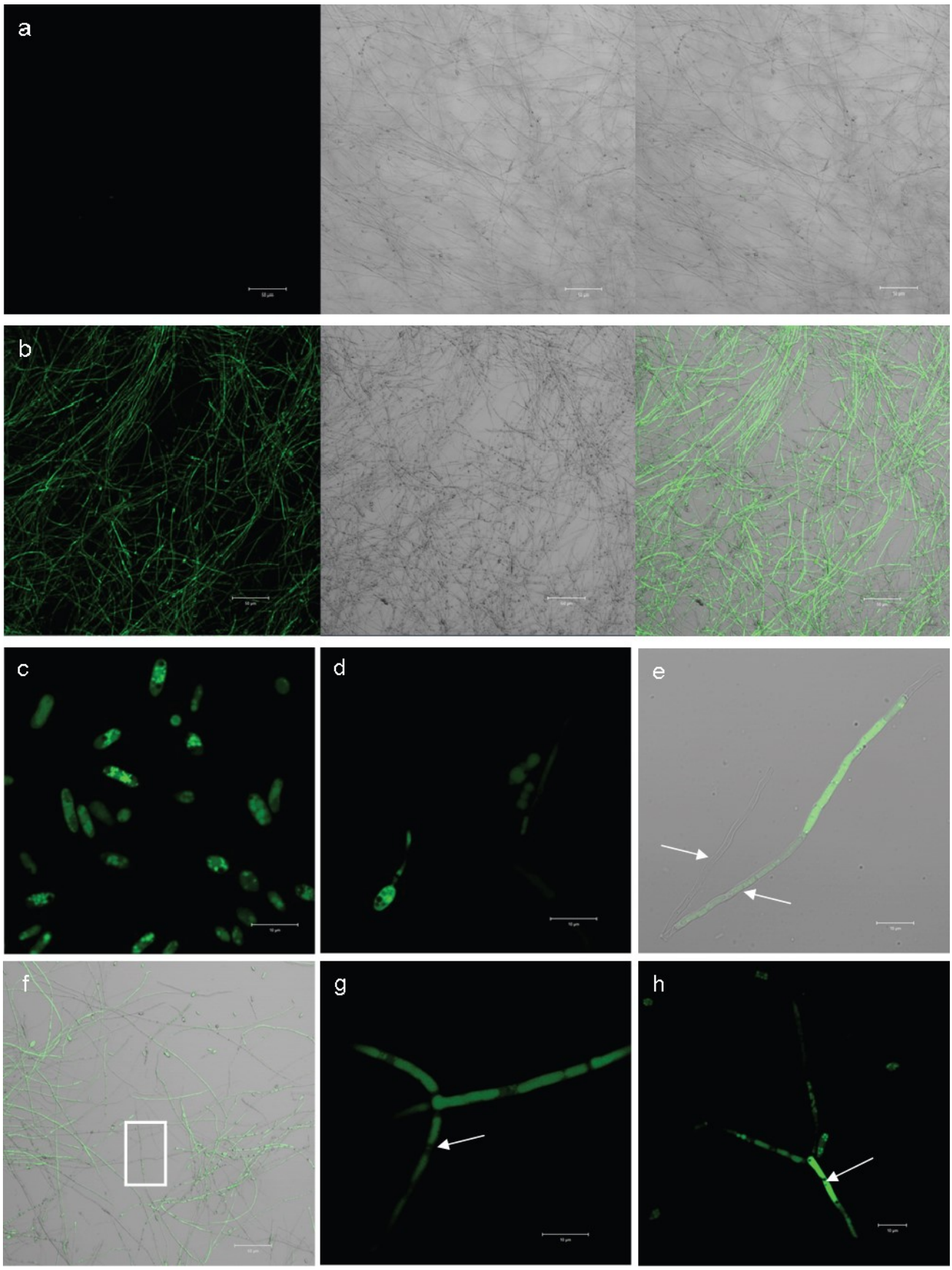

Fig. 5. Expression of eGFP during different development stages of fungus. Mycelia and conidia were removed from an actively growing colony, suspended in sterile water, and examined microscopically. (a) Hyphae of the untransformed wild-type strain. (b) Free-living mycelium of *V. dahliae* transformant. Strong fluorescence due to GFP expression was detected in the mycelium; (c-d) GFP images of spores (Fig. 5c) and germinated spores (Fig. 5d); (e-f) Parts of hyphae showing reduced or no reporter gene expression. The arrows and box indicate the mycelia were displaying very weak or no GFP fluorescence; (g-h) GFP images showing the dendritic structure of *V. dahliae* transformants. Arrows indicate hyphal knot (g) and vacuoles (h), which showed reduced fluorescence.

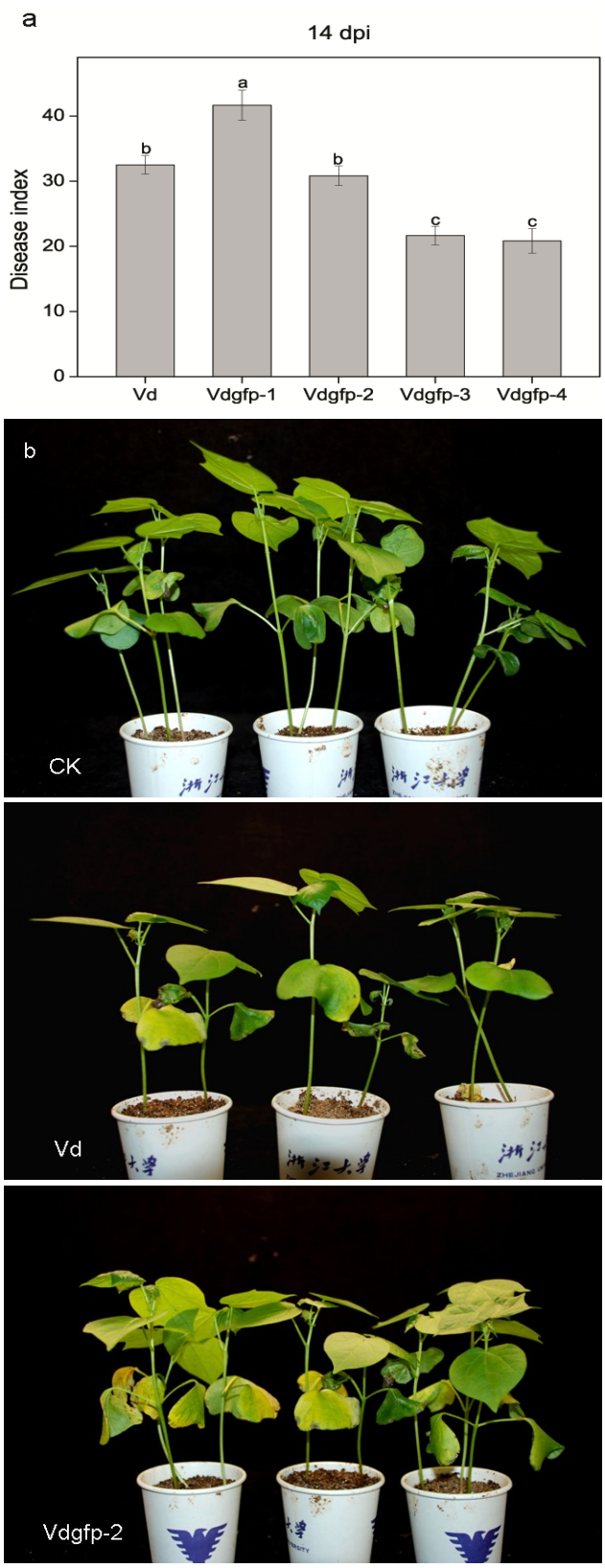

Fig. 6. Pathogenicity analysis of fungus transformants. (a) Disease severity was recorded at 14 dpi, expressed as disease index (DI). Observations were based on a representative of three independent experiments using 10 plants per experiment. Different letters denote statistically significant differences at $p<0.05$. (b) Typical disease symptoms of cotton seedlings inoculated with *V. dahliae* wild type and transformant Vdgfp-2 at 14 dpi. Other transformants caused similar results. For mock inoculation, cotton seedlings were infected with Czapek's medium.

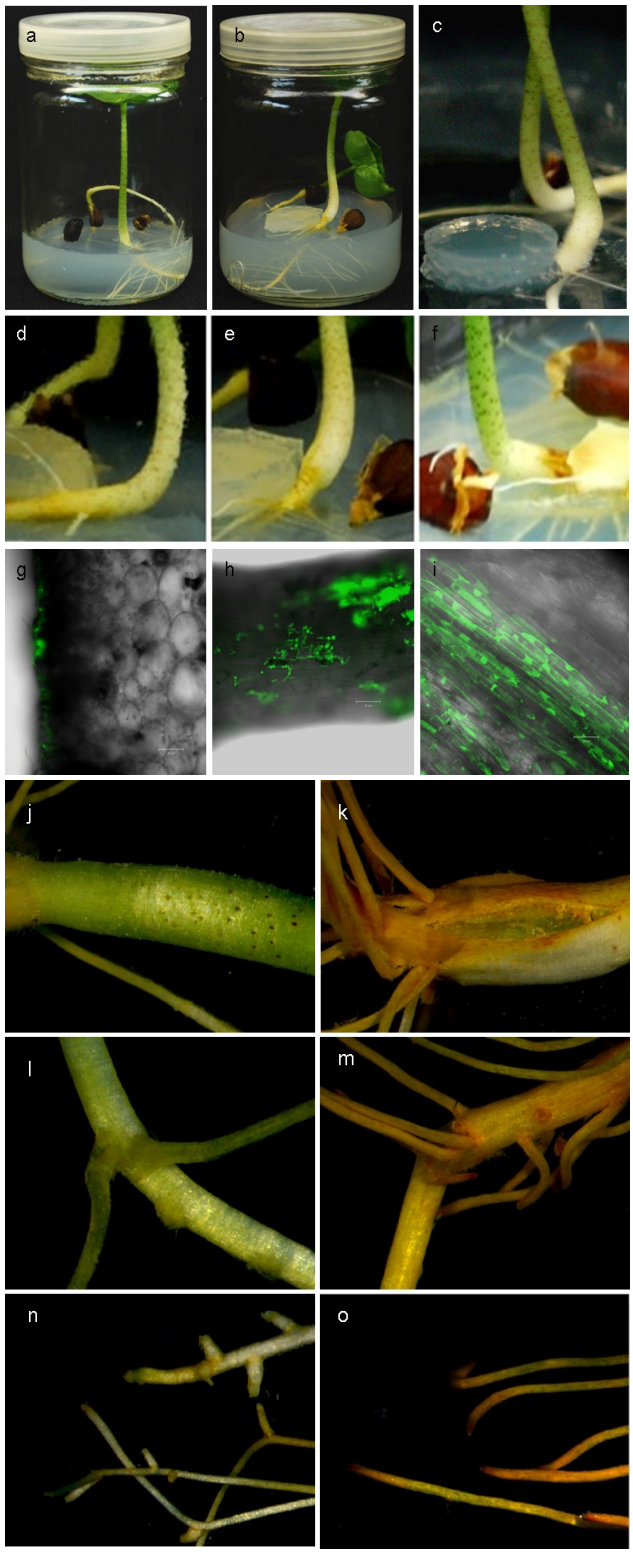

Fig. 7. The transformant expressing eGFP was observed together with cotton plant. (a-b) Growth condition of seedlings and the inoculation method applied. (c) Mock inoculation of seedlings. (d-f) The pathological changes of seedlings after 2-day, 4-day and 6-day inoculation, respectively. (g-i) Corresponding fungus infection processes under LSM microscope observation after 2-day, 4-day and 6-day inoculation, respectively. (j-o) Difference of morphology between uninoculated seedlings (j, l and n) and 6-dpi seedlings (k, m and o) under the observation of stereomicroscope. The upper (j-k), central (l-m) and lower panel (n-o) were showing the stem, taproot and lateral root of the seedling, respectively.

Discussion

For gene transformation, antibiotics screening of the stable transformants is one of the most important steps. Hygromycin B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus*, which is effective against both prokaryotes and eukaryotes (Blochlinger & Diggelmann, 1984). It has been used as a selective marker for specific selection of stable transformants. In the present study we found that the transformants of *V. dahliae* cannot maintain hygromycin B resistance in the consecutive cell generations and showed hyperplasia compared with the wild type. Previous studies also reported this phenomenon and might be due to formation of extra chromosomal plasmids transformation without incorporating the hph gene into the host genome (Hua *et al*., 2000), or gene deletion or mutation (Dobrowolska *et al*., 2009). Furthermore, there is also a possibility of the transient expression of transformed hph gene in the unstable transformants (Irie *et al*., 2001). In our study we speculate the reason of epigenetic modification of the transgene locus. As reported, epigenetic variations can be induced and transmitted between generations, suggesting that persistent exposure to a new environment can have cumulative effects (Jablonka, 2012). Progressive and cumulative epigenetic modification during recultivation of fungus transformants over successive generations can result in changes in gene expression, thus causes the fungus unstable resistance to hygromycin B treatment (Alien *et al*., 1990; Wang *et al*., 2004). The further work to verify our hypothesis is now in progress in our lab.

The growth rate, colony morphology and pathogenicity of fungus transformants all displayed differences compared with wild-type strain in our present study. Heterogeneous gene insertion may bring interventions for the growth and development of fungus. Similarly, a break into the host genome can be caused by gene integration, and original host gene combination can be disturbed. In addition, altered metabolism of additional proteins due to transformation could be another cause as mentioned previously (Riedel *et al*., 2009).

In our study, GFP expression level among the fungus transformants showed significant difference. A number of factors can be responsible for different transgenic expression levels (Riedel *et al*., 2009). As mentioned previously, the expression strength of GFP may depend on the integration locus inside the recipient host genome, rather than the copy number of these genes (Soanes *et al*., 2002; Eckert *et al*., 2005). Different sites of integration into the fungal genome may cause positional effects, thus affect the expression strength of the GFP gene. Although the GFP transcript levels of fungus transformants are different, direct observation of GFP fluorescence under LSM microscope was not influenced by low GFP expression level. They all showed a strong fluorescence and the GFP fluorescence of transformants can be easily distinguished with autofluorescence of both fungus and plant tissue examined.

Establishing an effective approach is very necessary to monitor the interaction between the plant and microbes. The inoculation method, inoculum concentration, and state of the plant tissue play major roles on the disease development (Berruyer *et al*., 2006). Methods such as soil drench, root dip and stem prick use spore suspensions as the inoculum for vascular fungus inoculation (Vallad & Subbarao, 2008; Gao *et al*., 2011). In the present study, we inoculated the aseptic cotton seedlings with agar plugs carrying *V. dahliae* mycelium. No matter wounds exist or not, early events during cotton colonization by *V. dahliae* can be observed (data not shown). Besides, our observations showed clear plant pathological changes without the influences of soil and other microbes. As shown in Fig. 7, symptoms of tissue wilting, discoloration followed by necrosis and stem dehiscence can be clearly observed after 6-day inoculation. Microscopic observations of *V. dahliae* infection showed the rapid formation of a mycelial network on the surface of the stems. The fungus penetrated tissue through tiny openings in the cell wall, then quickly spread into the intercellular spaces along the junctions of stem epidermal cells, showing the same infection mode as described previously (Ramírez-Suero *et al*., 2010; Li *et al*., 2011). The early events during cotton colonization by *V. dahliae* can be successfully observed in 10 days including plant growth period. Because of limited cultural space in bottle, longer growth and incubation period for cotton may not be suitable. However, compared with conventional soil-grown plant inoculation assays, the method used in our study can give a rapid disease development and is feasible in small cultivation space. Moreover, this methodology will prevent the spread of pathogens, which can be caused by the poor experimental conditions control like unreasonable use of spore suspensions and untreated polluted soil.

In conclusion, GFP-labeled strain has proved to be a valuable tool in studying the interaction between plants and fungus, together with the inoculation method elaborated above, it can provide a higher efficiency to check the fungus colonization process and identify the plants resistance and defensive ability for our study on resistant germplasm resources development and resistance breeding.

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