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Green fluorescent protein (GFP) as a vital marker for studying the interaction of *Phytophthora sojae* and soybean

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Transgenic *Phytophthora sojae* strains that produce green fluorescent protein (GFP) were obtained after stable DNA integration using the *Hsp70* promoter and the *Ham34* terminator of *Bremia lactucae*. The expression of GFP during different developmental stages of *P. sojae* was observed using fluorescent microscopy. Based on this reporter system, the histopathologic events caused by the pathogen in soybean leaves, hypocotyls and roots were monitored. Meanwhile, the difference in resistance between different soybean cultivars against *P. sojae* was analyzed microscopically in roots. The results indicate that GFP can be stably expressed in zoosporangia, zoospores, cysts, hyphae and oospores of *P. sojae*. Using the GFP marker, the infecting pathogens in leaves, hypocotyls and roots of host could be distinctly visualized. The germ tube length of cysts germinating on the roots of resistant cultivar Nannong 8848 was longer than that on the roots of susceptible cultivar Hefeng 35. These results show for the first time that this eukaryotic reporter can be used in *P. sojae* as a stable and vital marker, allowing the study of genetics of this hemibiotrophic pathogen.

Phytophthora sojae, soybean, green fluorescent protein (GFP), genetic transformation, disease resistance

The group of eukaryotic microbes known as oomycetes includes plant and animal pathogens responsible for many economically important diseases. The genus *Phytophthora*, for example, includes devastating pathogens of most crops, responsible for multibillion dollar losses per year throughout the world[1,2]. Oomycetes are a group of "fungus-like" mycelial organisms that belong to the kingdom Straminopila, but represent a unique evolutionary line distant from true fungi, more closely related to brown algae and diatoms[3]. Significant resources currently are being devoted to large-scale sequencing and research but due to the diploid nature of oomycetes and lack of high-throughput genetic tools, the functional gene analysis of these organisms is still a major challenge[4,5].

Phytophthora sojae (Kaufmann and Gerdemann) is an oomycete pathogen of soybean (*Glycine max* L.(Merr)) and can cause "damping off" of seedlings and root rot of older plants, with an annual cost worldwide of $1—2 billion. Due to its economic importance, this species, along with *Phytophthora infestans*, has been developed as a model species for the study of oomycete plant pathogens[6].

The developmental stages associated with pathogenicity of *Phytophthora* spp. include production of zoospores, germination and growth of cysts, formation of appressoria or appressoria-like structures, an initial biotrophic phase after infection, and following necrotrophic growth[7]. In previous cytological studies of soybean hypocotyl tissues infected with *P. sojae*, the

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interaction of soybean with *P. sojae* was examined by light and electron microscopy[8–10]. Ward et al.[8] observed the interaction of soybean cv. Harosoy 63 with *P. sojae* race 1 (incompatible) and race 4 (compatible) and of the near-isogenic cv. Harosoy with these races (compatible) by light and electron microscopy in hypocotyl tissues at 2, 3, 5, and 7 h after inoculation. The extent and rate of colonization were similar in both incompatible and compatible interactions. However, there were major differences in host cell responses. Similarly, by light and electron microscopy, compatible and incompatible interactions of two soybean isolines containing either *Rps1a* or *Rps1b* resistance genes with races 2 and 8 of *P. sojae* were examined from 30 min to 20 h post-inoculation (hpi)[9]. Zoospore encystment, germination and infection occurred within 30 min in all interactions. No evidence of appressorium formation was found. Differences between compatible and incompatible interactions became evident as early as 4 hpi. In compatible interactions, *P. sojae* exhibited a short biotrophic phase with the establishment of many haustoria without triggering visible plant responses. The incompatible interaction was characterized by nearly complete absence of haustoria, rapid host cell necrosis, and formation of many cell wall appositions. Zuo et al.[10] reported the similar results by light and electron microscopy.

Judelson et al.[11] first developed a $CaCl_2$-polyethylene-glycol-based (PEG) transformation protocol for the late blight pathogen *Phytophthora infestans*. Oomycete molecular biology had just taken its first steps. This method has been successfully used for the expression of marker genes such as β-glucuronidase gene (*gus*) in *P. infestans*[12,13] and green fluorescent protein gene (*gfp*) in *Phytophthora palmivora*[14], *Phytophthora parasitica* var. *nicotianae*[15,16] and *Phytophthora brassicae*[17], which provide two excellent reporter systems for studying the development, gene expression and infection events of oomycetes. To determine the location and time of *in planta*-induced gene *ipiO* expression, cytological assays were performed using a *P. infestans* transformant expressing a transcriptional fusion between the *ipiO* promoter and *gus* gene[18]. The development of gametangial associations in *P. infestans* was examined using matings between strains containing *gus* transgene and nontransformed strains[19]. The *gus* gene enabled the facile assessment of whether oospores resulted from hybridization between isolates or from self-interactions and allowed measurements of the sexual preference of strains. Kamoun et al.[13] used a transgenic *P. infestans* strain that constitutively produces and secretes GUS in detached leaf assays to quantify the levels of resistance to late blight in five potato cultivars. Another reporter gene, *gfp* gene was used in *P. parasitica* var. *nicotianae*[15,16] and *P. palmivora*[14], both as a quantitative reporter of gene induction and as a vital marker allowing the study of development of oomycetes *in vitro* and in the host plants. Remarkably, induced resistance was studied in the model pathosystem *Arabidopsis-P. brassicae* in comparison with the late blight disease of potato using the *gfp* gene[17].

The quantification of induced disease resistance is often performed with methods such as estimation of diseased area or evaluation of sporulation. Depending on the pathosystem, these measurements can be rather subjective and time-consuming[17]. The transformation of *Cladosporium fulvum* with the *gus* gene allowed researchers to follow the fungal infection at the microscopic level as well as the photometric quantification of GUS activity as a measure of fungal biomass[20].

Here, we transformed *P. sojae* with a newly constructed transformation vector containing, as a reporter, the *gfp* gene. The GFP-expressing *P. sojae* transformants were characterized and used to monitor the infection of the pathogen in soybean, and to develop methods for the quantification of soybean cultivar resistance to *P. sojae* in plant roots, which acknowledges the mechanism underlying the host disease resistance to *Phytophthora*. The establishment of the GFP reporter gene system provides a strong molecular tool for studying the genetics of *P. sojae*.

1 Materials and methods

1.1 Strains and soybean cultivars

The *P. sojae* strain P6497 with the genome fully sequenced was kindly provided by Dr. Brett M. Tyler (Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA) and preserved in the Fungal Lab, Department of Plant Pathology, Nanjing Agricultural University. P6497 was grown on V8 juice agar (10% Campbell's V8 juice, 0.02% $CaCO_3$, 1.5% Bacto-agar). To obtain zoosporangia, hyphal tip plugs were used to inoculate 20 mL of sterile clarified 10 % V8 broth in 90-mm Petri dishes. Stationary mycelial cultures were incubated at 25℃ in darkness for 3 days. Then, mycelia were transferred to an empty sterile Petri

dish using forceps. 15 mL of sterile tap water was added into the dish, and the dish was incubated at 25℃ in darkness for 12 h. The tap water was replaced by 15 mL of new sterile tap water and zoosporangia were produced after 15 h. To encourage the release of zoospores, the culture was placed at 4℃ for 30 min, and then incubated at room temperature for another 30 min. The zoosporangia and zoospores were checked under a Nikon light microscope. To prepare oospore suspension, mycelial plugs near inoculum on lima bean agar (LBA) (extract of 60 g/L frozen lima beans solidified with 1.5% Bacto-agar) were cut from 30-day-old plates and placed into sterile distilled water to make oospore suspension using a muller[21].

The soybean cultivars Hefeng 35 and Williams were susceptible to P6497, while Nannong 8848 and Williams 82 were resistant to the strain. Seeds of Hefeng 35 and Nannong 8848 were a gift from National Centre for Soybean Improvement, Nanjing, Jiangsu Province, China. Williams and Williams 82 were provided graciously by Dr. Brett M. Tyler. Seeds were sown in sterilized soil in 15 cm plastic pots (1 seed per pot). Seedlings were allowed to grow for 10 days in a greenhouse with natural sunlight, a 22－28℃ day/night temperature before assay, and watering every other day.

1.2 Transformation vectors

Plasmid pTH210[11] was kindly presented by Dr. Howard S. Judelson (University of California, Riverside, CA92521, USA). Selectable plasmid pHSPN generously provided by Dr. Brett M. Tyler, is a derivative of pTH210, which contains the neomycin phosphotransferase gene (*nptII*) in place of the hygromycin B phosphotransferase gene (*hpt*) of pTH210 besides *Hsp70* promoter and *Ham34* terminator from *Bremia lactucae*.

The g*fp* gene sequence was amplified from plasmid pACGFP1 (Clontech Laboratories, Inc., CA 94043, USA) using a pair of primers 5′-ATG GTG AGC AAG GGC GCC G-3′ and 5′-TCA CTT GTA CAG CTC ATC CAT G-3′. A Pyrobest DNA Polymerase (DR005A) (TaKaRa, Dalian, China) was employed to produce the DNA products with blunt ends. Cycling conditions for 30 cycles were 95℃ for 15 s, 52℃ for 30 s and 72℃ for 1 min, with pre-denaturalization at 94℃ for 5 min and prolongation at 72℃ for 5 min subsequently. The PCR fragment was purified from the gel with the Wizard DNA Clear-Up System (Promega, Madison, USA) and cloned into the *Sma*I-digested vector pTH210. The produced vector with sense orientation of *gfp* gene was designated p210ACGFP and sequenced to confirm the correct sequence and inserted *gfp* orientation.

Selectable plasmid pHSPN and non-selectable p210ACGFP for co-transformation experiments were prepared in *Escherichia coli* DH5α and purified by extraction with Endofree Plasmid Maxi Kit10 (Qiagen, CA91355, USA).

1.3 Transformation of *P. sojae*

Stable co-transformation of P6497 was performed by following the method described by McLeod et al.[22]. For co-transformation 25 μg p210ACGFP and 5－10 μg pHSPN were mixed with *P. sojae* protoplasts in 1 mL (2×10^6/mL). Transformation controls were obtained by transforming protoplasts only with 10 μg of selectable plasmid pHSPN. Colonies appeared within 3－4 days and were propagated on an LBA medium containing 50 μg/mL G418 (geneticin) (Sangon, Shanghai, China).

1.4 Screening and characterizing of GFP transformants

All transformants appearing on selective LBA medium (containing 50 μg/mL G418) were determined using a laser microscope system, Leica DC300F equipped with a Leica DC100DC350F digital camera (Leica Microsystems GmbH, Wetzlar, Germany) (excitation wavelength 474/40 nm, absorption wavelength 505/40 nm). Single-zoospore progenies were obtained from GFP-expressing transformants according to the method described by Zheng[21]. These single-zoospore progenies were grown on the LBA medium containing 50 μg/mL G418 and determined by the laser microscope system.

The expression of GFP in mycelia, zoosporangia, zoospores, germinating cysts and oospores of single-zoospore progenies was determined by the laser microscope system. The wild-type isolate P6497 was used as the control.

1.5 Biological assays

Before assays, all the tested strains including single-zoospore progenies, transformation controls and P6497 were grown for 4 d on a nonselective LBA medium, and then transferred again to another nonselective LBA medium for another 4 d to get rid of G418.

Inoculations for pathogenicity assay were performed by the standard hypocotyl method[23], using 1 mm×3 mm pieces of mycelial plugs, and the wound was covered

with petrolatum to prevent desiccation of the inoculum and host tissue. After 2 days, the length (cm) of the lesion from the inoculation point upwards along the stem was measured. In this assay, virulence of isolates was evaluated by measuring the length of the lesion[24]. All observations and measurements were obtained from at least 10 seedlings per isolate. Controls were the inoculations with P6497 and transformation controls.

Colony appearance (shape and color), growth rate, yields of sporangia, and oospores of single-zoospore progenies were compared to P6497 and transformation controls. Growth rate was measured according to the previously described procedure[25] and the experiment was repeated 3 times. To count the number of sporangia, the cultures were grown in 90-mm Petri dishes containing 15 ml LBA for 7 days at 25℃ according to Rutherford et al.[26]. Ten mycelial masses (10 mm × 10 mm) were transferred to Petri dishes containing 30 mL sterile distilled water to induce sporangia as described by Bumbieris[27]. Every mass was observed to count the amount of sporangia using a Nikon microscope at 150 × magnification. To measure the number of oospores, the cultures were grown in 90-mm Petri dishes containing 15 mL LBA for 30 days at 25℃ according to Bhat and Schmitthenner[28,29]. Ten mycelial plugs (40 mm × 40 mm) with LBA were excised from the middle of the plate, and was put into a bottle with 20 mL sterile distilled water to produce a suspension of oospores as described by Bhat and Schmitthenner[28,29]. The oospores in the suspension were counted using a Nikon light microscope. Average sporangial or oospore production by single-zoospore progenies was compared with that of P6497 and transformation, controls and the experiment was repeated three times.

1.6 Infection observation of GFP-expressing strains

The zoospore suspensions of single-zoospore progenies and P6497 were prepared as described above.

Leaves were excised from 10-day old plants of Hefeng35 and placed on moist filter paper in Petri dishes with the adaxial surface facing up. 20 μL of zoospore suspension containing 0.05% Tween 20 (containing approx. 1000 zoospores) was placed on the detached leaves. The Petri dishes were sealed with Parafilm and placed at 25℃ in darkness for 24 h. The leaves were subsequently boiled for 20 min in 80% ethanol. The areas around the inoculation point were observed using a Leica laser scanning confocal microscope system, Leica TCS SP2 (Leica Microsystems GmbH, Wetzlar, Germany) (LSCM). The leaves inoculated with P6497 zoospores were used as controls.

Inoculation of Hefeng35 hypocotyls with *P. sojae* zoospores was carried out using the standard hypocotyl method[23] with some modifications. For wounding, seedlings were cut with a razor blade by searing the surface 0.5 mm long along the hypocotyls. Treatments were performed by placing 20 μL of the zoospore suspension (containing approx. 1000 zoospores) on soybean hypocotyls. Control seedlings were inoculated with P6497 zoospores. All treated seedlings were incubated in the dark at 25℃ for 24 h. The epidermis around the inoculated point was excised for observation using LSCM.

The roots of 10-day old Hefeng35, Nannong 8848, Williams and Williams 82 were cut and dipped into the zoospore suspension (5×10^4 zoospores /mL). All treated roots were incubated in the dark at 25℃ for 2 h. The germ tube length of germinating cysts was measured using an eye micrometer as previously described[30]. For each cultivar, 20 germinating cysts were measured randomly under a Nikon light microscope.

2 Results

2.1 Transformation of *P. sojae* with *GFP* gene construct

A construct containing the *hsp70* promoter of the oomycete *B. lactucae* fused to the coding sequence of the *gfp* gene, p210ACGFP, was introduced into the *P. sojae* strain P6497 by co-transformation with pHSPN. The latter construct contains the geneticin resistance gene (*nptII*) fused to the *hsp70* promoter of *B. lactucae*, as a selection marker (Figure 1). Thirty-seven G418-resistant

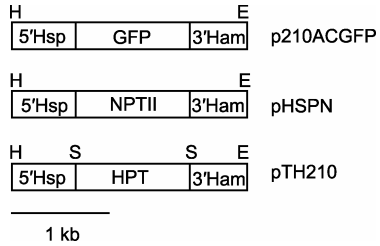

Figure 1 Transformation constructs used in reporter gene studies. Vector pTH210 contains the coding sequence of the *hpt* gene fused to the *hsp70* promoter (5′-Hsp) and *ham34* terminator (3′-Ham) of *B. lactucae*. For pHSPN, the coding sequence of *nptII* gene was fused to the *hsp70* promoter and *ham34* terminator. P210ACGFP consists of the coding sequence of the mammalian codon optimized GFP gene fused to the *hsp70* promoter and *ham34* terminator. H, *Hin*d III; E, *Eco*R I; S, *Sma* I.

transformants were obtained, called GFP1-GFP37. Only two of them, GFP1 and GFP2, obviously fluoresced under the laser microscope.

2.2 Genetic analysis of *hsp70-gfp* transformants

The condition of containing nuclei of oomycete protoplasts digested from mycelia is complicated probably due to less septum and multi-nuclei of mycelia[23]. Additionally, co-transformation of the selection marker and *gfp* gene on separate vectors could cause the low ratio of transformants expressing GFP in all G418-resistant transformants. The *hsp70-gfp* transformants GFP1 and GFP2 were tested for GFP production by analyzing mycelia with a Leica DC300F laser microscope. GFP produced in the transgenic strains fluoresce bright-green upon exposure to excitation at 475 nm. However, not all mycelia of GFP1 or GFP2 can fluoresce bright-green. So, single-zoospore progenies from GFP1 and GFP2 were isolated. In all, T1 and T2 from GFP1, T3, T4 and T5 from GFP2, were obtained (Table 1). All 5 zoospore progenies were tested for GFP production by analyzing mycelia, sporangia, zoospores, germinating cysts and oospores with a laser microscope. GFP produced in these 5 strains fluoresces bright-green after excitation (Figure 2), which proved that the five transgenic strains were homokaryotic.

Table 1 The numbers of *gfp* transformants and single-zoospore progenies[a)]

Sorts	Numbers
G418-resistant transformants	37
GFP-expressing transformants	2
Single-zoospore progenies	216
GFP-expressing single-zoospore progenies from *gfp* transformants	5

a) GFP1 and GFP2 were GFP-expressing transformants; single-zoospore progenies were obtained from GFP1 and GFP2, 108 for each transformant; GFP-expressing T1 and T2 were single-zoospore progenies of GFP1 while T3, T4 and T5 from GFP2.

The colony appearance, growth rate, and yields of sporangia and oospores of T1—T5 were compared with those of P6497 and transformation controls (Table 2). There were no significant differences in all tested characteristics between transgenic isolates and P6497 or transformation controls.

2.3 *In planta* detection of GFP-fluorescent *P. sojae*

Transgenic isolate T1, which retained pathogenicity and other morphological characteristics in various assays, was used for microscopy studies (Figures 3 and 4).

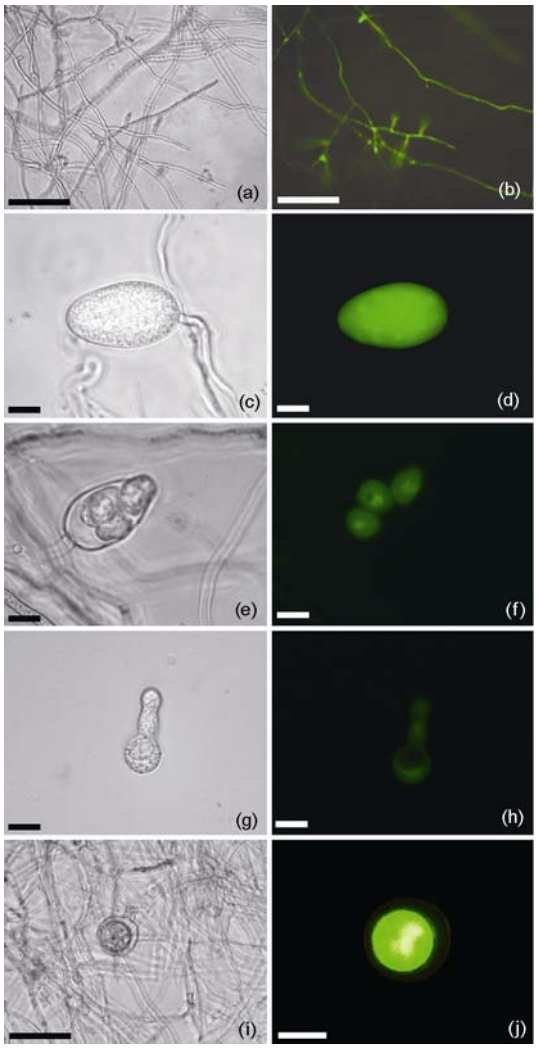

Figure 2 Expression of *gfp* in the isolate T1 at different stages of development. T1 was the single-zoospore progeny of transformant GFP1. (a) and (b) Vegetative mycelia; (c) and (d) a sporangium; (e) and (f) a sporangium releasing zoospores; (g) and (h) a germinating cyst; (i) and (j) an oospore. The right panels show GFP fluorescence images and the left panels show bright field images of the same cell types as in right panels. Bars shows 15 μm.

When infected soybean tissues (leaves and hypocotyls) were observed, the GFP-labeled strain was readily detectable (Figures 3 and 4). In leaves, hyphal proliferation was observed (Figure 3(b) and (c)). In hypocotyls, even intercellular hyphae were readily observable (Figure 3(e) and (f)). By light microscopic analysis, the parasite was barely detectable in the same samples (Figure 3(a) and (d)). *P. sojae* was present as germinating cysts on the root surface (2 hpi) (Figure 4(a) and (b)) and as straight infecting hypha within the root (4 hpi) (Figure 4(c) and (d)). Thus, labeling *P. sojae* with GFP allows straightforward detection of the parasite even in plant parts.

Table 2 Comparison of the growth rate, pathogenicity to soybean cultivar Hefeng35 between transgenic and receipt strain of *P. sojae* P6497[a)]

Strains	Growth rate (mm/d)[b)]	Lesion (cm)[b)]
P6497 (control)	5.7 a (0.129)	5.23 a (0.099)
T1	5.5 a (0.128)	5.03 a (0.115)
T2	5.2 a (0.163)	5.14 a (0.119)
T3	5.4 a (0.216)	4.96 a (0.155)
T4	5.5 a (0.214)	5.20 a (0.200)
T5	5.1 a (0.177)	5.18 a (0.144)

a) The growth rate was compared based on 5 technical replicates for each isolate and 3 biological repeats. The pathogenicity was represented by geometric average of lesion lengths from 10 seedlings for each isolate and the experiment was repeated biologically three times. b) standard deviation, given in parentheses. a, according to Duncan's new multiple range test, letters stand for significant difference at 0.05 level. T1 and T2, the single-zoospore progenies of GFP1; T3, T4 and T5, the single-zoospore progenies of GFP2.

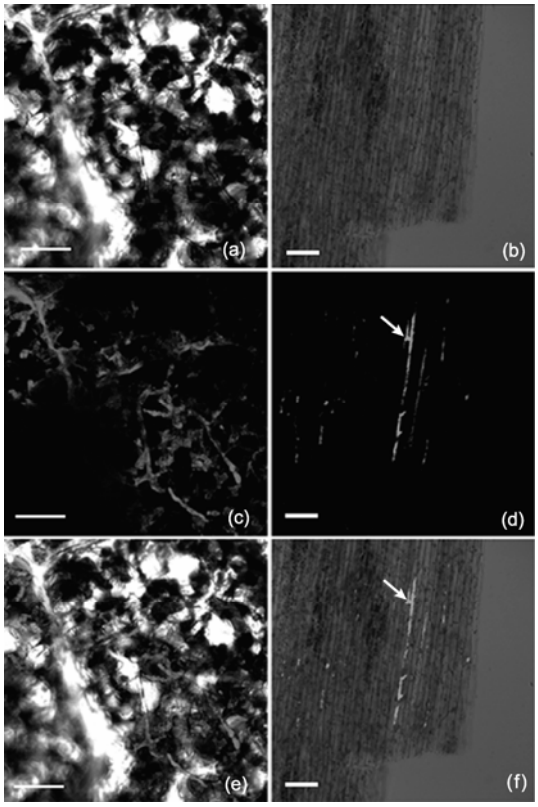

Figure 3 Detection of the isolate T1 expressing *gfp* in infected soybean tissues using LSCM. (a) and (b) Bright field micrograph of a leaf and a hypocotyl, respectively; (c) and (e) the mycelium of *P. sojae* inside the leaf of susceptible *Glycine max* cv. Hefeng 35. Inside the plant cells, the green fluorescent is visible; (d) and (f) the mycelia of *P. sojae* inside the hypocotyl of Hefeng 35. Inside the plant cells, the intercellular hyphae (arrows indicated) expressing *gfp* is visible; (c) and (e), (d) and (f) the same as (a) and (b), respectively. (c) and (d) Micrographs taken under blue light excitation; (e) and (f) micrographs taken under blue light excitation overlapped with bright field. (a), (d) and (e) Bar = 40 μm; (b), (d) and (f), bar = 80 μm.

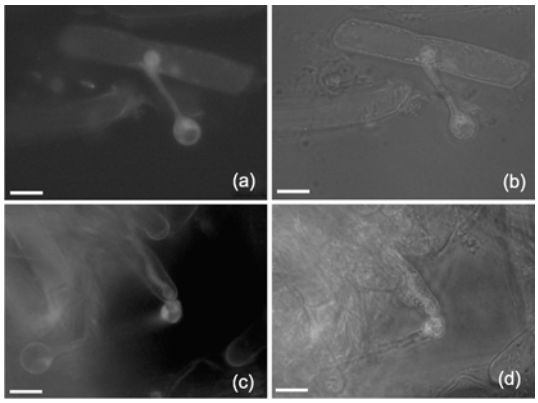

Figure 4 The infection of the isolate T1 expressing *gfp* in roots of soybean cv. Hefeng 35. (a) and (b) Micrographs taken at 2 h post inoculation (hpi); (c) and (d) micrographs taken at 4 hpi; (a) and (c) micrographs taken under blue light excitation; (b) and (d) micrographs taken under blue light excitation overlapped with bright field. Bars indicate the magnifications: 200×.

2.4 The length of germ tubes of cysts germinating on susceptible and resistant cultivars

The *hsp70* promoter driving *gfp* expression was demonstrated to be constitutively active in *Phytophthora* species[15]. It conferred high GFP expression in the zoospores, germinating cysts, hyphae and sporangia (Figure 2) both *in vitro* and *in planta*, thus providing a basis for the use of the visual marker for disease quantification.

A comparison of the germ tube length of the cysts of *hsp70-gfp* transformant T1 germinating on the roots of Hefeng35 and Nannong8848 revealed obvious differences (Figure 5). At 2 hpi, the germ tube length of cysts germinating on roots of resistant cultivar Nannong 8848 is 36.7 ± 11.10 μm, longer than 14.8 ± 2.98 μm which was produced on roots of susceptible cultivar Hefeng 35. Meanwhile, latter germ tubes appeared larger in diameter than the former ones. Similar results were observed between resistant cultivar Williams 82 (30.9 ± 11.80 μm) and susceptible cultivar Williams (15.7 ± 6.28 μm).

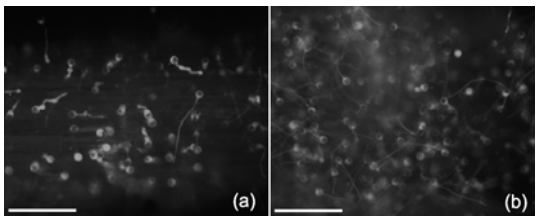

Figure 5 Resistance differences of two soybean cultivars to *P. sojae* in roots. All inoculations were made with *P. sojae gfp*-expressing isolate T1, and photographs were taken at 2 hpi. (a) Microscope view of a root surface of susceptible cultivar Hefeng 35 infected by T1; (b) microscope view of a root surface of resistant cultivar Nannong 8848 infected by T1. Bars indicate the magnifications: 100×.

3 Discussion

The utility of GFP has been demonstrated in several *Phytophthora* species including *P. palmivora*[14], *P. parasitica* var. *nicotianae*[15,16] and *P. brassicae*[17]. In this paper we extend the use of GFP to *P. sojae* using the $CaCl_2$/PEG-mediated transformation method. GFP expression in different developmental stages of *P. sojae* was analyzed. GFP-fluorescent *P. sojae* can be readily detectable *in planta* including in leaves, hypocotyls and roots of soybean plants. Meanwhile, difference in resistance of soybean cultivars can be measured in roots using GFP-fluorescent *P. sojae*. The ability to tag *P. sojae* with GFP provides a stable molecular marker for studying the genetics of the parasite, but also a means to track *P. sojae* and analyze the gene expression of transient stages such as the zoospore stage and induced gene expression.

Owing to its economic effect on soybean production, researchers have constantly been focusing on *P. sojae*. With an increasing amount of EST data becoming public and the draft genome sequences release in 2004, *P. sojae* has been developed as one of model species for the study of the development and pathogenicity mechanisms of oomycete plant pathogens[6,31,32].

Currently the most stable method for DNA introduction into *Phytophthora* is the standard transformation protocol based on $CaCl_2$/PEG-mediated transformation of protoplasts developed by Judelson and his colleagues[11]. This method has been successfully used for the expression of marker genes such as *gus* and *gfp* in *Phytophthora* spp.[12–18]. Here, we describe the first successful transformation of the oomycete *P. sojae* with the reporter gene *gfp*.

We use a construct containing the open reading frame of a mammalian codon-optimized GFP (http://www.clontech.com/images/pt/PT3933-5.pdf) fused to the constitutively expressed *hsp70* promoter from *B. lactucae*. GFP fluoresces bright-green upon exposure to excitation light within minutes, unlike other bioluminescent reporters which require additional proteins, substrates, cofactors or even enzyme reaction to emit light[33]. GFP fluorescence was stable enough to analyze *P. sojae* cells without photo-bleaching. Green fluorescence was detected predominantly in living cells. This can be an advantage for the analysis of single cells. However, it can also be a major disadvantage in those cases when a population of cells or cell-cell interactions is studied. The GUS reporter gene system is another widely used reporter gene system, especially for macroscopic studies[18]. The blue stain was visualized easily with the naked eye. The viability of *P. sojae* cells cannot be monitored with the GUS reporter gene system, because the enzymatic staining reaction must be performed on dead tissues. The ability to employ living material is especially useful for analyzing GFP production in zoospores. GFP will be suitable for the analysis of genes expressed during this and other life-cycle stages of *P. sojae*.

Here, transgenic *P. sojae* strains that produce constitutively GFP were obtained after stable DNA integration using the $CaCl_2$/PEG-based transformation protocol. GFP was monitored during several stages of the life cycle of *P. sojae* to evaluate its use in molecular study. GFP was not toxic to *P. sojae* and stably expressed in several developmental stages. GFP-fluorescent *P. sojae* was readily detected in infected leaves, hypocotyls and roots of soybean plants.

The quantification of induced disease resistance is often performed with methods such as estimation of the lesion area or evaluation of sporulation, which can be rather subjective and time-consuming[17]. Wubben et al. transformed *C. fulvum* with the *gus* gene and measured the fungal biomass by photometrically quantifying GUS activity[20]. In this study, the difference of resistance of different cultivars was determined using GFP reporter gene. It was found that the germ tubes of cysts germinating on roots of resistant cultivars were longer and thinner than those on susceptible cultivars. This is the first report about cultivars' resistance to *P. sojae* reflected by laser microscopy. Similarly, Bignell[34] and Lapwood[35] reported that potato cultivars with higher resistance delayed the formation of appressoria and caused longer germ tubes of *P. infestans*. This could help researchers to elucidate the mechanisms underlying the resistance of host plants to *Phytophthora*.

We conclude that the ability to tag *P. sojae* with GFP will accelerate the genetic research of the pathogen. Real time analyses of gene expression in cell types or structures that are unique to oomycete microbes, such as the transient stage represented by motile zoospores, will be facilitated by the use of GFP as a reporter. It also provides a means to track genetically modified strains and analyze the spatial distribution of gene expression in soil or aquatic natural systems[36]. The results from this study also could facilitate quantifying the resistance level of host plants to *Phytophthora*[17].

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