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A dsRNA mycovirus causes hypovirulence of *Fusarium graminearum* to wheat and maize

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Abstract The association of *Fusarium graminearum* isolate China-9 with the dsRNA mycovirus FgV-ch9 was evaluated for hypovirulence-related traits. Single conidia-originating cultures of China-9 isolate can be associated either with high, medium or low amounts of the viral dsRNAs. At high and medium dsRNA levels, China-9 isolates exhibit reduced mycelia growth rate and conidiation capacity, abnormal colony morphology, disorganized cytoplasm, as well as reduced virulence for wheat and maize plants. At low dsRNA levels the fungus shows no symptoms, however, the RNA segments can be detected by RT-PCR. Transfection of the virulent *F. graminearum* PH-1 isolate with purified Virus-like Particles (VLPs) of FgV-ch9 reduced its conidiation capacity, perithecia formation, and pathogenicity for wheat and maize several folds. These results further demonstrate that FgV-ch9 is associated with hypovirulence of *F. graminearum.*

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Abbreviations
FgV-ch9 Fusarium graminearum Virus-china9

Introduction

The fungus *Fusarium graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schwein.) Petch] is the major causal agent of Fusarium head blight (FHB) of small-grain cereals like wheat and barley and the stalk and ear rot of corn (Goswami and Kistler 2004). The economic losses and health consequences associated with FHB are mainly due to the reduction of the kernels weight and the capability of *F. graminearum* to produce mycotoxins such as deoxynivalenol (DON), nivalenol (NIV), zearalenone, fusarin C and aurofusarin (Trail 2009; Bennett and Klich 2003; Gilbert and Tekauz 1995).

Reduced or debilitated fungal virulence (hypovirulence) due to mycovirus infections has been reported for isolates of several phytopathogenic fungi such as, *Botrytis cinerea* (Castro et al. 2003), *Helminthosporium victoriae* (Ghabrial 2001), *Sclerotinia sclerotiorum* (Zhang et al. 2009; Yu et al. 2010), *Ophiostoma novo-ulmi* (Hong et al. 1999), and the white root rot *Rosellinia*

necatrix (Chiba et al. 2009). Hypovirulent strains of phytopathogenic fungi and their associated mycoviruses are model systems to examine the mechanisms of fungal pathogenesis and might serve as or lead to biocontrol. For example, hypovirulent strains of the chestnut blight fungus *Cryphonectria parasitica* were used effectively to control this disease in Europe (Anagnostakis 1982).

Mycovirus-associated hypovirulence is largely unknown in the genus *Fusarium,* although mycoviral dsRNAs have been reported for *F. poae* (Compel et al. 1999), *F. solani* (Nogawa et al. 1996), *F. oxysporum* (Kilic and Griffin 1998), and *F. graminearum* (Chu et al. 2002; 2004); the association of these viruses with host-hypovirulent traits was suggested only for a few isolates including *F. graminearum* strain DK21 (Chu et al. 2002).

In this study, we present data that the recently described FgV-ch9 (Darissa et al. 2011) provokes a set of discernible hypovirulence-associated traits in *F. graminearum.* These include reduced fungal mycelia growth rate, reduced vegetative and sexual reproduction and reduced pathogenicity to wheat and maize.

Material and methods

Source and characterization of FgV-ch9

The source and molecular characterization of FgV-ch9 were described previously (Darissa et al. 2011). Levels of dsRNA per g fresh weight mycelium were determined after isolation and digestion of the extracted total nucleic acid material with DNAse and RNAse (Darissa et al. 2010) photometrically.

Media and culture conditions of *F. graminearum*

F. graminearum china 9 and PH-1 isolates were preserved on SNA plates as described by Nirenberg (1981) or stored in water at −70 °C. While the propagation conditions of the PH-1 isolate were on CM medium (Leach et al. 1982) at 28 °C under shaking (180 rpm) in the dark, those for china 9 isolate were slightly different (25 °C and 120 rpm).

To induce conidiation, either a mycelia plug (0.5 cm^2) or a drop of conidia suspension was placed on SNA plates for 2 weeks at 18 °C under near-UV light (TLD 36 W-08; Philips, Eindhoven, The Netherlands) and white light (TL 40 W-33 RS; Philips) with a 12 h photoperiod.

Statistical treatment of data

For statistical evaluation of our bioassay data we used Student's *t*-test, a two-tailed distribution and a significance level of $p<0.05$. The statistics were calculated using Microsoft Office Excel 2007.

Production of perithecia

Carrot agar plates were inoculated with 10 µl of *F. graminearum* conidia suspension (~100 conidia) and incubated at 25 °C in the dark for up to 1 week until the plate was covered with aerial hyphae. The mycelia were vigorously pressed down with 1 ml of sterile 2.5 % (v/v) Tween 60 solution using a sterile glass rod while spreading the solution all over the plate. Remaining mycelia clumps were removed and the plates were incubated at 25 °C in the dark or under a mixture of near UV and white light with a 12 h photoperiod up to three weeks. Produced perithecia were counted under a stereo microscope. The carrot agar medium was prepared according to Klittich and Leslie (1988) with some modifications. Briefly, 400 g of fresh carrots were washed, peeled, cut into small pieces and boiled in 400 ml of H_2O in a microwave for 10 min. The carrots were further homogenized in a blender and the slurry was strained through cheesecloth. Before autoclaving for 50 min at 121 °C, 20 g of granulated agar were added and the medium volume was brought to 1 l with ddH_2O.

Ultrastructural study

Fresh 3-days old mycelia cultures on CM-plates of *F. graminearum* china 9 originating from single conidia were used for ultrastructural studies. Fixation of the mycelia was carried out by covering the plates with 2 % paraformaldehyde (PFA) in 50 mM MSB buffer pH 6.8 for 30 min at RT, then for 3 h on ice. Lipid saturated membranes were preserved with 0.5 % OsO4 for 1 h on ice. The dehydration of the tissues and the infiltration of the embedding medium were performed with ethanol according to standard protocols. Embedding was carried out with LR-White medium and polymerization in gelatin capsules for 2 h at RT followed by 36 h at 50 °C in O_2 free atmosphere.

Using an ultramicrotome (Reichert & Jung, Ultracut E) with a diamond knife, ultrathin sections (~70 nm) were cut and mounted on Mowital-coated nickel grids (150 mesh). The sections were observed in a TEM (LEO 906E, 100 kV) and documented with a CCD Gatan-Camera and the software Digital Micrograph 3.3.4.

Virulence assay on wheat heads

Wheat plants, cv. Nandu (Lochow-Petkus, Bergen-Wohlde, Germany), were grown under greenhouse conditions till the flowering stage. Infection of the plants took place in an infection chamber with a 12 h photoperiod, 20 °C and 70 % humidity. At the anthesis stage, each of two alternate central spikelets was inoculated with a 10-µl suspension containing 500 conidia. Inoculated spikes were covered with plastic bags and misted with water during the first 3 days. Spikes were collected and results evaluated 3 weeks post-infection. For each treatment, 30 spikes were inoculated.

Virulence assay on maize cobs

Maize, inbred line A188 (Green and Phillips 1975), was used for virulence assays. Plants were grown and infected in a green house at 26°–30 °C, 60–80 % humidity, and natural daily photoperiod. During short-day conditions, artificial light was supplemented. Pollination of the maize silks was manually performed 3 days before inoculation. Four ml conidia suspension (10^4 conidia/ml) was injected into the silk channel of the primary ears of each maize cob using a syringe with cannula. Inoculated cobs were covered with plastic bags and misted with water for the first 3 days. Disease severity was determined 5 weeks after inoculation as described by Reid and Hamilton (1995).

Preparation of *F. graminearum* protoplasts

Protoplasts from *F. graminearum* were prepared as described by Maier et al. (2005). Briefly about 1×10^6 conidia were inoculated in 100 ml YEPD medium and incubated overnight in the dark at 150 rpm RT. Grown mycelium was harvested by filtration through a 200 µm Wilson-sieve, washed with 200 ml sterile water and dried on a stack of sterile filter paper. Enzyme solution (2.5 % (w/v) Driselase, 0.5 % (w/v) lysing enzymes and 1.2 M KCl) was stirred for 30 min, centrifuged at 5000 rpm at RT, and the supernatant was sterilized through 0.2 µm pore filters. About 1 g of the dried mycelium was mixed with 20 ml of the enzyme solution and incubated for 90 min at 30 °C with gentle shaking. The protoplasts were filtered twice sequentially through 100 µm and 40 µm Wilson-sieves, pelleted at 2000 rpm for 10 min at RT and washed with 10 ml STC medium. Finally protoplasts were resuspended in STC solution at a concentration of 1×10^7 protoplasts per ml.

Protoplast transfection with FgV-ch9

Protoplast transfection was carried out as described by Kanematsu et al. 2004, and Sasaki et al. 2006 with some modifications; CsCl-purified VLPs were filtrated through a 0.2 µm Millipore sterile filter and eluted in 300 µl of 0.01 M Na-phosphate buffer, pH 7.0. The purified VLPs contained 60 ng/µl of dsRNA as was measured with a pico-drop spectrophotometer. In a 15 ml Falcon tube, 100 µl protoplasts (1×10^7 cells) were gently mixed with 3–5 µg of VLPs and incubated on ice for 30 min. To induce transformation, 500 µl of sterile 60 % (w/v) PEG_{4000} supplemented with 10 mM of MOPS pH 7 and 10 mM of $CaCl_2$ were added, the contents mixed gently and incubated at 25 °C for 25 min. Cell wall regeneration was induced by adding 2 ml TB3 medium (0.3 % yeast extract, 0.3 % casamino acids, and 20 % sucrose) and incubating the tube overnight at RT with no shaking. Protoplasts were pelleted at 4000 rpm for 10 min at RT and resuspended in 300 µl of TB3 medium. Protoplasts were cultured on CM plates (100 µl/plate) at 25 °C in the dark for 5 days until the mycelium covered the whole plate. From each plate, 3–4 mycelial plugs (~0.5 cm^2) were cut out, transferred separately to a cellophane-CM plate and incubated for 4–5 days at 25 °C in the dark. Parts of the mycelia were used for dsRNA isolation to assay for viral infection.

Results

Cultures of *F. graminearum* China 9 originating from single conidia were found associated with high, medium or low amounts of FgV-ch9 dsRNAs. Cultures with a high amount contain 50–150 µg, those with medium amount 8–30 µg viral dsRNA/g fresh weight

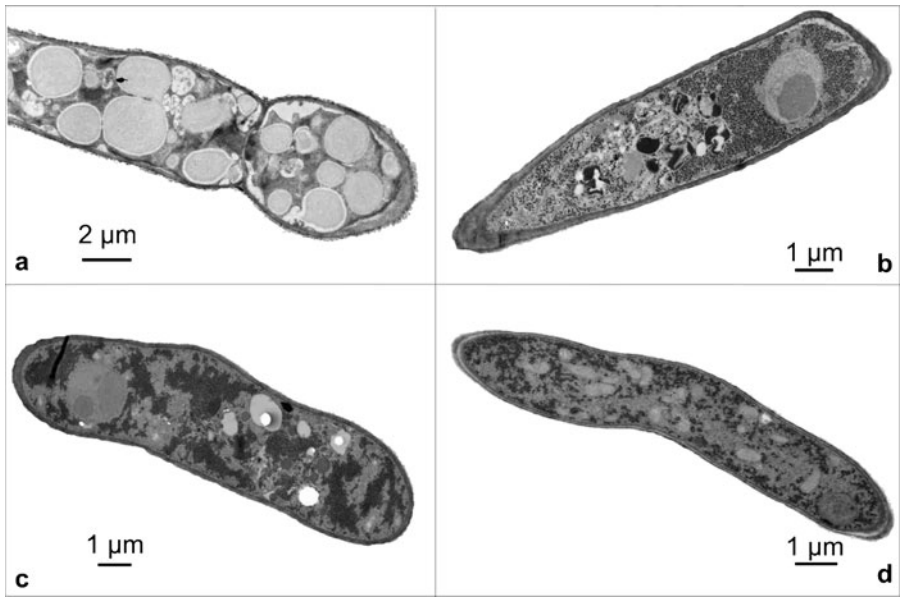

Fig. 1 Transmission electron micrographs of *F. graminearum* China 9 associated with different levels of FgV-ch9 dsRNA. **a** China 9 with high dsRNA level possesses abnormal cytoplasm with many large vacuoles that lack electron density. **b** China 9 with medium dsRNA level showing normal nucleus, abundance of ribosomes, but also a disorganized part of the cytoplasm. **c** China 9 with low dsRNA level has normal cytoplasm and nucleus. **d** not infected wildtype isolate PH-1

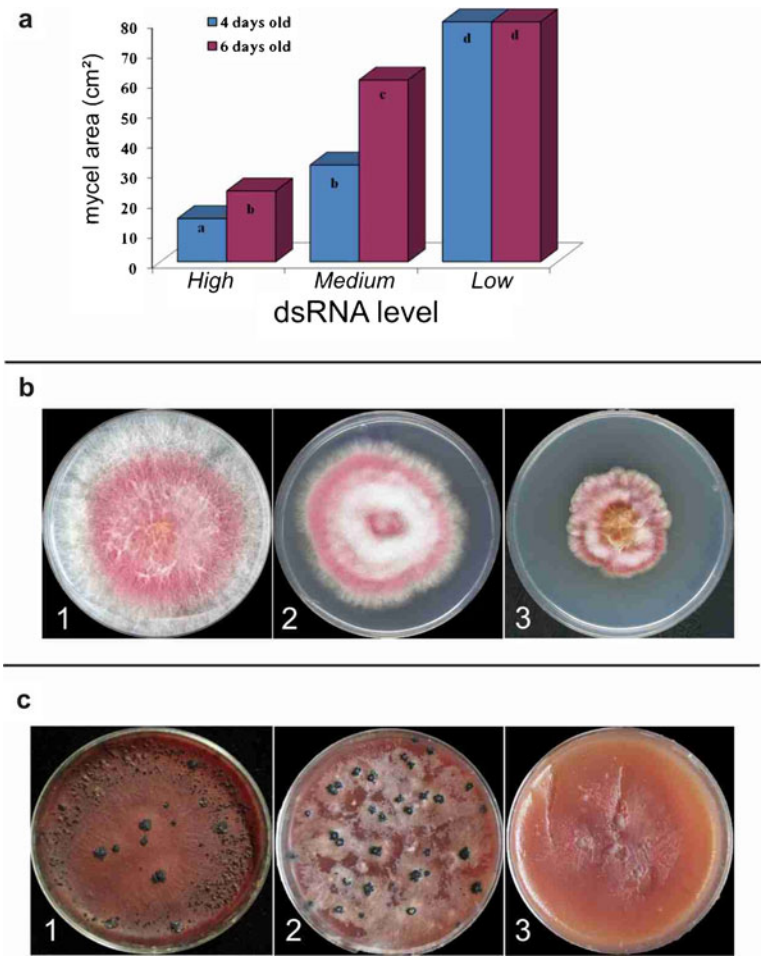

Fig. 2 Growth rate and colony morphology of *F. graminearum* China 9 cultures associated with different titres of FgV-ch9. **a** Two dimensional colony growth. The colony area was measured 4 and 6 days after inoculation. The fungus was grown on solid CM medium at 25 °C in the dark. Values are the average area of 6 colonies from 2 independent experiments. Different letters indicate statistically significant differences. **b** Macroscopic colony growth of China 9 with different dsRNA level. **b1:** China 9+ low dsRNA, **b2** China 9+ medium dsRNA, **b3** China 9+ high dsRNA. The cultures were photographed after 4 days of growth. **c** Perithecia formation dependent on FgV-ch9. **c1:** PH-1untreated perithecia formation, **c2** PH-1 transfected with FgV-ch9 perithecia formation, **c3** China 9 high level dsRNA perithecia formation

mycelium. Viral dsRNA in mycelia with low dsRNA level is detectable by means of RT-PCR only (the RT-PCR conditions were as described by Darissa et al. 2010; 2011). In correlation to the amount of the viral dsRNA, the three culture types of China 9 exhibited also differences in their cellular ultrastructure, colony morphology and growth rate, conidiation capacity, and virulence for wheat and maize plants.

Ultrastructural examination of China 9 isolate revealed presence of VLPs in membrane-bound vesicles and vacuole-like structures (results not shown). In cultures with high dsRNA levels severe cellular degeneration and cytoplasmic regions with decreased electron density were frequent (Fig. 1a). Most evident was the presence of many large vacuole-like structures and disintegration of nuclear and mitochondrial membranes. Although most of the examined cells of China 9 with medium dsRNA level had a normal nucleus and abundance of ribosomes, many of them showed partially disorganized cytoplasm (Fig. 1b). On the other hand, the majority of the cells of China 9 with low dsRNA level appeared normal with organized cytoplasm of usual electron density and a nucleus with a smooth nuclear membrane (Fig. 1c) like that observed in the dsRNA-free wildtype isolate PH1 (Fig. 1d).

In addition to subcellular symptoms, the growth rate of China 9 isolate on CM was significantly reduced when cultures were associated with high and medium dsRNA levels (Fig. 2a, b2 and b3). The colony area of 6 day-old cultures associated with high or medium dsRNA levels were 23.6 and 60.6 cm^2, respectively. At low dsRNA level, the mycelia of 4 day-old cultures of *F. graminearum* China 9 covered the whole Petri-dishes (81 cm^2, Fig. 2a and b1). Besides the restricted growth rate in cultures associated with high dsRNA level, such cultures also exhibited abnormal growth phenotypes including dark pigmentation and altered (uneven) colony morphology with the frequent formation of ear-like structures at the colony margin (Fig. 2b3).

The capacity of *F. graminearum* China 9 to produce conidia was significantly reduced in cultures associated with a high to medium level of viral dsRNA. In negative correlation to the dsRNA level, the production of conidia per plate SNA medium was reduced between 16 % and 60 % for high and low, respectively (Table 1). However, as a trend, the capacity for conidiation in all of the *F. graminearum* China 9 cultures was significantly lower than that of the PH-1 isolate (0.3–2.4 million in China 9 in comparison to 7–10 million conidia per plate in PH-1).

Table 1 Effect of FgV-ch9 on the conidiation capacities of *F. graminearum* isolates china 9 and PH-1

Treatment	Conidia per plate ($x10^3$)
A- dsRNA level in China 9 isolates:	
High	384.5[a]
Moderate	1427.5[b]
Low	2355.5[c]
B- Transfection:	
PH-1 (control)	7380[a]
PH-1VLP1.1[a]	135[b]
PH-1VLP1.2[b]	980[c]
PH-1VLP11.1[a]	63[d]
PH-1VLP11.2[b]	780[c]

[a] 1.1 and 11.1 are the first conidial generation of the transfected colonies PH-1VLP1 and PH-1VLP11, respectively

[b] 1.2 and 11.2 are the second conidial generation of the transfected colonies PH-1VLP1 and PH-1VLP11, respectively

Conidiation was initiated in SNA medium at 18 °C for 2 weeks. Figures are the average of 5 independent experiments. In each set of treatments (A and B), the different letters behind conidial counts indicate statistically significant differences

Infection of wheat spikes with conidia originating from cultures with high and medium dsRNA level remained restricted to the inoculated spikelets (Fig. 3a, b). However, when conidia with low dsRNA level were inoculated the symptoms spread and became visible in about 27 % of kernels (Table 2 and Fig. 3c). Inoculations with the PH-1 conidia (Fig. 3d) resulted in 87 % infected kernels, whereas negative water controls remained symptomless.

Less than 6 % of the maize kernels developed *F. graminearum* symptoms 5 weeks after inoculation with China-9 conidia originating from high and medium dsRNA level cultures (Table 2 and Fig. 4b). On the other hand, infection of maize cobs with conidia from low dsRNA level cultures increased the percentage of infected kernels to 28 % (Table 2 and Fig. 4a). While cobs inoculated with water, (negative control) showed no disease symptoms, those infected with the PH-1 conidia produced kernels with severe symptoms (Table 2 and Fig. 4d, c, respectively). All data were confirmed to be significantly different as indicated in Tables 1 and 2.

In order to corroborate that the hypovirulent features of China 9 isolate are due to its association with

Fig. 3 Effect of different dsRNA levels in *F. graminearum* China 9 on its pathogenicity for wheat. Two central spikelets of wheat spikes were each inoculated with 500 conidia of the PH-1 isolate (**d**) or China 9 harbouring **a** high dsRNA, **b** medium dsRNA, **c** low dsRNA. The infection was monitored for 3 weeks *post inoculation*. Arrowheads indicate inoculation sites

FgV-ch9, purified VLPs of FgV-ch9 were transfected into protoplasts of the PH-1 isolate. Several mycelial plugs, originating from transfected protoplasts, were checked for the presence of FgV-ch9 by means of RT-PCR and two of the successfully transfected plugs (PH-1VLP1 and PH-1VLP11) were used for further studies.

The conidiation capacities of the successfully transfected PH-1 plugs were significantly reduced. In comparison to the PH-1 isolate, the produced first generation of conidia of PH-1VLP1 and PH-1VLP11 were reduced by 54 and 117 folds, respectively, while those of the second generation were reduced by 7.5 and 9.5 folds, respectively (Table 1). In addition, the virus transfected PH-1 cultures exhibited significant reduction in perithecia production. The average number of produced perithecia in nine plates representing three independent experiments was 45 for the virus transfected PH-1 (Fig. 2c2) in comparison to 220 in the PH-1 isolate (Fig. 2, c1). China 9 isolate did not produce any perithecia under the described conditions (Fig. 2c3).

When wheat plants were inoculated with the first generation of conidia produced from PH-1VLP1 and PH-1VLP11, the percent of symptomatic kernels was reduced in comparison to the PH-1 isolate. While the reduction in diseased kernels reaches 50 % in case of PH-1VLP1, it was less than 20 % for PH-1VLP11 (Table 2). Symptoms developed due to infections with the second generation of conidia were comparable to those produced by the PH-1 isolate (Table 2). Similar results were observed when maize cobs were inoculated with PH-1VLP1 and PH-1VLP11 conidia, 68 % and 82 % of the kernels, respectively, were diseased in comparison to 100 % for those inoculated with the PH-1 conidia (Table 2).

Discussion

We showed that fungal isolates from China 9, containing high dsRNA levels, exhibit abnormal and disorganized subcellular structures. Such observations were reported for several mycoviruses including reports on

Table 2 Effect of FgV-ch9 on the pathogenicity of *F. graminearum* isolates china 9 and PH-1 for wheat and maize plants

Treatment	% Infected kernels	
	Wheat	Maize
A- dsRNA level in China 9 isolate:		
High	10[a]	5.7[a]
Moderate	10[a]	5.6[a]
Low	27[b]	28.2[b]
B- Transfection:		
PH-1 (control)	100[c]	100[b]
PH-1VLP1.1[a]	48.4[a]	68.0[a]
PH-1VLP1.2[b]	86b[c]	ND[1]
PH-1VLP11.1[a]	82.2[b]	81.6[a]
PH-1VLP11.2[b]	94.5[c]	ND

[a] 1.1 and 11.1 are the first conidial generation of the transfected colonies PH-1VLP1 and PH-1VLP11, respectively

[b] 1.2 and 11.2 are the second conidial generation of the transfected colonies PH-1VLP1 and PH-1VLP11, respectively

[1] *ND* Not determined.

Results are the percent of infected kernels of 25–30 wheat spikes or 10 maize cobs per treatment. The results were collected 3 and 5 weeks after inoculation of wheat and maize, respectively. In each set of treatment (A and B) for each plant type, different letters behind data indicate statistically significant differences

the accumulation of vacuoles (Kwon et al. 2007), disintegration of nuclei and mitochondrial membranes (Zhang et al. 2009) and cytoplasm of light electron density (Kwon et al. 2007; Zhang et al. 2009). Moreover, the aggregation of mycovirus particles in membranous structures or vesicles was observed in fungal species including *Sclerotinia sclerotiorum* (Zhang et al. 2009), *Penicillium chrysogenum* (Yamashita et al. 1973), *A. bisporus* (Albouy 1972), and *Saccharomyces cerevisiae* (Border 1972).

Reduced growth rate and altered macroscopic morphology of fungal colonies due to the presence of viruses in their hyphae was reported for several fungal species (Jiang et al. 1998; Xie et al. 2006; Zhang et al. 1993; van Diepeningen et al. 2006) including *F. graminearum* (Kwon et al. 2007). In the case of FgV-ch9, such alterations are consistently correlated with the viral dsRNA level in hyphal cells. The association of China 9 cultures originating from single conidia with different levels of FgV-ch9 dsRNA might be due to the unequal encapsidation of the different virus dsRNAs (Darissa et al. 2011) which may lead to their unequal representation in different single conidia. Moreover, the reduction in the growth rate of *F. graminearum* cultures was observed also for PH-1 transfected cultures (data not shown).

More important, however, is the association of FgV-ch9 with significant reduction on the conidiation

Fig. 4 Effect of different dsRNA levels in *F. graminearum* China 9 on its pathogenicity for maize. At the stage of early kernel formation, the maize cobs were injected with the conidia of **a** China 9 with low dsRNA, **b** China 9 with high and medium dsRNA, **c** PH-1 isolate (positive control), **d** water (negative control). In each treatment, 10 cobs were injected. Cobs were photographed 5 weeks *post infection*

capacity of its fungal host. The fungal capacity to produce conidia is negatively correlated with the viral dsRNA level. The same was also observed in FgV-ch9 transfected cultures of PH-1. In addition to that, transfection of the PH-1 isolate with FgV-ch9 significantly reduced its capacity to produce perithecia. Conidia and ascospores of *F. graminearum* play a key role for the spread of the fungus in the field, which would contribute to the disease epidemics. In addition, perithecia can constitute an infectious dose in future culturing seasons of cereals because of their ability to survive and overwinter. Reduced conidiation capacity of some fungi due to their association with mycoviruses was reported for several species (Castro et al. 2003; Deng and Nuss 2008; van Diepeningen et al. 2006).

The association of FgV-ch9 with hypovirulence of its fungal host was observed in wheat and maize plants inoculated with the fungal conidia. The ability of China 9 conidia to infect wheat and maize depends on the dsRNA level found in the mycelium. The higher the dsRNA load, the less virulent is the fungal isolate. The positive correlation between the threshold of hypovirulence and FgV-ch9 titres is a strong indication that the virus is involved in the former process. Moreover, the percentages of the infected wheat and maize kernels in the plants inoculated with the first generation of conidia produced from FgV-ch9 transfected PH-1 cells were significantly reduced compared to the non-transfected PH-1 controls. However, the inoculation of the plants with the second generation of conidia produced from FgV-ch9 transfected PH-1 results in disease symptoms comparable to those of the non-transfected WT PH-1 isolate. This change in virulence/infectivity was in all cases associated with a drastically reduced dsRNA level. Since the same curing of a plant pathogenic fungus after successful transfection, including the resulting loss of hypovirulence, was already described for *Rosellinia necatrix* mycoreovirus 3 (Sasaki et al. 2006), our results are another indication that FgV-ch9 is infectious and is responsible for hypovirulence of *F. graminearum*.

China-9 *F. graminearum* has been verified taxonomically by PCR (Darissa et al. 2011), like the isolate PH1. Nevertheless, the two isolates behave highly differently when compared against each other in growth rate, sporulation capacity, and infectivity. Even Fg-ch9 with a dsRNA level only detectable by PCR exhibits a reduced fitness and virulence compared to isolate PH1. This might reflect a general feature of China 9 isolate, or might be due to the remaining viral particles, as we were unable to isolate a truly virus free China 9 mycelium. Since isolate PH 1 lost the transfected dsRNA rather quickly, different fungal genetic backgrounds might be responsible for the successful viral infection and maintenance in isolate China 9. As described by Hammond et al. (2008), downregulation of dsRNA levels may be due to RNA silencing effects. These may be inefficient in China-9 whereas in the PH-1 isolate such silencing effect might be highly efficient and lead to a curing from virus infection. This possibility could be checked by means of quantitative RT-PCR and will be subject of further studies.

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