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Apple cultivar Regia possessing both *Rvi2* and *Rvi4* resistance genes is the source of a new race of *Venturia inaequalis*

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Abstract The apple cultivar Regia, bred in Dresden-Pillnitz, Germany, is one of the few cultivars originating from a different scab (*Venturia inaequalis*) resistance background than *Malus floribunda* 821, the source of *Rvi6*. Cv. Regia is a descendant of Russian apple R12740-7A and has been proved to contain the two scab resistance genes *Rvi2* and *Rvi4*. The cultivar itself has been grown in Dresden-Pillnitz since the early 1970s, while seedling populations derived from cv. Regia have been raised in fungicide-free plots since at least 1982. In 2011, small scab lesions were found for the first time on leaves of cv. Regia trees in an experimental unsprayed orchard in Dresden-Pillnitz. Single spore isolate Regia2 (R2) was cultured from these scab lesions and propagated, and then inoculated on scions of the VINQUEST scab differential host set comprising host (0) to host (15), host (17) and cv. Regia, grafted on rootstock M9, in a greenhouse. Strong sporulation was observed on hosts (0), (1), (2), (8), (9), (10) and cv. Regia, and weak sporulation on hosts (3), (4), (13) and (17), suggesting that the new isolate possesses the respective virulences. The implications of these results are discussed in relation to the natural infection of the differential hosts in the orchard and the artificial inoculation of the grafted differential hosts with inoculum gained from in vitro culture of R2.

Keywords Scab · Hosts · Virulence

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Apple scab, caused by the ascomycete *Venturia inaequalis* (Cooke) Wint., is one of the major apple diseases worldwide. Since orchard management practices aimed at disrupting the disease cycle are not sufficient to effectively control the disease, fungicide applications are required each growing season. Nevertheless, even 10–18 applications can be insufficient in producing apples with an acceptable level of scabbed fruits (MacHardy et al. 2001). Therefore, growing resistant cultivars is regarded as an important aspect in the management strategy of apple scab, thus preventing abundant applications of fungicides and hence mitigating the threat of strains of *V. inaequalis* developing resistance to fungicides, an increasing problem in apple production worldwide (Chapman et al. 2011; Mondino et al. 2015; Villani et al. 2015). This approach also reflects the socio-economic and environmental impacts of a more sustainable production preferred by today's consumers. Historically, breeding for scab resistance has largely relied on the major resistance gene *Rvi6* (*Vf*), derived from the wild species accession *Malus floribunda* 821

(Hough et al. 1953), with an abundant number of *Rvi6*-resistant cultivars having been released to date (Baumgartner et al. 2015). The use of this single scab resistance gene suffered a setback when Parisi et al. (1993) reported a new race of *V. inaequalis* overcoming *Rvi6*, with the race subsequently reported to have spread to other regions in Europe (Parisi et al. 2004). A race is regarded as a group of genotypes (strains) with the same race spectrum, i.e. they are all able to overcome the same R-genes, whereas a strain is a genotype with a specific race spectrum.

Lemaire et al. (2016) demonstrated a common origin of *V. inaequalis* populations infecting *Rvi6* cultivars and *M. floribunda* at the European scale. These authors prefer the hypothesis that the *Rvi6*-virulent lineage separated several thousand years ago from populations infecting non-*Rvi6* hosts from standing variation present on the non-agricultural progenitor of the *Rvi6* resistance *M. floribunda*. Nevertheless, although *Rvi6* resistance is overcome and virulent lineages spread progressively, the planting of *Rvi6* cultivars remains an important feature for sustainable apple production (Peil et al. 2014) and can facilitate a sustainable management of scab control in the orchard (Didelot et al. 2016) as not all *Rvi6*-cultivars are equally susceptible. The breakdown of scab resistance controlled by *Rvi6* re-emphasized the need to breed apple cultivars with more durable resistance through the pyramiding of functionally different resistance genes (MacHardy et al. 2001), ideally without *Rvi6* when race (6) is present. In 2011, small scab lesions were observed for the first time on apple cv. Regia carrying a pyramid of two major scab resistance genes, *Rvi2* and *Rvi4* (Peil et al. 2011). The existence of race (2) has been reported previously (Shay and Williams 1956), while the reports on race (4) have been spurious to date (Bus et al. 2011). Since 2012, the plots with scab resistant breeding material in Dresden have been sprayed once or twice with fungicide during spring in the attempt to prevent the dissemination of this virulent strain of *V. inaequalis*, and to minimize the selection of strains with new virulences. The objective of this research was to confirm the virulences of scab strain Regia2 (R2) and the impacts on resistance breeding.

A single spore isolate of R2 was prepared from scab lesions of cv. Regia to determine the race status of that isolate. Therefore, sporulating scab lesions were cut from an infected leaf with a scalpel and transferred into Eppendorf tubes (2 ml) with 300–400 μl of tap water. Lesions were rubbed with a stirring rod and vortexed for 2 min. Depending on their concentration, conidial suspensions were diluted with tap water and plated onto sterilized water agar medium consisting of 2% agar and tap water. Single germinated conidia were picked off with a small needle after overnight incubation and transferred to a medium containing 1.5% malt extract, 1.3% agar and 200 μg/ml kanamycin for 6 weeks of culturing at room temperature. Afterwards, proliferation of sporulating strains was performed on the same malt extract agar medium, but without kanamycin, at 21 °C for the first 7–14 days, followed by a treatment with ultraviolet light to stimulate conidia production, for 12 h/day at 18 °C for at least 7 days. For the production of conidia of R2 for inoculation, a modified protocol of Parker et al. (1995) was used. Sterile cellophane membranes were laid over the surface of malt extract agar (1.5% malt extract, 1.3% agar). Small sections of mycelium bearing high numbers of well-formed conidia were blended in sterilized tap water, and the suspension was spread onto the membrane. Dishes were sealed with parafilm and incubated in the dark at 21 °C. Depending on mycelial growth and initial conidia production, dishes were transferred after 7–14 days to ultraviolet light (12 h/day) and 18 °C. After 3–7 days of culturing under ultraviolet light, membranes were removed from the medium and dried for 7–14 days in unsealed sterile plastic petri dishes at 21 °C (1 membrane/dish). For storage at −20 °C, dry membranes were placed in new petri dishes (2–5 membranes/dish) and sealed with parafilm. For inoculation, conidia were washed from the membranes and diluted with water to a concentration of about 1.5–2.5×10^5 conidia/ml.

For artificial inoculation, each host was grafted on rootstock M9 for each year of the trial (2013–2016) and grown in a ventilated greenhouse (18 °C day/15 °C night), and afterwards, artificial inoculation with R2 was performed. The youngest leaves of actively growing shoots of the scab differential hosts and cv. Regia (Table 1) were spray-inoculated in the greenhouse, wrapped with wet paper and put in a plastic bag for at least 48 h at a ventilation temperature of the greenhouse set at 18 °C. After removing the bags and paper, the plants were kept in a greenhouse chamber with a minimum of 70% relative humidity and a ventilation temperature set at 18 °C. Windows were screen shaded. Inoculation of up to five trees each of scab hosts (1) to (15) (Table 1) were performed in 2013 to 2016 with R2. Host (17) was screened in 2014 to 2016, and cv. Regia only in 2015. Scoring of symptoms was performed at

Table 1 Differential host set[a] used to determine virulences of *Venturia inaequalis* isolate Regia2, R-genes present in the differential hosts, scab severity in the orchard (highest score in the years 2012 to 2016) and highest artificial scab infection score in the years 2013 to 2016 in the greenhouse of each differential host

Hosts	Genotypes	Names of R-gene		Highest score for scab				
		Historical[b]	New[b]	orchard[c]	greenhouse[d]			
					2013	2014	2015	2016
h(0)	Gala			8	4	4	4	4
h(1)	Golden Delicious	*Vg*	*Rvi1*	8	4	4	4	4
h(2)	TSR34T15	*Vh2*	*Rvi2*	3	4	4	4	4
h(3)	Q71	*Vh3.1*	*Rvi3*	8	4	2	4	3a
h(4)	TSR33T239	*Vh4*	*Rvi4*	2	4	4	4	4
h(5)	9-AR2T196	*Vm*	*Rvi5*	2	0	0	0	2
h(6)	Priscilla	*Vf*	*Rvi6*	5	2	2	2	2
h(7)	*M. floribunda* 821[e]	*Vf/Vfh*	*Rvi6/7*	8	0	0	0	0
h(8)	B45	*Vh8*	*Rvi8*	7	4	4	4	4
h(9)	J34	*Vdg*	*Rvi9*	2	4	4	4	4
h(10)	A723–6	*Va*	*Rvi10*	5	4	4	4	4
h(11)	*M. baccata jackii*	*Vbj*	*Rvi11*	1	0	2	0	0
h(12)	Hansen's baccata 2	*Vbj*	*Rvi12*	1	1	0	1	1
h(13)	Durello die Forli	*Vdg*	*Rvi13*	3	4	4	4	4
h(14)	Dülmener Rosenapfel	*Vdr1*	*Rvi14*	1	0	2	2	2
h(15)	GMAL2473	*Vr2*	*Rvi15*	1	0	2	2	2
h(17)[f]	04214/79	*Va1*	*Rvi17*	1		4	2	4
	Regia[g]		*Rvi2/4*	3	–	–	4	–

[a] Adapted from http://www.vinquest.ch/monitoring/establishing_network.htm

[b] Before (historical) and after (new) the new nomenclature of scab resistance genes (Bus et al. 2011)

[c] According to the scale presented in Table 2

[d] According to Chevalier et al. (1991)

[e] Since h(7) only carrying *Rvi7* from *M. floribunda* 821 was not available, *M. floribunda* 821, which is h(6,7), was used

[f] h(17) was grafted onto placeholder trees of cv. Golden Delicious in the trap orchard in 2014 and 2015

[g] Scab on Regia was observed in a different plot in the experimental orchard in Dresden-Pillnitz in 2011

least two times, three and four weeks after inoculation according to Chevalier et al. (1991). Score values were: 0 – no symptoms; 1 – pin point pits; 2 – chlorosis; 3a – necrotic and some chlorotic lesions with very slight occasional sporulation; 3b – clearly sporulating chlorotic and necrotic lesions; 4 – sporulating lesions. Classes 1 to 3b were regarded as resistance reactions and class 4 as fully susceptible.

Additionally, trees of the differential host set growing on rootstock M9 in five randomized blocks in a trap orchard (fungicides were applied at most twice during spring) were scored twice a year (beginning of June and August) in 2012 to 2015 for scab infections according to the scheme presented in http://www.vinquest.ch/monitoring/collection.htm (Table 2).

Susceptibility (score 4) was observed after inoculation with R2 in the greenhouse for most replicates in all years for hosts (0), (1), (2), (8), (9) and (10). For hosts (3), (4), (13) and (17), and cv. Regia, susceptibility of replicates was not always consistent. Some plants of these four differential hosts showed beside sporulation (class 4), also no symptoms (class 0) and some only chlorosis (score 2). Chlorosis, but never sporulation, was observed on hosts (5), (6), (11), (14) and (15), and pin-point pits for hypersensitivity on host (12), but also not consistently. Figure 1 shows a set of leaves of the

Table 2 Evaluation scale for apple scab severity in the orchard in Dresden-Pillnitz

Score	Definition of the symtpoms	Proportion of affected organs
0	no observation	–
1	no visible symptoms	0%
2	one or very few lesions detectable on close scrutiny of the tree	0 to 1%
3	immediately apparent lesions in general clustered in few parts of the tree	1 to 5%
4	intermediate	×
5	numerous lesions widespread over a large part of the tree	± 25%
6	intermediate	×
7	severe infection with half of the leaves badly infected by multiple lesions	± 50%
8	intermediate	± 75%
9	tree completely affected with (nearly) all the leaves badly infected by multiple lesions	> 90%

Adapted from Lateur and Populer (1994)

hosts after scoring in 2015. No macroscopic symptoms were visible on hosts (5), (6), (7) and (14), whereas the others expressed clear symptoms. The highest score determined after scab inoculation for each genotype is listed in Table 1.

Table 1 also shows the highest score for each host in the trap orchard following natural infection after screening twice a year in 2012 to 2016. On all hosts, except host (17), which showed sporulation after artificial inoculation in the greenhouse, sporulation could be found in the orchard after natural infection. The highest severity in the field was detected for hosts (0), (1), (3), (7), (8) and (10), whereas hosts (2), (4), (5), (9) and (13) were less affected.

Since the *Rvi6*-mediated scab resistance has been overcome, breeders have increased their efforts to pyramid scab resistance genes in order to obtain more durable resistance (Peil et al. 2008; Kellerhals et al. 2008). Scab-resistant cv. Regia, bred in Dresden-Pillnitz, Germany, possesses a pyramid of two major scab resistance genes, which was confirmed by the 3:1 (resistant to susceptible) segregation ratios in progenies of cv. Regia crossed with susceptible cultivars (Fischer 2002). Screening with molecular markers confirmed the findings of Peil et al. (2011) that cv. Regia contains the two major race-specific genes *Rvi2* and *Rvi4* (Baumgartner et al. 2015).

In 2011, small scab lesions were observed for the first time on leaves of cv. Regia in a fungicide-free plot (no application for eight years) of the research orchard at the Julius Kühn-Institut (JKI) in Dresden-Pillnitz. Cv. Regia is a descendant of the Russian apple R12740-7A, highly resistant to scab, which is reported to carry a naturally pyramided complex involving three major effect genes: one race-nonspecific gene and two race-specific genes (Bus et al. 2005). This largely agrees with the original genetic analysis of three major effect genes and two minor effect genes (Dayton et al. 1953). Since scab lesions were found on cv. Regia leaves, it is probable that strains of *V. inaequalis* present in the orchard were

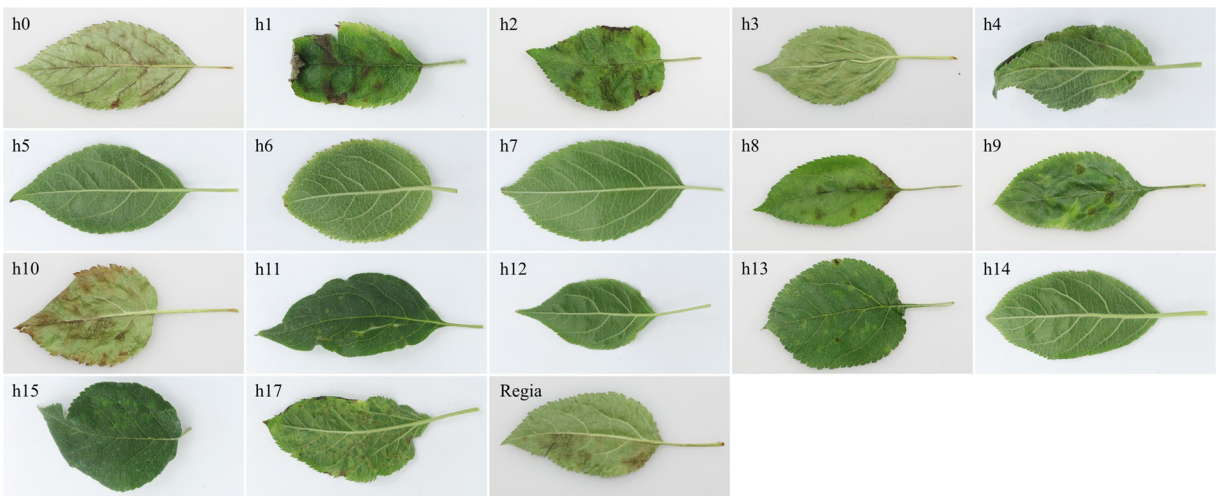

Fig. 1 Leaves of differential apple hosts (1) to (15) and, (17) [in pictures: h0 – h15, h17] and cv. Regia after artificial inoculation with strain R2 of *V. inaequalis* in the greenhouse in 2015

able to overcome the gene pyramid. The single spore isolate R2 was prepared to verify its ability to overcome the scab resistances of both genes, and to determine whether it has additional virulences. Artificial inoculations of the differential host set were performed in four consecutive years, and leaves of hosts (2) and (4) showed sporulating lesions in all four years. The incidence of scab and the conformity of results were higher on host (2) than on host (4). Some replicates of host (4) showed no sporulating lesions, but occasionally showed chlorosis. This was also true for cv. Regia, with some replicates showing no symptoms, whereas others showed sporulating lesions without any resistance reactions. These differences could be caused by the plant material, since not all replicates were at an optimal condition at the time of inoculation. Caffier et al. (2015) reported that interpretation of symptoms after artificial inoculation of the differential host set of *Malus* with *V. inaequalis* reference isolates was not always straightforward. One scab isolate induced a resistance reaction on host (13), but sporulation was also evident on the same host (Caffier et al. 2015). These authors regarded this interaction as partially resistant, whereas an interaction showing no resistance reaction, but only limited sporulation was regarded as compatible. The scab symptom scale by Chevalier et al. (1991) identifies distinct classes based on characteristic symptoms, but the large variation in individual symptoms rather makes the resistance-to-susceptibility scale continuous. In the current study, while the sporulation of host (4) as well as of 'Regia' after artificial inoculation with R2 was limited compared with that on host (2), they were both regarded as compatible interactions. The variability in infection response of the replicates of the hosts after artificial inoculation can be attributed to variation in the developmental stage of the individual plants and their interaction with the environmental conditions during the experiments.

The natural infection of hosts (2) and (4) and cv. Regia in the orchard suggests the presence of scab strains in the orchard of JKI able to overcome their resistances. However, while there were clear scab lesions on the trees, the level of infection was variable across years on all three hosts and remained low on both differential hosts as their scores were 3 (Table 1), i.e. only 1 to 5% of affected organs (Table 2), in the year with the highest incidence. The lesions may be the result of the presence of *V. inaequalis* strains able to overcome the resistance genes *Rvi2* and *4*, but at a low proportion of the pathogen population, since a high proportion would be expected to also lead to high incidence scores, such as those for e.g. hosts (1), (3) and (8) showing more than 50% of the tree leaves being highly infected (Table 1). For host (1), it was demonstrated that for 87% of the population, the complementary race (1) is predominant in the European *V. inaequalis* population (Parisi et al. 2004), which explains the high susceptible status of cv. Golden Delicious, rendering *Rvi1* ineffective as a resistance gene. The same could be said for *M. floribunda* 821, the host carrying *Rvi6* and *Rvi7*, however, the very high incidence score is in contrast with that of the less infected host (6) cv. Priscilla, for which the presence of unknown ephemeral resistance gene(s) (MacHardy et al. 2001) or QTLs may be hypothesized (Table 1).

In addition to the races causing infection of the differential hosts, some lesions may also be the result of opportunistic infection by (avirulent) strains of *V. inaequalis*. Even the strongest resistances are not absolute and can be overcome under favourable conditions for the pathogen, i.e. infection levels increase when infection periods are more severe (Martinez-Bilbao et al. 2012). Analysis of a random sample of scab lesions will be required to quantify the proportions of true virulent and opportunistic strains, and hence the distribution of race isolates like R2.

The greenhouse experiments indicated that isolate R2 carries at least five virulences. It shows compatible interactions with the hosts carrying *Rvi1*, *Rvi2*, *Rvi4*, *Rvi8* or *Rvi10*, making it a race (1,2,4,8,10). The interactions with hosts (3), (9), (13) and (17) were not straightforward as the sporulation on these hosts was limited, and hosts (9), (13) and (17) displayed clear chlorosis, too, an indication of an active resistance response that is common for this type of resistance gene. Divergence from the usual stellate necrotic (SN) resistance reactions conditioned by *Rvi9* and *Rvi13* suggests that they have been reduced to partial resistance, as was previously demonstrated for *Rvi4* (Bus et al. 2011). This raises the question whether this partial resistance is the new status quo or only an intermediate step towards being fully overcome, i.e. *V. inaequalis* developing towards complete virulence. As hosts (1), (2), (3), (8), (9), (10) and (13) consistently showed compatible reactions with R2, we assign a race (1,2,3,4,8,9,10,13,17) status to this isolate, which re-confirms that *V. inaequalis* has the evolutionary potential to develop highly virulent strains as demonstrated previously by isolates EU-NL24 and

174 (Caffier et al. 2015). In this report, the scab isolates tested on an incomplete differential host set were found to carry on average three virulences. Only isolates 413 and 1639 were virulent on host (2). Isolate 413, which originated from host (2) in France, was able to overcome *Rvi1*, *Rvi2* and *Rvi10*, hence identified as race (1,2,10), but it was not tested on hosts (3) and (9) (Caffier et al. 2015). Isolate 1639 also originating from host (2) in 2001 in France (Bus et al. 2005), was determined to be race (1,2,8,9). Neither isolate was able to overcome *Rvi4* like this new isolate R2.

Observations on the natural infection of the differential host set of *Malus* over four years in the research orchard of JKI proved the presence of virulences to scab resistance genes *Rvi1*, *Rvi2*, *Rvi3*, *Rvi4*, *Rvi5*, *Rvi6*, *Rvi7*, *Rvi8*, *Rvi9*, *Rvi10* and *Rvi13* (Table 1). No scab has been observed on hosts (11), (12), (14), (15) and (17) to date. Scab incidence was very low on hosts (4), (5), and low on hosts (2) and (13) (Table 1).

The virulence pattern of R2 raises the question of how this scab race has developed. It may have arisen from the sequential accumulation of mutations by a single strain, or have been the product from sexual mating between two isolates each carrying one of the virulences. Based on the *Rvi6* breaking lineage (Lemaire et al. 2016), current thinking assumes that mutations to virulence occurred several thousands of years ago. While the development and selection of virulences needs further elucidation, it is clear that the loss or mutation of many avirulence genes in the same isolate, as demonstrated here for R2, is possible. This, together with the indication that scab strains on average carry three virulences (Caffier et al. 2015), has a considerable impact on the strategy of pyramiding scab resistances. An understanding of what makes certain genes or gene combinations more durable than others is required for the long-term durability of resistance breeding itself, which may be enhanced by the possibility of creating new resistance genes through gene manipulation techniques, such as CRISPR/Cas9 (Charpentier and Marraffini 2014). In the meantime, the evaluation of virulence patterns of scab isolates for their presence and distribution by the VINQUEST project (www.vinquest.ch) provides some guidance of which resistance genes are the best candidates for achieving more durable resistance in gene pyramids based on the absence of the corresponding virulences in *V. inaequalis* populations in the different apple production regions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. The manuscript was prepared under compliance with ethical standards.

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