Effect of polyploidisation on the response of apple (*Malus × domestica* Borkh.) to *Venturia inaequalis* infection

Niek Hias*, nick.hias@kuleuven.be

Anze Svara*, anze.svara@kuleuven.be

Johan (Wannes) Keulemans, wannes.keulemans@kuleuven.be, +32 16 32 26 63**

***equal contribution**

****corresponding author**

Department of Biosystems, KU Leuven,

W. De Croylaan 42, 3001 Leuven, Belgium

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Abstract

Development of new and durable strategies to improve the apple plant's resistance against apple scab (*Venturia inaequalis*) remains a major challenge. Polyploids or organisms with three or more complete chromosome sets often possess properties superior to their diploid counterparts. Studies reported that polyploidy confers an increased resistance to biotic and abiotic stress factors. However, the potential use of polyploidy to increase disease resistance is still insufficiently investigated. We determined the influence of artificial genome doubling on the response of three *Malus × domestica* genotypes, with a variable level of susceptibility to apple scab. Based on visual symptom evaluation and real-time PCR quantification of the *V. inaequalis* DNA in apple leaves, an increased resistance was observed in the neotetraploid form of the monogenic resistant genotype compared to its diploid progenitor. No pronounced effects were observed comparing ploidy levels of the susceptible genotypes. These results suggest a potential role for polyploidisation in apple scab resistance, but it seems to

depend on the degree of disease susceptibility of the genotype. Still, further research on the effects of polyploidy might offer potential in the development of new and durable resistance strategies.

Key words: apple scab, *Venturia inaequalis*, *Malus × domestica*, non-model fruit crop, polyploidy, resistance

Introduction

Apple scab, caused by the ascomycete *Venturia inaequalis* (Cooke) Wint., is considered as the most destructive disease in apple culture (*Malus × domestica* Borkh.). If left untreated, an infection results in severe commercial yield loss, sometimes up to 70 percent (Bowen et al. 2011). Currently the main approach for controlling this disease is by multiple fungicide applications (Bus et al. 2011; Gessler and Pertot 2012). The long-term extensive application of these fungicides has, however, resulted in resistance of *V. inaequalis* populations to various fungicide classes (dodine, kresoxim-methyl, myclobutanil and thiophanate-methyl, benzimidazole, demethylation inhibitor and quinone outside inhibitor) (Hildebrand et al. 1988; Bowen et al. 2011; Chapman et al. 2011). Together with the increased amount of fungicide restrictions and limited development of new fungicides researchers are forced to develop alternative strategies to control apple scab (Bowen et al. 2011; Chapman et al. 2011). The development of scab resistant varieties, by introgression of disease resistance genes derived from wild *Malus* germplasm into *Malus × domestica* germplasm gained much importance and led to a number of scab resistant cultivars (Gessler et al. 2006; Bus et al. 2011). The breeding process is, however, very time consuming and entails several pseudo-backcrosses to eliminate undesirable traits (Gessler and Pertot 2012). Additionally, the predominant use of monoculture orchards stimulated the development of *V. inaequalis* races that have overcome this monogenic resistance (Bus et al. 2011). It has indeed been shown that the *Rvi6* resistance gene, which is the gene of choice in breeding for apple scab resistance, has been overcome by some pathotypes (Gessler and Pertot 2012).

One of the more durable solutions to reduce apple scab incidence suggested by MacHardy et al. (2001) is a combination of three practices (i) increasing host genetic diversity in orchards, based on a better understanding of apple scab race evolution in the orchard, (ii) advanced sanitation practices aimed at reducing the primary inoculum and (iii) strengthening the host's natural (horizontal) resistance (MacHardy et al. 2001).

An integrated approach incorporating these management options might significantly reduce the amount of necessary fungicide treatments and strengthen the concept of an environment-friendly approach in apple production (Holb 2007 and 2009). Nevertheless, a durable host resistance remains an important aspect in this

integrated pest management system. A more durable resistance frequently reported is quantitative or multigenic resistance, conditioned by a multitude of genes each conferring partial resistance (Dewdney et al. 2003; Didelot et al. 2016). Although partial resistance by itself does not confer complete resistance, integrated with other management options, such as improved and more specific fungicide spraying schedules, sanitation treatments, e.g. leaf removal, might significantly reduce the amount of necessary fungicide treatments (Holb 2007; Holb 2008; MacHardy 2001). Since multigenic or partial resistance against apple scab is difficult to breed for, alternative strategies to improve the horizontal resistance of apple plants might be very beneficial.

Polyploidy is ubiquitous throughout the plant kingdom, it is stated that nearly 70% of all angiosperms had experienced one or more episodes of polyploidy in their ancestry (Soltis et al. 2009). Obvious reasons for this high incidence is that ploidy confers several advantages over their diploid predecessors in adverse environments (Te Beest et al. 2012). It has been reported that in some cases increased resistance to pathogens can be linked to polyploidy. For instance, tetraploid *Trifolium pratense* is more resistant to clover rot than its diploid progenitor (Vestad 1960; Vleugels et al. 2013), and tetraploid *Glycine tabacina* was found to be more resistant to the leaf rust pathogen *Phakopsora pachyrhizi* than its diploid progenitor (Burdon and Marshall 1981). Recent hypotheses suggest that ploidy can influence plant defenses through an increased genetic adaptability towards pathogens through sub- or neofunctionalization in resistance genes and plant defense related genes (Innes et al. 2008; King et al. 2012). However, this theory mainly unfolds over an evolutionary scale and is less relevant for neo-autopolyploids. Nevertheless, other studies also suggest a potential role for neoploidy to influence the host-parasitic relation and induce resistance towards the pathogen (Stover and Buddenhagen 1986; Oswald and Nuismer 2007). Genome duplication can directly drive genetic modifications through gene silencing, and genetic or epigenetic interactions, able to significantly change the host-pathogen interaction (Vleugels et al. 2013). It has also been suggested that an additional genome is often associated with increased gene expression which in turn may generate a higher amount of proteins involved in plant immunity (Vleugels et al. 2013). Indeed, some studies could correlate these higher expression levels with increased resistance (De Godoy and Olsen 2008; Coate and Doyle 2010; King et al. 2012). King et al. (2012), however, stated that certain loci or even whole genomes (generally in allopolyploids) are up- or downregulated (or even silenced) as polyploidy increases, making it difficult to predict how polyploidy will affect gene expression. On the other hand studies comparing gene expression levels between artificially produced polyploids with their progenitor proved only a limited differential gene expression, but could still correlate it with significant anatomical and

physiological changes (Yu et al. 2010; Allario et al. 2011; Tan et al. 2015) and increased abiotic resistance (del Pozo and Ramirez-Parra 2014; Allario et al. 2013). In a tetraploid citrus rootstock compared to its diploid progenitor, polyploidy had a limited effect on the transcriptome, nevertheless the metabolic profile showed an upregulation of many stress-related metabolites in tetraploid plants; suggesting a clear potential for artificial polyploidy to strengthen the host's natural resistance (Tan et al. 2015). To conclude, there is evidence that ploidy-induced phenotypic and physiological changes significantly alter the interaction between the host and the pathogen (Segraves and Anneberg 2016). Nevertheless, the amount of studies comparing neopolyploids with their diploid progenitor are still very limited. We report on the comparison of three tetraploid apple genotypes with their diploid progenitors for their resistance against apple scab. The research might offer an alternative technique for improving host resistance to apple scab and permits us to shed some more light on the topic of ploidy and biotic resistance.

Material and methods

Plant material

Based on a previous scab resistance screening experiment, three diploid genotypes (G40, 'Gala' and G58) were selected each showing a different susceptibility level to apple scab (Table 1). G58 contains the *Rvi6* resistance gene-locus, which was confirmed by markers (AL07-SCAR and M18-CAPS; Tartarini et al. 1999), Based on our visual observations G58 was classified in Chevalier class 2, characterized by chlorosis and necrotic lesions considered resistant against *V. inaequalis* race 1. During the same screening experiment 'Gala' and G40 could be classified respectively in Chevalier class 3b, susceptible and class 4, very susceptible.

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Tetraploid plants were obtained after chromosome doubling in tissue culture by stirring axillary buds during 2 days in a MS multiplication medium supplemented with colchicine according to Hias et al. (2017).

Ploidy analyses were performed after induction of polyploidy in tissue culture and, as a second control, on the rooted greenhouse plants on a flow cytometer Cyflow Space (Partec, Münster, Germany) as described by Dhooghe et al. (2009). After *ex vitro* acclimatisation, plantlets were transported to the greenhouse and grown under controlled greenhouse conditions: a day/night temperature 16/14 °C and a day/night relative humidity (RH) of 70/60%. Artificial lighting (25 W/m²) from high-pressure sodium lamps (400-W SON-T-AGRO, Philips, Amsterdam, Nederland) during the day was applied when the light intensity dropped below 100 W/m².

After artificial genome doubling, di- and tetraploid plants of these three genotypes were compared in two experiments: April 2014 and May 2015.

For each infection experiment scions were collected near the end of the winter dormancy period from dormant G40 and 'Gala' genotypes and grafted on M.9 rootstocks. Because of acclimatisation issues during the first year of *ex vitro* growth, shoot growth of genotype G58 was limited and no scions could be collected for the experiment in April 2014 and own-rooted plants were used. In 2014 shoot growth for G58 was normal and scions were collected and grafted on M.9 for the experiment in May 2015. All plants were left to grow in a 2,5 L container with ready-made soil (DCM Pepi 3, Grobbendonk, Belgium) for one month under controlled greenhouse conditions as mentioned above. One week before the inoculation plants were placed according to a completely randomized block design. Thereafter, depending on the weather conditions, the most optimal time point for inoculation was chosen.

Fungal material and artificial inoculation

In order to determine the effect of polyploidisation on the response to apple scab in a susceptible and resistant variety all inoculation experiments were performed using *V. inaequalis* strain 104, the reference strain of race (1). Race (1) is chosen, because it is avirulent to all hosts carrying any resistance gene with exception of the *Rvi1* resistance gene (Bus et al. 2011). Hence G58, which is an *Rvi6* monogenic resistant genotype will exhibit an incompatible interaction, while both 'Gala' and G40 will have a compatible interaction.

V. inaequalis spores for the conidial inoculum were collected from previously infected leaves, dried and stored at -20 °C as described by Daniëls et al. (2012). All apical shoot leaves were spray-inoculated on the adaxial leaf side until run-off with a *V. inaequalis* conidial suspension at a concentration of 2×10^5 spores/ml (diluted in distilled water and 0.01% of Tween 20). Inoculation was performed on minimum five to maximum ten, a month-old actively growing apple shoots. To standardize future visual evaluation and sampling, the youngest fully unfolded leaf was labelled 0, the second leaf 1 and leaf younger than leaf 0 (not yet fully unfolded) was labelled -1. Optimal germination conditions were achieved by incubating the plants for 48h in the dark in a micro-environment with 16 – 20 °C and 95 – 100% RH, to ensure constant leaf wetness. This micro-climate was achieved by setting up an inoculation tunnel with plastic foil and active air humidifiers (Boneco 7135, Widnau, Switzerland). To discriminate for symptoms possibly caused by abiotic stress under the tunnel, mock-inoculated plants (sprayed only with water and Tween 20) were included in each experiment. After the incubation, plants

were placed under controlled greenhouse conditions as previously described with continuous sprinkling for 15 seconds every 5 minutes for 10 consecutive days to ensure leaf wetness.

Visual evaluation

Quantification of the susceptibility is based on macroscopic symptom evaluation every second days, starting from 5 days post inoculation (dpi) until approximately 25 dpi of the three leaves labelled before the inoculation. Leaf symptoms were evaluated according to three characteristics i.e. chlorosis, sporulation, and necrosis. Sporulation was determined as a percentage of leaf surface and divided into 8 classes (classes 0–7: respectively 0, 0–1, 2–5, 6–10, 11–25, 26–50, 51–75, >75%), while the degree of chlorosis as a percentage of leaf surface is divided over 4 classes (classes 0–4: respectively 0, 1–25, 26–50, 51–75, >75%) according to Croxall et al. (1952) and modified by Parisi et al. (1993). Additionally, to the experiment of April 2014, during the May 2015 experiment, we evaluated, in addition to the amount of chlorosis and sporulation, necrosis, using the similar grading scale of 8 classes.

All three classifications (chlorosis, sporulation, and necrosis) were integrated via the Townsend-Heuberger formula (i) for each plant at each time point. As such the overall symptom severity per plant can be determined (Townsend and Heuberger 1943; Juhásová et al. 2004) was calculated.

$$TH = \frac{\sum n_i \cdot i}{N \cdot I} \cdot 100\% \quad (i)$$

Where:

n_i = number of leaves in class i

i = class (0-I)

N = total number of leaves

I = number of symptom showing classes

Leaf sampling, DNA extraction and real-time PCR for the quantification of *V. inaequalis* in infected leaves

At the designated time points all three labelled leaves (-1, 0 and 1) of five plants for each condition and genotype were harvested individually and stored at -80 °C until further processing. Before DNA extraction, leaves were lyophilized (Freezone 4.5, Labconco) and the whole leaf was grinded. Total genomic DNA (gDNA) was

extracted from 20 – 30 mg lyophilized leaf tissue (from each labelled leaf separately) using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. DNA concentrations and purities were determined (NanoDrop 2000c, Thermo Scientific).

Real-time PCR analysis was performed on each labelled leaf of the five plants per condition and genotype according to the protocol developed by Daniëls et al. (2012). Fungal DNA was quantified by means of a SYBR Green assay with external fungal and plant gDNA standard curves. Normalization was performed to a plant housekeeping gene coding translation initiation factor eIF-4A (Genbank ID: AY347787; Zubini et al. 2007).

Statistics

Statistical analyses were performed with the statistical software JMP® Pro (version 12.1.0). To determine significant differences in pathogen DNA and symptoms, results were subjected to the Wilcoxon each pair test.

Results

Inoculation experiments with *V. inaequalis* were performed in April 2014 and May 2015 on two susceptible (G40 and 'Gala') and one resistant genotype (G58). For both experiments, symptoms were visually evaluated, while in the second experiment growth of the fungus was additionally confirmed by real-time PCR.

Visual evaluation

Foliar disease symptoms for the two levels of ploidy of the *Rvi6*-resistant genotype G58 and scab susceptible genotypes 'Gala' and G40 were evaluated up to 25 dpi. Results are expressed as the mean TH-values for all individual plants per genotype and ploidy level describing the amount of chlorosis and sporulation for the susceptible varieties and only chlorosis for the *Rvi6*-resistant genotype, since no sporulation was observed. During the May 2015 experiment also the degree of necrosis was evaluated for all genotypes and ploidy levels. During the April 2014 experiment at 6 dpi few chlorotic symptoms could be observed on both ploidy levels of the *Rvi6*-resistant genotype G58 (Figure 1a). Between 6 and 10 dpi there is a steep increase in chlorotic lesions on leaves of both diploid (G58-2x) and tetraploid (G58-4x). At 10 dpi there is a relatively higher degree of chlorosis in G58-2x compared to its tetraploid variant G58-4x. Between 10 and 22 dpi chlorosis is slowly increasing for G58-2x, while for G58-4x a plateau has been reached at 10 dpi, resulting in a 15% higher degree of chlorosis on the leaves of G58-2x plants.

In May 2015 a similar trend has been noticed; a steep increase between 5 and 10 dpi with a significantly higher degree of chlorosis on the leaves of G58-2x from 12 dpi on (Figure 1a). The difference is however limited to around 5% and both ploidy levels approximately stabilize after 15 dpi. There is a noticeable difference in severity between 2014 and 2015, especially the tetraploid plants were more affected in May 2015 compared to April 2014.

In May 2015 the amount of necrosis was determined throughout the experiment (Figure 1b). For both G58-2x and G58-4x necrotic lesions appeared between 8 and 10 dpi, followed a steep increase between 10 and 12 dpi and reached a plateau after 15 dpi. Although almost no significant differences were observed between the ploidy levels, Figure 1b shows a trend towards a higher amount of necrosis in the leaves of the diploid variant, with significantly higher degree (6%) in G58-2x at 20 dpi. In addition to the higher amount of chlorosis and necrosis compared to the tetraploid leaves, the diploid leaves also displayed characteristic chlorotic ‘dents’ in the leaf, which were less or almost completely absent on the tetraploid plants (Figure 2).

To investigate the influence of leaf age on the susceptibility to the fungus, the TH-Chlorosis and TH-Necrosis values were plotted for the three susceptible leaves near the end of the May 2015 infection experiment (22 dpi, Figure 1c, d). For both G58-2x and G58-4x the oldest leaf (leaf 1) exhibited the lowest susceptibility. Differences between the G58-2x and -4x plants generally increased with leaf age and were predominantly associated with the oldest leaf. Similar results were obtained for the April 2014 experiment (Supplemental Figure 2).

During the April 2014 experiment, chlorosis increased steeply between 5 and 10 dpi, for ‘Gala’-2x and -4x (Figure 3a). At 10 dpi ‘Gala’-4x manifested a significantly increased area of chlorosis relative to ‘Gala’-2x and reached a peak at 20 dpi with approximately 20% difference between ‘Gala’-2x and ‘Gala’-4x. After 20 dpi less chlorosis was visible due to increasing sporulation which covered the chlorotic lesions. A similar change could be observed for the evolution of sporulation, which increased sharply between 10 and 15 dpi followed by a plateau (Figure 3c). From 15 dpi on ‘Gala’-4x leaves showed 20% more leaf surface with sporulation compared to ‘Gala’-2x.

For Gala, the experiment in May 2015 could however not confirm the differences observed in April 2014 and both ploidy levels expressed largely the same degree of symptoms (Figure 3a). Both chlorosis and sporulation increased sharply between 8 and 10 dpi. The amount of chlorosis reached a peak at 20 dpi, while sporulation kept increasing and no plateau was reached at the end of the experiment.

In 2014, similar to ‘Gala’, chlorosis increased sharply between 5 and 10 dpi for both ploidy levels of G40 (Figure 3b). At 16 dpi chlorosis reached a peak and started to decline, probably due to the masking of chlorotic spots by the increasing sporulation. Between both ploidy levels no significant differences were observed. Describing the visual trends, G40-2x exhibited marginally more chlorosis than G40-4x. However, this was much more pronounced in the experiment of May 2015. Starting from 12 dpi diploid plants exhibited a significantly increasing amount of chlorosis compared to G40-4x variant, with a maximum difference of almost 40% at 20 dpi (Figure 3b). In both experiments, similar to ‘Gala’, sporulation increased sharply between 5 and 10 dpi, however no significant differences could be observed between the ploidy levels (Figure 3c, d).

During the May experiment necrosis was evaluated for both ploidy levels of ‘Gala’ and G40. Necrosis increased for both ploidy levels after 15 dpi. At 20 dpi G40-2x accumulated 25% more necrosis due to the delayed response of the tetraploid variant (Figure 3e).

Real-time PCR evaluation

During the experiment of May 2015 samples for real-time PCR were collected during three time points, which were chosen based on the symptom evolution graphs of the April 2014 experiment (Figures 1a and 3a-d). These time points include a latent phase where no symptoms are observed (5 dpi), a growth phase associated with a steep increase in symptoms (10 dpi) and a time point near the end, when symptoms are around their maximum (25 dpi).

Between 5 and 10 dpi fungal DNA increased 6-fold for G58-2x, and 12-fold for G58-4x. This increase coincides with the steep increase in chlorosis. Between 10 dpi and 25 dpi G58-2x still exhibited a 3-fold increase in fungal DNA, while no increase was observed in G58-4x. Comparing di- and tetraploid plants of G58, no significant differences were observed at the early time points (5 and 10 dpi, Figure 4a). The last time point, however, shows a substantially higher amount of fungal DNA in the G58-2x.

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268 For both ploidy levels of the susceptible genotypes, a significant amount of fungal DNA was already present at 5
269 dpi (Figure 4b). This amount increased approximately 50-fold between 5 and 10 dpi, coinciding the steep
270 increase in chlorosis and start of sporulation, and 10-fold between 10 and 25 dpi for 'Gala'-2x and 'Gala'-4x.
271 Relative concentration of fungal DNA in G40 leaves increased around 30-fold between 5 and 10 dpi and 7-fold
272 between 10 and 25 dpi. Although the increase in fungal DNA was steeper in 'Gala', comparing the ploidy levels
273 for both 'Gala' and G40 revealed no significant differences in the amount of fungal DNA in the leaves.

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275 Discussion

276 In this study we investigated the influence of neopolyploidy in apple on the response to apple scab of two
277 susceptible genotypes and one *Rvi6* resistant genotype; their di- and tetraploid form were visually and
278 molecularly characterized after a controlled inoculation with *V. inaequalis* conidiospores.

279 It was hypothesized that due to a potential increase in gene expression, an up-regulation of stress-related
280 metabolites and an altered morphology, neopolyploids are able to confer a higher biotic resistance compared to
281 their diploid progenitors (Segraves and Anneberg 2016; Vleugels 2013). Research on this topic has nevertheless
282 been very limited (Segraves and Anneberg 2016).

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284 The phenotypic evaluation, through a visual and a real-time PCR based quantification showed no consistent
285 influence of genome doubling on the investigated susceptible genotypes G40 and 'Gala'. Questionable trends
286 were an increased chlorosis and sporulation in 'Gala'-4x relative to 'Gala'-2x and increased sporulation in G40-
287 4x relative to G40-2x observed in April 2014. This would suggest chromosome doubling in the susceptible
288 genotypes resulted in an increased susceptibility to the fungus, but this visual evaluation could not be repeated in
289 May 2015, and was not confirmed by real-time PCR based quantification. Based on these observations we
290 assume that polyploidisation did not have an influence on the response to apple scab in our susceptible
291 genotypes.

292 The response of the resistant genotype G58, on the other hand, was significantly affected by the polyploidisation
293 event. Tetraploid plants of G58 were found to show less symptoms (chlorosis) and limited growth of the fungus
294 compared to the diploid G58 plants.

295 Our observations are not fully consistent with other studies on polyploid plants who point towards an increased
296 resistance to pathogens (Segraves and Anneberg 2016; King et al. 2012). As mentioned earlier autotetraploid *T.*

297 *pratense* was found more resistant to clover rot (*Sclerotinia* spp.) than its diploid progenitor (Vleugels 2013;
 298 Öhberg et al. 2005) and tetraploid *G. tabacina* was found to be more resistant to the leaf rust pathogen *P.*
 299 *pachyrhizi* than its diploid progenitor (Burdon and Marshall 1981). However this pattern of increased resistance
 300 is not always observed. Tetraploid *Glycine tomentella* showed similar resistance to soybean leaf rust
 301 (*Phakopsora pachyrhizi*) compared to diploid plants (Schoen et al. 1992). Most studies comparing the influence
 302 of polyploidy on disease resistance also focussed on established auto- and allopolyploid plants where the
 303 difference in resistance between the ploidy levels can, in addition to the direct consequences of genome
 304 duplication itself, also be caused by evolutionary genetic changes following genome duplication (King et al.
 305 2012; Segraves 2016). All these results combined fit with the opinion formulated by King et al. (2012) who
 306 stated that the interaction between polyploidy and disease resistance remains very complex.
 307
 308 Still our results suggest that polyploidy has an influence on the resistance, the effect however, seems to depend
 309 on the sensitivity of the genotype. But in order to check this genotypic dependence the experiment should be
 310 repeated with more apple genotypes. Between the two experiments we also observed a substantial difference in
 311 infection levels, i.e. the highest levels were observed in April 2014 followed by May 2015. According to Turner
 312 et al. (1986) and Lian et al. (2007), this variance can be explained by the influence of temperature. Based on the
 313 optimum germination and growth temperatures for *V. inaequalis* (16 – 20 °C) (Ross 1974; MacHardy 1996) and
 314 the temperature data of the greenhouse we observed that the experiment in April 2014 spend a longer time in this
 315 optimal growth temperature range compared to the experiment in May 2015 (Supplemental Figure 1).
 316 Considering the resistant genotype G58 this temperature effect will be less important since the defence response
 317 initiated after recognition has an even more limiting effect on growth. Although, we did not investigate the
 318 mechanisms behind the altered tolerance of genotype G58, some hypotheses could be drawn based on our
 319 observations and the literature. One hypothesis is the potential gene-dosage effect of the *Rvi6* resistance gene. It
 320 has indeed been shown that genotypes homozygous for the *Rvi6* gene confer a higher tolerance to apple scab
 321 than heterozygous genotypes (Belfanti et al. 2004). It could of course be argued that the increased amount of
 322 gene product due to the gene doubling is compensated by an increase of the cell volume. Then again the *Rvi6*
 323 gene is known to encode an extracellular receptor with a transmembrane region and the membrane surface does
 324 not increase at the same amount as the cell volume, e.g. doubling the volume of a sphere results only in a $1.6 \times$
 325 larger surface area. If this is the case, then a doubling of the gene product effectively results in a higher
 326 concentration of receptors on the membrane able to recognize the pathogen. Counter arguments are however the

signal cascade in the cytoplasm, where the ploidy-induced increased amount of gene products are compensated by the increased cell volume and the irregular cell shapes, which makes it difficult to evaluate how the cell surface area changes due to genome doubling. However further research is needed to clarify the above hypothesis. In the case of our susceptible cultivars which did not exhibit an increased resistance to apple scab after genome duplication, experiments are being set up to investigate if a combination of defense induction through elicitors and ploidy has a similar effect as observed in the tetraploid *Rvi6* resistant genotype.

In all apple genotypes an increase in resistance with an increasing leaf age (ontogenic resistance) also plays an important role in inhibiting fungal growth (Gessler et al. 2006; Gusberti et al. 2013). A theory based on this ontogenic development, is a potentially faster leaf maturation in the tetraploid plants, reaching the ontogenic resistance stage earlier than the diploid plants. During both experiments in May 2014 and April 2015 the difference in symptom manifestations between the di- and tetraploid G58 plants was mainly limited to the oldest leaf. Also the symptom progression rate of the tetraploid G58 plants decreased substantially after 10 dpi, the latter observation was also confirmed with real-time PCR. This could mean that after 10 dpi fungal growth in the tetraploid G58 plants is inhibited by the cumulative effect of ontogenic resistance and *Rvi6* induced resistance. This supposedly ploidy-induced ontogenic effect is however not observed in the investigated susceptible genotypes. A possible explanation is the plant's response to the genome doubling, which for various characteristics, such as leaf morphology and growth proves to be significantly depending on the genotype (Riddle et al. 2006; Hias et al. 2017).

For now we can only speculate on the reasons behind an altered resistance and this has to be investigated further. Nevertheless based on the available literature on different crops, polyploidy works on potentially increasing resistance against pathogens on multiple fronts (physiology, morphology and genetics (Segraves and Anneberg 2016; King et al. 2012; Vleugels et al. 2013). All partially contributing to a final resistance, the pathogen is less able to break through, as opposed to monogenic resistant varieties. This kind of resistance resembles to some extent the frequently reported partial resistance, which is considered more durable than a monogenic one (Didelot et al. 2016). Combined with other apple scab management strategies (e.g. sanitation, increasing host diversity in the orchard) polyploidy could significantly limit primary apple scab inoculum, reduce the amount of frequent fungicide treatments and significantly improve environmentally-friendly apple production and could also fit in integrated or organic orchards (Holb 2009).

In conclusion, it seems fairly evident that genome doubling has a genotype dependent influence on the susceptibility to apple scab. In our study, investigated neopolyploids of susceptible genotypes were analysed but were not or arguably affected by the polyploidisation event and further research is necessary to make solid conclusions whether all susceptible genotypes react in the same way. On the other hand, the tetraploid variant of the *Rvi6* resistant genotype proved to significantly limit symptom expression and fungal growth compared to their diploid progenitor. Possible explanations for this altered resistance are the effect of gene-dosage and/or an earlier onset of ontogenic resistance. Although there is still a lot to be confirmed and investigated, overall polyploidisation seems to offer a great potential in strengthening the host natural plant defense response, which is one of three earlier mentioned goals that are strived for to reduce apple scab incidence. Provided knowledge and incorporation of artificial polyploidisation in apple breeding programs might offer great potential towards reducing the amount of fungicide sprays.

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Table 1 Genotypes with corresponding apple scab resistance level and symptom expression, determined on diploid plants in previous screening experiments (results not shown).

Genotype	Resistance level	Symptoms
G40	very susceptible	chlorosis, heavy sporulation, necrosis
'Gala'	susceptible	chlorosis, moderate sporulation, necrosis
G58	resistant (<i>Rvi6</i>)	chlorosis, denting, very rare sporulation

Fig. 1 a Evolution of the mean TH-Chlorosis values for the di- (2x) and tetraploid (4x) variant of the *Rvi6*-resistant genotype G58 for two independent experiments: April 2014 and May 2015 (n = 5 – 10; $\pm$ SE; * p < 0.05; ** p < 0.01). **b** Evolution of the mean TH-Necrosis values for the di- (2x) and tetraploid (4x) variant of the *Rvi6*-resistant genotype G58 for May 2015 (n = 5 – 10; $\pm$ SE; * p < 0.05; ** p < 0.01). **c** Mean TH-Chlorosis

values for the youngest leaf (-1), one leaf older (0) and the oldest leaf (1) for the di- (2x) and tetraploid (4x) variant of G58 at 20 dpi for the experiment in May 2015 ($n = 7-10$; $\pm SE$; * $p < 0.05$; ** $p < 0.01$). **d** Mean TH-Necrosis values for the youngest leaf (-1), one leaf older (0) and the oldest leaf (1) for the di- (2x) and tetraploid (4x) variant of G58 at 20 dpi for the experiment in May 2015 ($n = 7-10$; $\pm SE$; * $p < 0.05$; ** $p < 0.01$)

Fig. 2 Pictures from inoculated leaves during thescab experiment in April 2014. Symptom expression on the leaves of the di- (2x) and tetraploid (4x) variant of the *Rvi6*-resistant genotype G58 and the susceptible genotype 'Gala'

Fig. 3 a-d Symptom manifestation in the susceptible genotypes 'Gala' and G40 for two independent experiments: April 2014 and May 2015 (n = 6 – 10; $\pm$ SE; * p < 0.05; * * p < 0.01). **a** Evolution of the mean TH-

Chlorosis values for the di- (2x) and tetraploid 4x variant of the susceptible cv 'Gala'. **b** Evolution of the mean TH-Chlorosis values for the di- (2x) and tetraploid 4x variant of the susceptible genotype G40 for two independent experiments. **c** Evolution of the mean TH-Sporulation values for the di- (2x) and tetraploid 4x variant of the susceptible cv 'Gala' for two independent experiments. **d** Evolution of the mean TH-Sporulation values for the di- (2x) and tetraploid 4x variant of the susceptible genotype G40 for two independent experiments. **e** Evolution of the mean TH-Necrosis values for the di- (2x) and tetraploid (4x) variant of the susceptible genotype G40 for May 2015 (n = 6 – 10; $\pm$ SE; * p < 0.05; ** p < 0.01)

Fig. 4 Amount of *Venturia inaequalis* DNA determined with real-time PCR in infected leaves of the di- (2x) and tetraploid (4x) variants **a** of the *Rvi6*-resistant genotype G58 at 5, 10 and 25 dpi for May 2015 (n = 5; $\pm$ SE; * p < 0.05; ** p < 0.01) and **b** of the susceptible genotypes 'Gala' and G40 at 5, 10 and 15 dpi for May 2015 (n = 5; $\pm$ SE; * p < 0.05; ** p < 0.01)

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527 **Supplemental Fig. 1** Boxplots of mean daily temperature for the experiments in April 2014, July 2014 and May

528 2015

Supplemental Fig. 2 Mean TH-Chlorosis values for the youngest leaf (-1), one leaf older (0) and the oldest leaf (1) for the di- (2x) and tetraploid (4x) variant of G58 at 20 dpi for the experiment in April 2014, July 2014 and May 2015 (n = 7–10; $\pm$ SE; * p < 0.05; ** p < 0.01)