Hungarian University of Agriculture and Life Sciences

Improvement of methods for resistance development against grape gray mould and black rot; role of stilbenes in resistance development

PhD thesis

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Gödöllő
2023

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INTRODUCTION AND OBJECTIVES

One of our most ancient agricultural crops is the vine, of which the largest part under cultivation is the *Vitis vinifera* L. grape, which is subject to a number of biotic stresses.

Resistance studies targeting fungal and oomycotic diseases are mainly focused on the biotrophs *Erysiphe necator* Schwein (grape powdery mildew) and *Plasmopara viticola* (Berk & Curtis) Berl. & De Toni (grape peronospora) and the necrotroph *Botrytis cinerea* Pers.

However, in recent decades, extreme weather conditions associated with climate change and the use of varieties resistant only to powdery mildew and downy mildew have all contributed to the increased emergence of secondary pathogens in vineyards, such as *Guignardia bidwellii* (Ellis) Viala et Ravaz, which causes black rot. An epidemic level of this hemibiotrophic ascomycete fungus was observed in Hungary in 2010 and 2014, causing significant yield losses mainly in organic and environmentally friendly cultivation without any chemical control.

Today, the need to take into account sustainable farming and adaptation to a changing climate in grapevine breeding is becoming increasingly urgent. This may require, on the one hand, exploiting or enhancing the natural resistance of the grapevine, exploring the biological basis of fungal-pathogenic relationships and, on the other hand, breeding new resistant varieties to traditional varieties.
In clonal selection, we can ensure yield security and wine quality by selecting progeny with favourable characteristics and resistance to various pathogens, thus contributing to the improvement and survival of grape varieties. On the other hand, with advances in biotechnology, it is now possible to inhibit pathogenic fungi and oomycetes by enhancing the expression of plant defence genes such as stilbene synthases and to increase protection by silencing plant disease

susceptibility genes. Secondary metabolites, such as stilbenes, which are considered biomarkers of resistance to pathogenic fungi in grapes, play a key role since they may confer preformed resistance as phytoanticipins or increase in abundance in response to infection as phytoalexins.

Due to the low support for transgenic plants, the aim is also to achieve resistance through genome editing techniques such as CRISPR/Cas9-based methods.

In the present work, I wanted to contribute to the control of some of the species causing fungal diseases of grapes, necrotrophs, biotrophs and especially hemibiotrophs. For the development of the biological basis for resistance breeding, the following specific objectives have been formulated.

1. To investigate control options against necrotrophic *Botrytis cinerea*, I planned to evaluate two less susceptible clones 'Juhfark' to gray mould in a clonal selection experiment.
2. To analyze the relationship between resistance to the hemibiotrophic pathogen of grapevine black rot and stilbenes, I set myself three objectives.
   2.1. Quantification of stilbene forms from healthy and infected leaves on plants grown under laboratory conditions.
   2.2. Determination of stilbene levels from the leaves of black rot resistant and susceptible cultivars in field experiments.
   2.3. Examination of stilbene levels and resistance in progeny derived from crosses between black rot resistant and susceptible genotypes. To verify the extent of the relationship between traits (stilbene content and black rot).
3. To analyse the RNA level response to infection with the hemibiotrophic pathogen of black rot, I set two objectives.
   3.1. Identification of genes showing differential expression changes in susceptible and resistant varieties in response to black rot infection.

3.2. Validation of genes associated with putative resistance to black rot in RT-qPCR expression experiments

4. In preparation for studies on the silencing of genes potentially involved in disease resistance, I also plan to:
   4.1. create a CRISPR construct to silence a few selected plant genes involved in the disease process
   4.2. develop an embryogenic callus induction from grapevine leaves and grapevine anthers for a CRISPR experiment

MATERIALS AND METHODS

Susceptibility to *Botrytis cinerea* in clones of the 'Juhfark' cultivar

In the clonal selection experiments, two selected clones of *Vitis vinifera* cv. 'Juhfark' (B.1. and B.2.) were grown at the Hungarian University of Agriculture and Life Sciences, Institute of Viticulture and Enology research station in Badacsony, Hungary.

12 years (2011–2022) of meteorological data (daily minimum, maximum and mean temperature; daily precipitation), the key phenological stages: bud burst (BBCH 09, EL 5), beginning of flowering (BBCH 61, EL 19), end of flowering (BBCH 69, EL 26), veraison (BBCH81, EL 35) harvest (BBCH 89, EL 38) and the rate of the *Botrytis cinerea* infection during the whole harvest were recorded. All data analysis were carried out using the R software package.

Examination of stilbene levels in pot and field experiments

The effect of the black rot pathogen on quantity of a different stilbene forms (trans-resveratrol, cis-resveratrol, trans-piceid, cis-piceid, trans-pterostilbene, trans-ε-viniferin, cis--ε-viniferin) was investigated in a black rot resistant interspecific hybrid 'Csillám' and a susceptible 'Csaba gyöngye' *Vitis vinifera* cultivar.

We obtained 'Csillám' from the Kecskemét Research Station of the NARIC Research Institute for Viticulture and Oenology and 'Csaba gyöngye' from Balatonboglár, and their two-bud cuttings were grown perlite for 3.5 months at 25 °C with 16 hours of light period in the growth chamber of the Department of Soil Science at Hungarian University of Agriculture and Life Sciences.

The isolate of *Guignardia bidwellii* was obtained from the Institute of Viticulture and Enology of Eszterházy Károly University, and was maintained for 4 months in oatmeal containing Furmint green berry juice at 25 °C under constant fluorescent illumination (50 % Tungsram 36 W F7 Daylight és 50 % Sylvania 36W T8 F Black light blue (UV-A)).

For infection, ~2×10^5 spores/ml of suspension were evenly applied to the top young leaves with sterile brushes, while control leaves were sprayed with sterile distilled water. Leaf samples were collected in 3 biological replicates at 6, 18, 36 h after inoculation and stored at -80 °C. Infection was validated on trypan blue stained leaf samples by light microscopy.

In field experimental grapevines, no artificial infection was carried out, and the levels of the following stilbenes (trans-resveratrol, cis-resveratrol, trans-piceid, cis-piceid, trans-pterostilbene, trans-ε-viniferin, cis-ε-viniferin, trans-ω-viniferin, cis-ω-viniferin, α-viniferin, trans-piceatannol) were studied.

From the vineyard of the Research Institute of Viticulture and Oenology of the University of Pécs, Szentmiklóshegy Experimental Station, different black rot resistant and susceptible grape varieties were selected at the beginning of flowering (BBCH 61, EL 19), the first to second 2,5-3 cm (small, "3 cm"), fourth to fifth 4,5-5,5 cm (large, "5 cm") diameter leaves were taken from the growing tip in 3 biological replicates.

We also studied the segregating generation of the parents of ’Csillám’ × ’SK001/7’ and ’Csillám’ × ’01-1-797’, which included black rot resistant (109, 147, 180, 187, 192) and susceptible (83, 106, 112, 113, 127, 163, 213, 234) hybrids, with biological replicates being each individuals.

Leaves >7.5 cm in diameter were also selected in the case of ’Csillám’, SK001/7, 01-1-797. The leaf samples were snap frozen in liquid nitrogen and stored at -80 °C. The measurement of stilbene levels was carried out by the

Department of Plant Biology of the Agricultural Research Institute of the Hungarian Academy of Sciences in Martonvásár, Hungary using UPLC-MS/MS.

Validation of the differentially expressed genes putatively involved in black rot resistance

To compare gene expression changes in response to *Guignardia bidwellii* infection, we worked with the black rot resistant ’Csillám’ and the susceptible 'Csaba gyöngye'.

Based on previous results from bioinformatics analysis of RNA sequencing (Kellner, 2022), I performed quality control of genes with differential expression at the ($p<0.01$) significance level of 6, 18, 36 hpi and selected some of them, which were subjected to RT-PCR to confirm the results.

For this reason, we designed primers for 8 selected genes (*Vv*STS10, *Vv*STS20, *Vv*STS21, *Vv*PAL12, *Vv*OPR2, *Vv*WRKY, *Vv*RPM1, *Vv*DMR6).

The grapevines were obtained from the Research Station of the NARIC Research Institute of Viticulture and Enology, Kecskemét, Hungary. The two-bud cuttings were planted in a 1:1 mixture of general potting soil and perlite and grown for 6 months at 21°C with 16 h of light period in the growth chamber of the Department of Soil Science at Hungarian University of Agriculture and Life Sciences.

The fungal culture of black rot was provided by the Research Institute of Viticulture and Enology of the University of Pécs. Cultures were incubated on PDA (potato dextrose) medium under constant fluorescent light (50% Tungsram 36 W F7 Daylight and 50% Sylvania 36W T8 F Black light blue (UV-A)) at 25°C for 41 days.

For infection, we used the half-leaf method based on Kellner et al. (2014), filter paper discs were soaked in a suspension of 10^4-10^5 spores/ml, while sterile distilled water discs served as controls.

Leaf samples were collected in 3 biological replicates at 6, 18, 36 hours post-infection and RNA was extracted using the modified CTAB-based method of Gambino (2008).

The RNA samples were digested with DNase and cDNA was synthesized then amplified the genes of interest by real-time PCR.

For relative quantification of gene expression, the comparative ΔΔCT method was used (Schmittgen and Livak, 2008). Statistical analysis of real-time PCR results was performed using GraphPad Prism software. Infection was validated by light microscopy on leaf samples stained with trypan blue (Várallyay et al., 2012).

Creation of CRISPR/Cas9 construct and induction of embryogenic calluses

CRISPR constructs were designed and embryogenic calluses were produced to test resistance to powdery mildew and black rot in *Vitis vinifera* cv. 'Furmint' by silencing MLO genes (*Vv*MLO6, 7, 11, 13) and DMR6 gene.

To design the guide RNAs, the coding region and mRNA sequences were extracted from the NCBI GenBank and the University of Padova V2.1 annotated database (https://genomes.cribi.unipd.it) and aligned to the 12x grapevine reference genome, the V2.1 annotated genome and our sequenced 'Furmint' genome using NCBI Blast.

Based on the Interpro database (https://www.ebi.ac.uk/interpro/), we selected protein domains that are essential for the function of MLO, DMR6 and designed guide RNAs using Geneious software.

The CRISPR constructs were generated using the pUC gRNA shuttle (Addgene: 47024) and p201N:Cas9 (Addgene: 59175) plasmids.

The MtU6 promoter and scaffold regions were amplified from the pUC gRNA shuttle vector and the MtU6 promoter, gRNA and scaffold sections were assembled using NEBuilder HiFi DNA Assembly.

To link the resulting gRNA cassettes and ligate them into the p201N-Cas vector, 5' and 3' overhanging ends were generated using Q5 polymerase.

We ligated our inserts, i.e. one gRNA cassette or the linked 2-3 gRNA cassettes, into the p201N-Cas vector, transformed them into a 10-beta strain of *E.coli* and cultivated these cells into media.

Colony PCR was used to verify the success of ligation in the clones, followed by further quality screening using Sanger sequencing, the results of which were evaluated using Geneious software.

Embryogenic callus induction was performed using *Vitis vinifera* cv. 'Furmint' anthers and leaves. The grape inflorescences were collected just before flowering from a plantation in Bicske, Hungary (GPS: 47°28'21.3"N 18°41'03.2"E). The leaves were derived from in vitro Furmint obtained from the NARIC Research Institute of Viticulture and Enology in Kecskemét, Hungary.

Dissected anthers from disinfected inflorescences were tested on MSE/2 and MST/2 media at 22+/-2 °C in the dark according to Oláh and coworkers (2009). Leaf sections were started on ½ MS medium containing 1-naphthyl acetic acid (NAA) and 6-benzylaminopurine (BAP) and on ½ MS medium containing indole-3-acetic acid (IAA), kinetin and incubated 16 h light /8 h dark periods at 22+/-2 °C under Polylux XL 36 W and Tungsram F7 Daylight 36 W light. Embryogenic capacity was tested on MSE/2 media supplemented with activated charcoal.

RESULTS

Botritisz ellenállóság vizsgálata szabadföldi ’Juhfark’ ültetvényben

The aim of the ’Juhfark’ clonal selection was to produce a looser clustered fruit, making it less susceptible to botrytis. To determine the clonal differences, we clustered the years into 3 groups using k-means clustering based on the meteorological data from the phenophases of each year.

Based on principal component analysis, the year groups can be clearly distinguished. ’Juhfark’ had an average yield of 1,23 kg/m^2 over 10 years, which is a good result for a native variety. There was no statistically significant difference in yield between clones or year groups.

The average botrytis infection rate over the years was 19.67%, which is high and typical for the variety. Significant differences were only found between botrytis affected vintage year groups.

In most years, the rate of grey mould was lower in the clones compared to the base variety, especially in the vintage groups where the total rot was quite low.

The relationship between black rot and stilbenes

Quantification of stilbene forms from healthy and infected leaves on plants grown under laboratory conditions

The effect of black rot infection on stilbene levels was investigated at ($p<0.05$) confidence level in the resistant ’Csillám’ (’Rayon d'or’ × ’Kékfrankos’) and the susceptible ’Csaba gyöngye’ in top, small leaves.

The ’Csaba gyöngye’ had lower levels of stilbenes in all time points and samples compared to the ' Csillám '. At time 0 hpi, the average stilbene level in ’Csaba gyöngye’ was 24454 ng/g FW, while in ' Csillám ' it was 2.4× higher.

There was no significant difference in stilbene levels between mock and infected leaves of 'Csillám', based on Kruskal-Wallis test p=0.2820 at the time points tested. Similarly, in the'Csaba gyöngye' cultivar, the difference in stilbene levels between mock and infected leaves in our sampling timepoints was not significant, based on Kruskal-Wallis test (p=0.2820).

However, in the case of the cultivar 'Csillám', the average total stilbene level at the initial 0 hpi was 59813 ng/g FW, which was 6.3× higher than in field samples (9489 ng/g FW). Similar to 'Csillám ', 'Csaba gyöngye' had 2.2× higher stilbene levels in laboratory conditions compared to the field. Despite the fact that fewer stilbene types were tested, higher total stilbene levels were observed under laboratory conditions.

Detection of stilbene levels in leaves of black rot resistant and susceptible cultivars in field experiment

The young, small leaves of the cultivars we studied contained less stilbene than the older ones under field conditions. The difference between the stilbene content of the two leaf sizes was significant (p=0.0006, df=14) by paired t-test.

Due to the different stilbene levels resulting from different leaf sizes, we further investigated the stilbene levels of small and large leaves separately. For the small leaves, the difference in stilbene content of each variety was significant (p=0.0024) at the confidence level (p<0.05) according to Kruskal-Wallis test.

Examination of stilbene content in leaves of cultivars and genotypes resistant and susceptible to black rot showed that *Vitis vinifera* cv. 'Furmint' had the lowest total stilbene levels (mean 2156 ng/g FW and 2944 ng/g FW) in both small and large leaves, but the black rot, powdery mildew and downy mildew resistant *Vitis vinifera* cv. 'Solaris' and 'Souvignier gries' also had surprisingly low stilbene levels at both leaf sizes.

Compared to the susceptible 'Furmint', only 1.3× higher stilbene levels are found in 'Solaris' and 2.4× in 'Souvignier gries' in small leaves. The small leaf size 'Csillám' had lower stilbene levels than ’Csaba gyöngye’, but the difference between them was not significant ($p=0.6062$) at ($p<0.05$) confidence level by the two sample t-test.

On the other hand, the stilbene level of ’Csaba gyöngye’ was lower in the large leaf size 'Csillám', but this was not a significant difference $p=0.4000$ either, based on Mann Whitney test.

When the lowest stilbene levels were studied, the average stilbene level was 1.6× higher in the resistant 'Solaris' compared to the susceptible 'Furmint' in the large leaves, while the difference was 1.7× in the susceptible 'Kékfrankos'.

The highest stilbene levels at both leaf sizes (mean 15180 and 17108.5 ng/g FW) were observed in a *Vitis amurensis* F2 hybrid. The mean total stilbene levels were almost identical in 'CSFT29' with 'Rayon d'Or' parent (6378.9 ng/g FW) and 'Merzling' with 'Rayon d'Or' grandparent (6414.4 ng/g FW) (Figure 1). For large leaves, the Kruskal-Wallis test showed a significant difference ($p=0.0016$) in the stilbene content of each variety at ($p<0.05$) confidence level.

Examination of stilbene levels and resistance in progeny derived from crosses between susceptible and resistant genotypes to black rot

In the case of parents of the segregating population, such as 'Csillám', 'SK001/7', '01-1-797', we also examined small, large and even older (more than 7.5 cm) leaves, as we also investigated hybrids in the segregating generations of ' Csillám ' × 'SK001/7' and 'Csillám' × '01-1-797'. As for the parent varieties, the difference in stilbene levels of SK001/7 is not significant ($p=0.3393$), is significant ($p=0.0425$) for '01-1-797', while in ' Csillám ' is not significant ($p=0.1679$) when comparing leaf sizes.

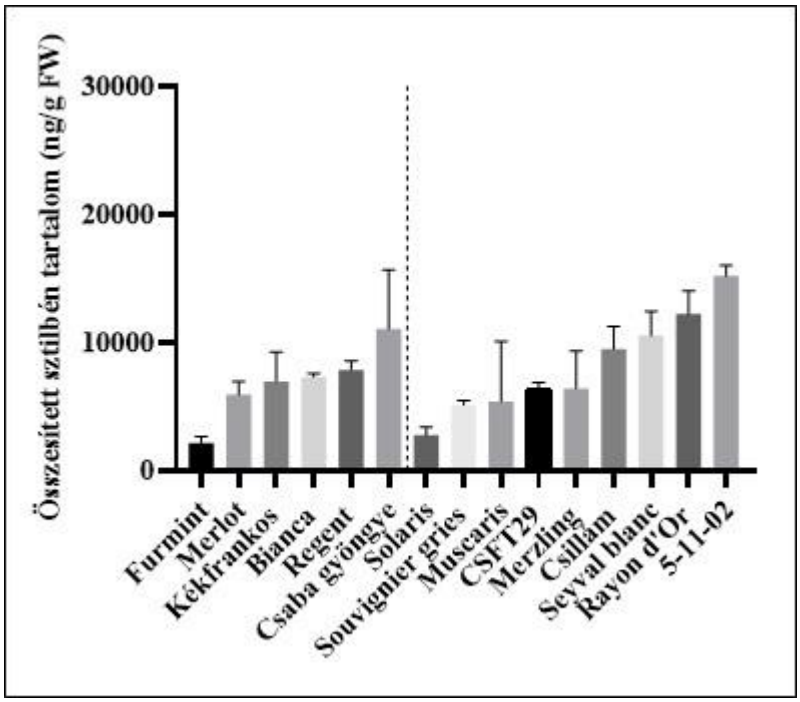

Figure.1

The small size ("3 cm") of the leaves and the total stilbene levels we measured in different grape varieties. To the right of the dashed line are indicated black rot resistant varieties and to the left susceptible varieties.

There was no significant difference in stilbene levels between the total stilbene content of small leaves of the parents according to Kruskal-Wallis test ($p=0.0857$), even less so for large leaves ($p=0.3821$) and no significant difference in stilbene levels for even older and even larger leaves according to one-way ANOVA test ($p=0.2328$) at the 95% confidence level.

Among the hybrids, 109,147,180,187 and 192 BR (black rot) are resistant. In case of small leaf, the hybrid with the lowest stilbene levels among the hybrids were BR resistant hybrids 180 and 187 with stilbene contents of 2713 ng/g FW and 3123 ng/g FW, respectively. However, the hybrid 147, also BR resistant, had the highest stilbene content (50284 ng/g FW). Hybrid 147 thus

outperformed the susceptible 'SK001/7' (3836.5 ng/g FW) and the BR resistant 'Csillám' (9489 ng/g FW) (Figure 2).

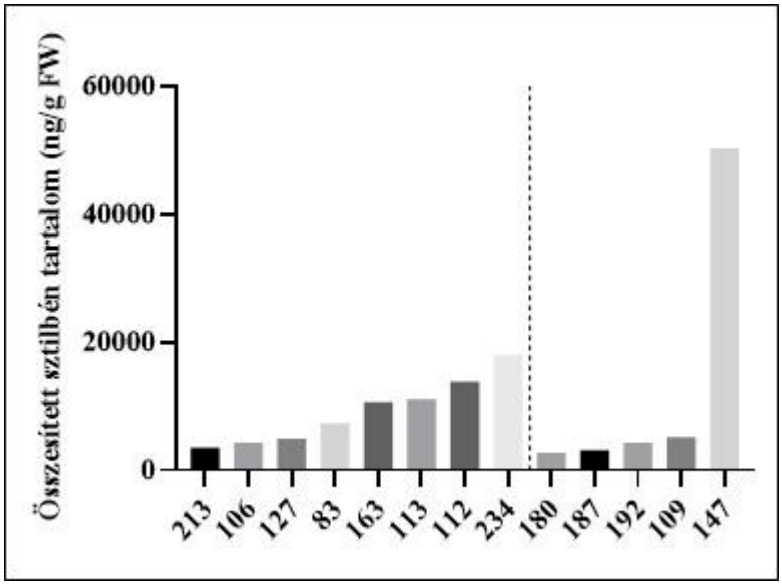

Figure 2.

Stilbene content in small ('3 cm') leaves of hybrids of the segregating *generation. To the right of the dashed line are the black rot resistant varieties and to the left the susceptible varieties.*

In the large leaf study, BR susceptible hybrid 213 had the lowest stilbene level among the hybrids (3835 ng/g FW), but BR resistant progeny 147 continued to have the highest stilbene level (39248 ng/g FW) among the hybrids.

When comparing small leaves of resistant hybrids and susceptible ones, there was no difference between them based on Mann Whitney test ($p=0.3543$), this was also true for large leaves ($p=0.7242$). Based on Mann-Whitney test, there was no significant difference ($p=0.3543$) between black rot resistant and susceptible hybrids ($p<0.05$) for '3 cm' leaves, but this was also seen for '5 cm' leaves ($p=0.7242$) in stilbene levels. This suggests that there is no link between resistance and stilbene levels and that these traits can be inherited independently.

Validation of the expression of differentially responding genes in cultivars susceptible and resistant to black rot

In grapevine, we investigated RNA level changes in the early cellular response to black rot attack (0, 6, 18, 36 hpi) in resistant 'Csillám' and susceptible 'Csaba gyöngye'. Based on the gene expression data, we presumed that stilbene synthase genes play a prominent role in the early response to black rot infection, as they were found among the DE (Differentially expressed) genes.

Total stilbene levels, however, showed no correlation with black rot disease and resistance in grapes. Therefore, I individually analysed the expression pattern of quality controlled DE genes (0,6,18,36 hpi) at time points ($p<0.01$), which allowed me to distinguish 32 groups with identical transcriptional patterns and 49 genes with unique expression in the two cultivars.

The results were evaluated at the $p<0.05$ significance level for each gene. Relative expression value indicates the gene expression value of the infected sample compared to the mock control.

The VvRPM1 gene in the susceptible cultivar 'Csaba gyöngye' was not expressed in untreated leaves and in leaves inoculated with the spore isolate at any of the time points we examined. However, the resistant cultivar 'Csillám' expressed the RPM1 gene in both mock inoculated and infected leaves. However, there was no significant difference ($p=0.7417$) between the time points (6, 18, 36 hpi) according to one-way ANOVA test.

In 'Csillám', at 6 hpi, mock expression decreased to 0.6 in the infected leaves, i.e. the uninfected sample had higher RPM1 expression. At 18 hpi, expression was already 1.1× higher in the infected leaves compared to the half-leaf control and this remained the same at 36 hpi, i.e. there was almost no difference at these time points, no significant induction of transcription occurred due to black rot infection.

When the VvDMR6 gene was tested, the difference between the two cultivars was not significant (p= 0.3381) by two-way ANOVA test in Geisser-Greenhouse analysis. The difference in expression between the mock and infected samples at each sampling time point in 'Csillám' was not significant (p= 0.6883) by one-way ANOVA test ($p<0.05$), while in 'Csaba gyöngye' it was significant (p=0.0470). In the 6 hpi sample, expression was lower (0.8×) in the infected sample, while it was 2.16× higher in 18 hpi and 1.23× higher in 36 hpi in the infected leaf compared to the mock.

VvDMR6 is a disease susceptibility gene, and higher expression levels were expected in the susceptible strain, which showed a significant increase at the 18 h time point.

When the STS (stilbene synthase) and PAL (phenylalanine ammonia-lyase) genes were tested in 3 biological replicates, the standard deviation was high at certain time points, so I could not draw any clear conclusions about their relative expression. For STS10 and STS21, a two-way ANOVA analysis showed no difference between the two strains (p=0.0629) and (p=0.2304), respectively, while for STS20 it was significant (p=0.0407). For PAL (p=0.6306), there was no significant difference between 'Csillám' and 'Csaba gyöngye'.

For OPR2 and WRKY gene, two-way ANOVA analysis showed that the expression difference between the two species was not significant (p=0.3497) and (p=0.3914), but the relative expression of OPR2 at the 36 h time point could not be evaluated due to the large standard deviation. The results of the transcriptomic data did not overlap with the relative expression data obtained by real-time PCR. One exception to this was RPM1, which was indeed a DE gene, expressed only in the resistant 'Csillám'.

Creation of CRISPR constructs and development of embryogenic callus induction in 'Furmint'

To date, three constructs have been prepared and are available for MLO genes involved in grapevine powdery mildew susceptibility, one targeting FuMLO11, one targeting FuMLO13 with one target each, while the third construct targets FuMLO6 and FuMLO7 genes at three points. In addition, one construct targeting the FuDMR6 gene is also available, targeting two sites of the gene simultaneously. However, in order to continue silencing, a plant regeneration system that is effective in the Furmint variety is needed and is also being developed.

Excised anthers of 4 different inflorescences (A, B, C, D) of the cultivar 'Furmint' were cultured on MSE/2 and MST/2 media with the following names: MSE_A, MSE_B, MST_C, MST_D. Anthers from inflorescences A and B were started on MSE/2, and those from inflorescences C and D on MST/2. After one month, all anthers were passed onto MSE/2 medium and incubated in the dark at 22+/-2°C. After one month of incubation, thus 2 months after the beginning of the experiment, pale and dark yellow calli appeared on most of the anthers and occasional white calli was observed.

In the 6th month after the start of the cultures, some of the calli were transferred from the MSE/2 media to media containing NAA, BAP and IAA, kinetin, respectively, and placed in a growth chamber under warm and cool white (Polylux XL 36W and Tungsram F7 Daylight 36W) light at 22+/-2°C for 16 h light and 8 h dark periods.

On MSE/2 media, we continued to maintain calli grown on anthers, which remained in the dark at 22+/-2°C. On the new NAA, BAP and IAA, kinetin media, 2.5 months later, the stems of the anthers became green, while the anthers on MSE/2 showed no change.

In our experiment, we successfully performed embryogenic calli induction from Furmint grape leaves on NAA, BAP containing MS/2 and MSE/2 medium containing activated charcoal. On IAA, kinetin containing media, the leaf margins turned brown after 30 days. After 80 days from the beginning of the experiment, a slight white calli formation was detected.

30 days after the start of the experiment, a white calli formation was observed on the NAA/BAP containing medium, and 60 days later, a pink calli were appeared next to the white calli region, so we passed calli to MSE/2 medium containing 0.05 g/l activated carbon to test embryogenic capacity. 50 days later, on these media a dark yellow, presumably embryogenic calli appeared and globular proembryos were formed, confirming the ability of 'Furmint' leaves to embryogenesis.

87 days after the beginning of the experiment, pale yellow embryogenic calli formation started on the calli left on the NAA/BAP medium, which intensively developed, turning yellowish approximately 180 days after the start of the culture.

CONCLUSIONS

Field testing of *Botrytis cinerea* resistance in a clonal experiment

'Juhfark' is highly susceptible to grey rot, and the characteristics of the bunch, such as the thickness of the berry skin and the compactness of the bunch, may all contribute to this. The results showed that in *Vitis vinifera* cv. 'Juhfark', the susceptibility to gray mould was related to the varietal characteristics. Both clones showed significantly different susceptibility to Botrytis infection in clusters of different vintages. In vintage group 2, higher values, i.e. worse results, were obtained than in the other vintage groups.

Bunch rot caused by *Botrytis cinerea* is strongly influenced by weather conditions during grape ripening. The results suggest that clonal selection is not a complete solution to avoid the negative effects of climate change.

Resistance genes and stilbene levels in black rot of grapes in Csillám and Csaba gyöngye cultivars under laboratory conditions

On the one hand, stilbene levels were studied because stilbene synthases were found among differentially expressed genes. Furthermore, the very first results of the mapping of black rot revealed a black rot QTL that overlapped with a resveratrol-degrading QTL on chromosome 16, which localizes stilbene synthase genes (VvSTS7-48) involved in the stress response (Vannozzi et al., 2012).

Similarly, our selected stilbene synthase genes (*Vv*STS10, 20 and 21), as chosen by transcriptome sequencing, were located on chromosome 16. To reveal their role in the black rot infection, stilbene levels can be analysed.

There were no significant differences in the expression of stilbene synthases STS10 and STS21 in the resistant and susceptible cultivars, whereas there was a substantial difference in STS20. Vezulli and coworkers (2019) found STS10, STS20, STS21 among DE genes in their study of grapevine downy mildew resistance and polyphenol content. The potential role of STS10 was associated with cis-piceid synthesis.

One of our candidate genes is the rpm1-like resistance gene, whose role in grape oomycosis has been described. The level of RPM1 is elevated in the downy mildew susceptible cultivar *Vitis vinifera* cv. 'Centennial Seedless' under Plasmopara viticola infection (Liu et al., 2020).

DMR6 is a powdery mildew susceptibility gene encoding an oxidoreductase of the 2-oxoglutarate (2OG)- Fe(II) oxygenase family. It was first shown to play an important role in the infection process in *Arabidopsis thaliana* in grapevine downy mildew studies (van Damme et al., 2008). In grapevine powdery experiments, Makovecz-Tóth detected this gene with differential expression.

Relationship of stilbene levels to leaf size in Csillám, Csaba gyöngye and other BR resistant and susceptible cultivars under field conditions

I tested the hypothesis that higher stilbene levels are indeed associated with higher resistance in leaves. Therefore, I first examined the effect of black rot in the uppermost leaves of laboratory-grown 'Csaba gyöngye' and 'Csillám' using the half-leaf method in early response (0-36 h).

In our study of the total stilbene levels in the cultivars, no significant differences were observed between the samples at 0, 6, 18, 36 h post infection in either 'Csillám' or 'Csaba gyöngye' cultivars.

No difference in stilbene levels was observed between the mock and its infected pair at each sampling time within the variety. This suggests that the infection did not induce a response which could significantly increase stilbene levels over the time interval studied.

However, the results confirmed the difference between the varieties, with higher stilbene levels in BR resistant 'Csillám' compared to 'Csaba gyöngye'.

In the field experiment, this was not the case for the small leaves, and stilbene levels were lower, despite the fact that fewer stilbene forms were tested in plants from growth chamber. We presume that two-bud cuttings of grapes grown under artificial light may not be suitable for this type of measurement because these laboratory conditions may induce stress effects.

To distinguish total stilbene levels by leaf size in all cultivars measured in field experiments, smaller leaf size is associated with lower stilbene levels, while larger leaf size is associated with higher stilbene levels. This may support the results described earlier on ontogenetic resistance.

Altogether, smaller leaves may be more susceptible partly due to lower stilbene levels. Whether high stilbene levels are a cause or a consequence of the development of ontogenetic resistance is not known. With increasing leaf size and stilbene levels, full resistance is established. Susceptibility and resistance can only be considered in young leaves.

Under field conditions, I did not have the opportunity to study the effect of infection, but we tested pre-infection conditions in several varieties besides 'Csaba gyöngye' and 'Csillám'. We also investigated the reasons behind the asymptomatic resistance of 'Csillám', based on differentially expressed genes and stilbene levels, and BR-induced stilbene levels.

Among the varieties tested was 'CSFT29', sister vine of 'Csillám', which also showed BR resistance, and 'Rayon d'Or' ('Seibel 4986'), the father vine of 'Csillám', which may have inherited its resistance to black rot from its ancestor

Vitis rupestris. On the other hand, the maternal parent of 'Csillám' is BR susceptible. Rayon d'Or' is also the grandparent of Merzling, which is also resistant to black rot.

We also tested the members of the segregating generation from the parents ' Csillám ' × 'SK001/7' and ' Csillám ' × '01-1-797', which included both black rot resistant and susceptible hybrids. One parent of 'Merzling' is the BR resistant 'Seyval blanc'.

In our field studies, we did not find a clear correlation between resistant and susceptible varieties in terms of resistance to black rot in the total stilbene levels we measured. However, the lowest stilbene level 'Furmint' is susceptible not only to hemibiotrophic grape black rot, but also to biotrophic grape powdery mildew, downy mildew and necrotrophic grape grey rot. Biotrophic pathogens generally prefer young, small leaves, while older, senescing tissues are the target of necrotrophs. This may suggest that grapevines need protection against biotrophic pathogens and hemibiotrophic (because they are initially biotrophic) pathogens on young leaves.

The black rot resistant ’5-11-02’ *Vitis amurensis* hybrid possessed the highest level of stilbenes but there is no data found regarding resistance to other pathogens. However, 'Rayon d'Or' and 'Seyval blanc', with high stilbene levels, are resistant to black rot, grape powdery mildew, downy mildew and less susceptible to *Botrytis cinerea*.

Contradictory results were found in the analysis of the 'Souvignier gris' variety, which has a high resistance to the aforementioned fungal diseases, yet relatively low stilbene levels were observed in small (5120 ng/g FW) and large (8761 ng/g FW) leaves, which recorded levels similar to those of the black rot susceptible 'Merlot' variety, which lacks resistance to powdery mildew and downy mildew.

The smaller BR sensitive leaves in BR resistant varieties had higher stilbene levels, but there are exceptions among the varieties.

When examining the parents 'Csillám' and the susceptible 'SK001/7' and '01-1-797', only in ’01-1-797’ was there a significant difference in stilbene content between the three leaf sizes.

By analyses of the segregating population, hybrids with diverse stilbene content were produced regardless of the BR susceptible parent and the degree of resistance. Different stilbenes may have different effects, which has not been fully discovered for grapes. The age of leaves has a profound effect on stilbene levels. In very young and old leaves, stilbene levels are not high, the stomata are not fully developed or are closed, which may limit the entry of certain pathogens and thus the induced synthesis of stilbene.

Of all the grape varieties we tested, the lowest total stilbene levels were measured in *Vitis vinifera* cv. 'Furmint', which is susceptible to grape powdery mildew, downy mildew, gray mould and black rot either. The stilbenes are important protective metabolites, inhibiting the development of fungi such as *Botrytis cinerea* (grey rot), *Plasmopara viticola* (downy mildew and *Erysiphe necator* (powdery mildew) in grapes. Higher stilbene levels were observed in mock inoculated leaves compared to infected ones in both 'Csaba gyöngye' and 'Csillám'.

Overall, no clear correlation was found between stilbene levels in leaves of a given size and black rot resistance. Stilbene levels solely does not explain resistance. And the segregating generation study demonstrated that high stilbene levels and high resistance may be independently heritable traits.

Both preformed tolerance (e.g. high stilbene levels) or induced resistance also play a role in resistance. In the case of 'Csillám', high stilbene content may contribute to outstanding resistance, but varieties with low stilbene levels, such as 'Solaris', may also be less resistant.

NEW SCIENTIFIC RESULTS

1. Stilbene levels in grape leaves have not yet been tested for resistance to grape black rot. I have found that total stilbene levels in young leaves with a diameter of 2.5-3 cm are lower than in more developed leaves with a diameter of 4.5-5 cm, in both susceptible and resistant cultivars, consistent with the emergence of ontogenetic resistance.

2. In a laboratory artificial infection experiment, the leaves of the susceptible 'Csaba gyöngye' cultivar had lower levels of stilbenes at all time points and in all samples compared to the resistant 'Csillám'.

3. There was no significant difference in stilbene levels between mock and infected leaves in 'Csaba gyöngye' and in 'Csillám' at each sampling time point, highlighting the role of stilbene as a phytoanticipin in preformed resistance.

4. There is no clear correlation between stilbene levels in black rot resistant and susceptible varieties and the resistance of varieties to black rot, and stilbene levels alone do not explain resistance.

5. In the offspring of crosses between moth-resistant and susceptible parents, moth resistance and stytenic acid levels can be inherited independently. The stilbene levels of susceptible and resistant individuals selected from a large population based on 3-year monitoring show no association with resistance.

6. To investigate the molecular background of resistance, I identified a gene (RPM1) that was expressed only in the resistant 'Csillám' variety according to the analysis of gene expression under artificial infection.

7. For the production of fungi resistant grapevines by targeted gene delivery, I produced CRISPR constructs with the genes I have studied and which are important in powdery mildew resistance (DMR6 and MLO) which play a role in fungi resistance and susceptibility (MLO) for plant transformation experiments.

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