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Sophia Müllner[1], Bianca Frommer[2], Daniela Holtgräwe[2], Prisca Viehöver[2], Bruno Hüttel[3], Reinhard Töpfer[1], Bernd Weisshaar[2], Eva Zyprian[1*]

Analysis of the *Rpv12* locus in a haplotype-separated grapevine genome sequence

Affiliations
[1]Julius Kühn Institute, Institute for Grapevine Breeding Geilweilerhof, Siebeldingen, Germany
[2] Bielefeld University, Genetics and Genomics of Plants, Faculty of Biology & Center for Biotechnology (CeBiTec), Bielefeld, Germany
[3]Max Planck-Institute for Plant Breeding Research, Max Planck-Genome-Centre Cologne, Cologne, Germany

Correspondence
Sophia Müllner: sophia.muellner@gmail.com, Bianca Frommer: frommer@CeBiTec.Uni-Bielefeld.de, Daniela Holtgräwe: dholtgra@CeBiTec.Uni-Bielefeld.de, Prisca Viehöfer: viehoeve@CeBiTec.Uni-Bielefeld.de, Bruno Hüttel: huettel@mpipz.mpg.de, Reinhard Töpfer: reinhard.toepfer@julius-kuehn.de, Bernd Weisshaar: bernd.weisshaar@Uni-Bielefeld.de, Eva Zyprian: eva.zyprian@julius-kuehn.de

Summary

Plasmopara viticola, the grapevine downy mildew pathogen, causes severe losses in viticulture if not counteracted by fungicide sprays that need to be repeatedly applied during each growing season. To reduce the amount of plant protection, modern grapevine breeding generates fungus-resistant grapevine cultivars by introgression of resistance loci from wild *Vitis spec*. sources. However, the presence of only a single resistance locus may provoke the emergence of pathogen races able to overcome the resistance trait of the host. Therefore, a combination of several, independently acting resistance loci is required for sustainable genetic resistance. Quite little is known about the resistance-conferring genes within the various grapevine resistance loci. To ameliorate this situation and make stacking of resistance loci more efficient, the *Rpv12* locus originating from the Asian *Vitis amurensis* was sequenced and characterized. The complete genome of breeding line Gf.99-03, carrying *Rpv12* in heterozygous state, was analyzed. Haplotypes were resolved by assigning the reads to one of the parents of Gf.99-03 using trio binning. Annotation of the resulting genomic sequences was based on RNA-Seq data and predicted gene models.

The haplotype carrying the *Rpv12* locus, delimited by markers UDV-014 and UDV-370 on chromosome 14 (Venuti *et al.*, 2013), diverges strongly from the susceptible haplotype as well as from the reference genome PN40024 12X.v2. It was found to contain two important gene clusters. One cluster includes pathogen-inducible genes similar to the gene *ACCELERATED CELL DEATH 6* (*A. thaliana*) likely involved in hypersensitive response upon pathogen attack. The second cluster comprises positional resistance candidate genes corresponding to typical *NLR*s (nucleotide binding site, leucine rich repeats), hypothesized to be involved in pathogen perception and cellular defense signalling.

Introduction

The oomycete *P. viticola* (Berk. & M. A. Curtis) Berl. & De Toni (*Peronosporales*) is an obligate biotrophic pathogen that causes significant problems in viticulture, especially under humid and warm weather conditions (Müller & Sleumer 1934; Kiefer *et al.*, 2002). Its potential damage is defeated by the extensive use of plant protection products (Eurostat, 2007, Massi *et al.*, 2021). In recent decades, fungus-resistant new varieties have been bred to reduce the heavy use of fungicides. Resistance breeding requires genetic analysis of resistance factors (QTL, quantitative resistance loci), which subsequently may be introduced by cross breeding and molecular selection into elite grapevine cultivars. For sustainable resistance, a combination of several independent resistance factors is required (pyramiding or stacking) (Eibach *et al.*, 2007).

Meanwhile, more than 30 QTL directed against *P. viticola* have been identified (Maul et al., 2023). In addition to the loci *Rpv1*, *Rpv3* and *Rpv10*, tagged with flanking genetic markers and frequently applied in breeding, the identification of the locus *Rpv12* in 2013 brought it into the focus of breeders, as it is already present in some grapevine cultivars (Venuti *et al.*, 2013). The resistance locus *Rpv12* was introgressed from the Asian wild grapevine species *V. amurensis* and is found in various cultivars such as 'Kunbarat', 'Kunleany', 'Michurinets' and 'Sauvignac' (Koleda, 1975; Venuti *et al.*, 2013, Wingerter *et al.*, 2021). *V. amurensis* is well known for its cold hardiness, withstanding temperatures down to -50 °C, the reason why it was used in grapevine cold resistance breeding already in early empirical approaches (Koleda, 1975; Wang *et al.*, 2021, Ren *et al.*, 2023).

Efficient grapevine breeding requires deep knowledge about grapevine varieties and their characteristics. Cytological studies provide insight into the cellular mechanisms involved. Detailed genetic information is obtained by genomic sequencing and the identification of genes associated with resistance traits or other desired characteristics. However, the functional confirmation of resistance gene candidates is still a demanding challenge in grapevine since transgenic approaches are very time consuming.

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Grapevines generally are diploid and largely heterozygous (Alleweldt & Possingham, 1988; Jaillon *et al.*, 2007). Classical sequencing of a grapevine genotype therefore usually results in a mosaic genome sequence that contains an intermixture of both haplotypes. A method developed in 2018 by Koren *et al.* allows to separate the two haplotypes of an interesting genotype to be sequenced by evaluating short sequence reads of both parental lines with its long reads (k-mer based trio binning).

In this work, an *Rpv12* carrier and its parental genotypes were sequenced, the read data of the targeted F1 were separated for the haplotypes and assembled individually. To assist the annotation of genes, a differential gene expression analysis was carried out after inoculation with *P. viticola*, in addition to the generation of RNA-Seq data from untreated tissues such as stems, shoots, leaves and roots. The locus itself was investigated in detail for its content of genes putatively involved in resistance. The efficiency of the resistance trait was studied by cytological follow-up of mycelial development in the *Rpv12* carrier Gf.99-03 as compared to a susceptible grapevine genotype after experimental inoculation.

Material and Methods

Plant Material

All plant material used in this work was obtained from the germplasm collection of the Julius Kühn Institute, Institute for Grapevine Breeding Geilweilerhof (49°12'54.1''N, 8°02'41.3''E) in Siebeldingen, Germany.

For the sequencing work, two-eye cuttings of genotypes 2014-099-0003 (Gf.99-03, *V*IVC 27131), Gf.2004-043-0021 (Gf.43-21, *V*IVC 27130) and genotype 65-153-18 (*V*IVC 41129) were grown in Jiffy-7® pots (Jiffy Products International BV, Zwijndrecht, The Netherlands) in 2019 in the greenhouse. Breeding lines Gf.43-21 and 65-153-18 are the parental genotypes of Gf.99-03. The *Rpv12* locus was transmitted from 65-153-18 to Gf.99-03.

Extraction of high molecular weight DNA of the genotypes Gf.99-03, Gf.43-21 and 65-153-18

High molecular weight genomic DNA of genotype Gf.99-03 was extracted from 2 × 2 g of leaf material and sequenced by PacBio long read technology.

For analysis of parental lines Gf.43-21 and 65-153-18 by Illumina short read sequencing, 2.5 g leaf tissue of both genotypes were harvested. The CTAB method according to Rosso *et al.* (2003) was used to extract the genomic DNA.

Generation of RNA-Seq data

Leaves to be used for RNA-Seq analysis were taken from greenhouse plants early in the morning to avoid accumulation of photosynthetic starch. At least two leaves (third and fourth apical insertion) from each of three plants of the same genotype were used. The leaves were washed in H_2O_{dest} before cutting out 1.5 cm diameter leaf discs with a surface-sterilised cork borer. A total of forty leaf discs per plant clone were punched out and positioned upside down in rectangular, translucent and lidded incubation plates (Corning 431111, Corning Incorporated, Corning, NY, USA) on 1 % agar in H_2O_{dest} (Gustav Essig GmbH & Co. KG, Mannheim, Germany).

Half of the leaf discs per clone were inoculated with 40 µl of a *P. viticola* zoospore solution, while the other half was mock inoculated with the same volume of H_2O_{dest}. The zoospores were separated from the sporangia according to Müllner & Zyprian (2022). Leaf discs were incubated at 16 h light, 8 h dark, 22 °C and 100 % humidity. At the respective time points (0, 6, 12, 24, 48 hpi), leaf discs with a diameter of 1.3 cm were cut out of four leaf discs at a time using a smaller cork borer and snap-frozen in liquid nitrogen. The leaf material was stored at -70 °C until further use.

The efficiency of inoculation and mycelial development was checked by fluorescence microscopy of additionally inoculated leaf material according to Hood and Shew (1996) as described in Müllner & Zyprian (2022).

RNA was isolated from inoculated leaf discs and whole non-infected leaves, tendrils and roots using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. The material was ground with a mortar and pestle (Jipo, Czech Republic) under the addition of liquid nitrogen and a spatula tip of PVP-40 (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) to inhibit plant phenolics. These steps were carried out at the Institute for Grapevine Breeding Geilweilerhof, Siebeldingen. The RNA obtained was subjected to Illumina RNA-Seq analyses.

Results

Rpv12 mediates efficient resistance to P. viticola

A comparison of mycelial development after experimental *P. viticola* inoculation of the susceptible genotype 'Italia' and the *Rpv12* carrier Gf.99-03 (Fig. 1) clearly showed a significant reduction in the resistant breeding line after 72 hpi.

Trio binning and genome assembly

By combining data from PacBio and Illumina sequencing for the genotypes Gf.99-03, Gf.43-21 and 65-153-18 and by application of the trio binning approach according to Koren *et al.* 2018, a well phase-separated genome assembly comprising a total of over 1,055 Mbp was created. The largest contig of Gf.99-03 had a length of 18.1 Mbp, the N50 length of the assembly is 3.76 Mbp. Roughly 35.000 protein-coding genes were identified in each haplotype.

Using BUSCO (Benchmarking Universal Single-Copy Orthologs; Waterhouse *et al.*, 2018) as a tool to evaluate assembly quality, the two haplotype assemblies Gf9921 and Gf9918 of genotype Gf.99-03 were found to be similarly complete as the reference genome sequence PN40024 12X.v2. Gf9921 and Gf9918 contain a few more duplicated genes. It can be concluded that the assemblies correspond to a largely complete representation of the diploid genome of Gf.99-03.

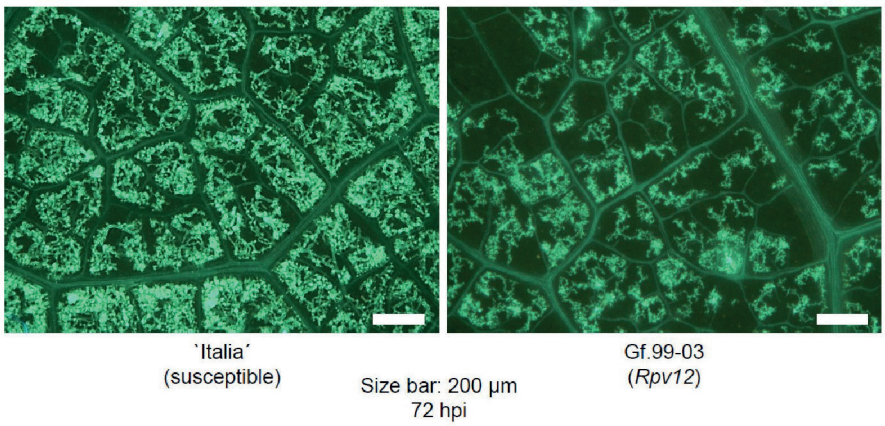

Fig. 1: Aniline blue staining of leaf discs inoculated with zoospores of *P. viticola* after 72 hpi. The pictures show the mycelial growth of *P. viticola* after 72 hpi on stained leaf discs of the susceptible genotype 'Italia' and the resistant *Rpv12* carrier Gf.99-03. The mycelial growth is strongly reduced in Gf.99-03 as compared to 'Italia'. The size reference bar corresponds to 200 µm.

Differential Gene Expression Analysis to assist Gene Annotation

To identify the relevant genes not only by ab initio prediction but also with RNA-Seq support, and to check possible gene regulation after P. viticola attack, a differential gene expression analysis was carried out at time points 0, 6, 12, 24 and 48 h after experimental inoculation (hpi, Fig. 2). The data were tested for grouping by principal component analysis (PCA). The untreated and treated samples grouped together in pairs at the 0 hpi time point as to be expected, while at the other time points a clear separation between untreated and treated samples was found and regulated genes were identified. This result also confirmed that the infection experiment was successful.

The complete set of annotated genes was searched for resistance gene analogs (RGAs). The biggest group was found for protein coding genes containing transmembrane and coiled coil- (TM-CC, more than 900), receptor like kinase (RLK, more than 700) and receptor-like protein (RLP, more than 200) domains in both haplotypes. Genes encoding the NBS type (with nucleotide binding site domain) and NBS-LRR (with nucleotide binding site and leucine rich repeat domains) appeared in rather low numbers of around 60 respectively 100 per haplotype. This is the kind of genes identified earlier as involved in *P. viticola* resistance (Feechan *et al.*, 2013).

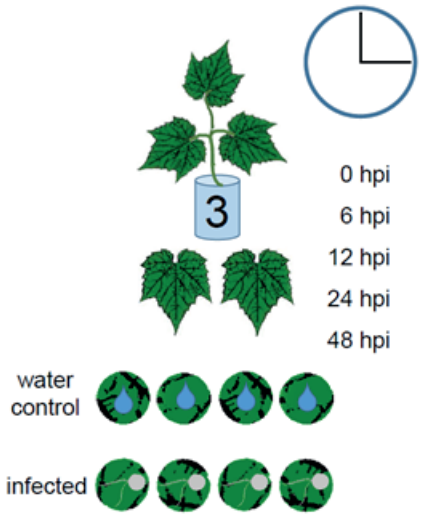

Fig. 2: Experimental set-up for differential gene expression analysis using RNA sequencing. Three clonally propagated grapevines of the same genotype (Gf.99-03) were used as biological replicates. Two leaves were taken from each plant and leaf discs were punched out of them. For each time point (0, 6, 12, 24, 48 hpi), four leaf discs per plant were incubated with H_2O_{dest} and four leaf discs were inoculated with zoospore suspension.

Looking particularly at the *Rpv12* locus on chromosome 14, as delimited by markers UDV-014 and UDV-370 (Venuti *et al.*, 2013), its overall size is comparable in both haplotypes and similar to the susceptible grapevine reference genome PN40024 12X.v2. However, the locus shows two specific gene clusters. Cluster 1 between markers GF14-21 and GF14-31 is larger in the *Rpv12* carrying haplotype Gf9918 (417 kb) than in the susceptible Gf9921 (158 kb) and differs also considerably from PN40024 12X.v2 (263 kb).

This cluster 1 includes four genes found induced after pathogen challenge with *P. viticola* in the RNA-Seq study consistently from 6 to 48 hpi. One of those encodes PGG (protein domain with PGG motif but of unknown function) and TM (transmembrane) domains, while three more have ankyrin repeats in addition to PGG and TM domains. They resemble *ACCELERATED CELL DEATH 6* genes (*ACD6*) known from *Arabidopsis thaliana*. These genes might be involved in hypersensitive responses to defeat the pathogen (Todesco *et al.*, 2010; Zhang *et al.*, 2019).

The second cluster of candidate resistance genes identified in the *Rpv12* locus encodes RGA proteins of RLP, CN (Coiled coil and nucleotide binding site), NL (nucleotide binding site, leucine rich repeat), TM-CC and CNL (CC, NBS and LRR domains) type. This cluster 2 is less extended in Gf9918 than in Gf9921 indicating significant absence/presence variation in the region. In the *Rpv12*-carrying haplotype it includes constitutively expressed genes of the *CNL* type that hypothetically may be involved in pathogen recognition and signal transduction to the cellular defense cascade.

Discussion and Conclusion

The *Rpv12* locus was found to work efficiently in conferring *P. viticola* resistance when present in heterozygous state, as here in the analyzed breeding line Gf.99-03. This breeding line was sequenced at genomic scale and both haplotypes were assembled at high quality. Within the area of the locus *Rpv12* extensive structural presence/absence variation was detected in comparison of both haplotypes. Between the locus delimiting markers

UDV-014 and UDV-370 two gene clusters were identified, that likely are involved in conferring resistance. One cluster contains RGA genes that could be involved in pathogen recognition and initiation of the plant defense pathway, while the second cluster covers pathogen-inducible genes probably related to hypersensitive response as defense reaction. This new knowledge provides the possibility of detailed molecular follow-up of the resistance genes in newly bred *Rpv12*-introgressed grapevines and may also be applied to characterize well-known *Rpv12* carriers such as 'Kunbarat' or 'Kunleany'. It is a cornerstone for grapevine breeding and more exact combination of resistance factors in stacked new cultivars with sustainable *P. viticola* resistance. Future work may confirm the function of the identified resistance gene candidates by their knock-out in the breeding line Gf.99-03 and other *Rpv12*-carrying cultivars.

Conflicts of interests

The authors declare that they do not have any conflicts of interest.

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