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Uncovering markers for downy mildew resistance in grapevine through mass spectrometry-based metabolomics

A descoberta de biomarcadores de resistência ao míldio através de metabolómica baseada em espectrometria de massa

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RESUMO

O míldio é uma ameaça importante à indústria vitivinícola e o desenvolvimento de híbridos entre *Vitis vinifera* e *Vitis* spp. selvagens, resistentes à doença, é uma estratégia promissora no combate a esta doença. A descoberta de marcadores metabólicos é essencial. Neste trabalho pretendemos acelerar o processo de seleção das características de resistência ao míldio utilizando metabolómica baseada em espectrometria de massa de elevada resolução para a descoberta de marcadores de resistência. Comparámos a cultivar suscetível 'Trincadeira' com a mais resistente 'Regent'. Ambas foram facilmente discriminadas com base no seu perfil metabólico, tendo sido encontrados vários picos exclusivos de cada cultivar.

Palavras-chave: Videira, metabolómica, míldio, resistência, biomarcadores

ABSTRACT

Downy mildew is a major threat to the wine industry and the development of hybrids between *Vitis vinifera* and wild resistant *Vitis* species is a promising strategy to cope with this disease. The discovery of metabolic markers associated with grapevine resistance is paramount. In this work we aim at speeding up the assignment of innate resistance to downy mildew in grapevine cultivars by applying high-resolution mass-spectrometry- based untargeted metabolomics as a profiling and resistance marker discovery method. We compared the susceptible cultivar 'Trincadeira' with the more resistant one 'Regent'. Both cultivars could be easily discriminated based on their metabolic profile and we found several peak features exclusive of each variety.

Keywords: Grapevine, metabolomics, mildew, resistance, biomarkers

INTRODUCTION

Vitis vinifera is one of the most important and cultivated fruit plant in the world, occupying a global area of 7.5 mha and with a global grape production of 7.8 mt in 2016 (OIV Statistical Report on World Vitiviniculture, 2017). Most of the produced grape is targeted for wine production, a highly strategic industry for the economy of several countries, Portugal included. With 14 wine producing

regions and 190 kha of vineyards, Portugal is the eleventh world wine producer and the fifth in Europe, accounting for ~700 million euros per year of exports (OIV Statistical Report on World Viticulture, 2017).

One major threat to the wine industry is downy mildew, caused by the biotrophic oomycete *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni, infecting all domesticated *V. vinifera* cultivars frequently used for wine production. This pathogen affects leaves, shoots, bunches and fruits, causing quality and yield reduction with significant production and financial losses (Gessler *et al*., 2011).

Currently used strategies to cope with downy mildew include the intensive use of fungicides, applied soon after the first leaves appear. However, the general recommendations of the European agricultural policy encourage the reduction of pesticides towards environmental sustainability and consumer health. A promising approach is the creation of new cultivars through breeding programs, combining the high degree of resistance against downy mildew from the wild *Vitis* spp., with the good berry quality of *V. vinifera*. One successful example already used in wine production is the interspecific hybrid 'Regent', developed from *V. vinifera* 'Diana' (a Silvaner x Müller-Thurgau cross) and from the interspecific hybrid 'Chambourcin', possessing a higher resistance to downy mildew and other relevant fungal diseases (Gessler *et al*., 2011).

The complete process of a new cultivar's breeding, from plant crossing to market release, can take about 25 to 30 years. Since grapevine is a perennial crop, the selection process concerning pathogen resistance is only possible 2 to 3 years after plant crossing, after which the more resistant seedlings are kept (Eibach and Töpfer, 2015). Considering the high number of newly developed seedlings, shortening this time would represent a considerable financial benefit to the producers. Hence, any new or advanced selection methods of these new cultivars can lead to a more efficient breeding process.

The discovery of mildew resistance-associated biomarkers in grapevine will allow a quick and accurate identification of the seedlings that inherited the resistant trait soon after germination. Disease-resistance genetic markers have been searched for the past 14 years and quantitative trait loci (QTLs) for resistance to downy mildew were identified (Gessler *et al*., 2011). Among these, the Resistance to *Plasmopara viticola* (RPV) loci were identified in several *Vitis* species. These loci can be passed into the offspring in cross-breeding programs between *V. vinifera* and other *Vitis* species, but their presence in the cross-bred cultivars does not guarantee resistance to downy mildew (Peressotti *et al*., 2010). Hence, the resistance of the wild *Vitis* species to *P. viticola* most probably goes beyond the presence of these DNA markers in their genome. Metabolic biomarkers have proven their value to predict phenotypical traits before they are observed (Wolfender *et al*., 2013). In this area, metabolomics is a powerful tool, for its ability to simultaneously characterize and quantify multiple metabolites (Shepherd *et al*., 2011). Recently, biomarkers associated with the defence response to downy mildew were identified in a resistant cultivar after leaf inoculation with *P. viticola* (Chitarrini *et al*., 2017). While most of these studies in grapevine are focused in the metabolic profile of host-pathogen interactions, little is known about the constitutive differences of resistant and susceptible cultivars that could undoubtedly discriminate both groups.

One of the first studies on the discrimination of downy mildew resistant and susceptible grapevine varieties followed a metabolic profiling approach using nuclear magnetic resonance (NMR) (Figueiredo *et al*., 2008). About 13 metabolites were identified and 7 were differentially accumulated in 'Regent' (more resistant to mildew) and 'Trincadeira' (susceptible). To substantially increase metabolome coverage, in the present work we analysed the same cultivars by Fourier Transform Ion Cyclotron Resonance mass spectrometry (FT-ICR-MS). Due to its ultra-high-resolution and ultra-high-mass accuracy, this technology is one of the best tools to fingerprint complex samples, allowing very high metabolome coverage (Maia *et al*., 2016). With this untargeted metabolomics analysis, we increased the number of features detected, significantly increased the ones exclusively present in 'Trincadeira' or 'Regent' and identified several metabolites that discriminate them.

MATERIALS AND METHODS

Plant Material

Vitis vinifera accessions 'Trincadeira' and 'Regent' were collected at the Portuguese Ampelographic Grapevine Collection (CAN, international code PRT051), INIAV-Dois Portos. For each accession, the third to fifth leaves (from the shoot apex) were harvested from 5 fully developed plants and combined in 1 biological replicate, being collected 3 biological replicates for analysis.

Metabolite extraction and FT-ICR-MS analysis

Metabolite extraction was performed as previously reported (Maia *et al*., 2016). Only the methanol fraction was analysed by direct infusion on the 7T-FT-ICR mass spectrometer (Brüker Daltonics), in positive electrospray ionization (ESI) mode. The internal standard leucine enkephalin (YGGFL, Sigma Aldrich Portugal) was added to all samples at a final concentration of 0.5 μg/mL, being considered a mass of $[M+H]^+ = 556.276575$ Da for analysis by ESI^+. Spectra were recorded between 100 and 1000 m/z. Spectra analysis and alignment were performed as previously reported (Maia *et al*., 2016).

Multivariate analysis

Multivariate analysis considering peak intensities (Principal Component Analysis (PCA) and Partial Least Squares - Discriminant Analysis (PLS-DA)) was performed using the program MetaboAnalyst (http://www.metaboanalyst.ca/, Xia *et al*., 2015). Missing value imputation was done by substitution by half of the minimum positive value found within the data. Intensity data were normalized to the internal standard (leucine enkephalin), generalized log transformed, and Pareto scaled prior to multivariate methods.

Metabolite identification

The search for candidate compounds among the peaks that clearly discriminate the 'Trincadeira' and 'Regent' cultivars was performed in the MassTRIX database (http://masstrix3.helmholtz-muenchen.de/, Suhre & Schmitt-Kopplin, 2008). Mass lists were searched in positive ionization mode, considering the adducts M+H, M+K and M+Na and 2 ppm as maximum m/z deviation from theoretical mass. *Vitis vinifera* was the selected organism and the search was performed in the combined database of KEGG (Kyoto Encyclopedia of Genes and Genomes) / HMDB (Human Metabolome Data Base) / LIPID MAPS without isotopes.

RESULTS AND DISCUSSION

Metabolomics has been widely used to discriminate samples based on the natural variance in metabolite content (Plumb *et al*., 2006; Gougeon *et al*., 2009; Rhourrhi-Frih *et al*., 2012; Becker *et al*., 2013). In this approach, metabolite identification is not a requirement for sample discrimination (Plumb *et al*., 2006; Becker *et al*., 2013). We performed an untargeted metabolomics analysis by FT-ICR-MS to compare the metabolome between two *V. vinifera* cultivars presenting different degrees of resistance against downy mildew, 'Trincadeira' and 'Regent'. A total of 1912 peaks were detected in *V. vinifera* 'Regent' and 1615 in 'Trincadeira'. Of these, 665 were exclusive to 'Regent' and 368 to 'Trincadeira'. When compared to NMR analysis of the same cultivars (Figueiredo *et al*., 2008), the metabolic profiling by FT-ICR-MS significantly increased metabolome coverage and the number of cultivar-specific features observed. PCA showed a clear separation between the two cultivars for the first two components (Figure 1A). This separation was confirmed by sample hierarchical clustering using the Euclidean distance for the most significantly different peaks (Figure 1B). Seeking the features most responsible for these differences, we obtained the top 15 of the most discriminatory peaks of a PLS-DA classification model using Variable Importance in Projection (VIP) scores (Figure 1C). Features with a higher VIP score (normally above 1) are regarded as significant in a given model. The top 15 most discriminatory peaks between 'Regent' and 'Trincadeira' present VIP scores between 2 and 3, being therefore highly important in this model.

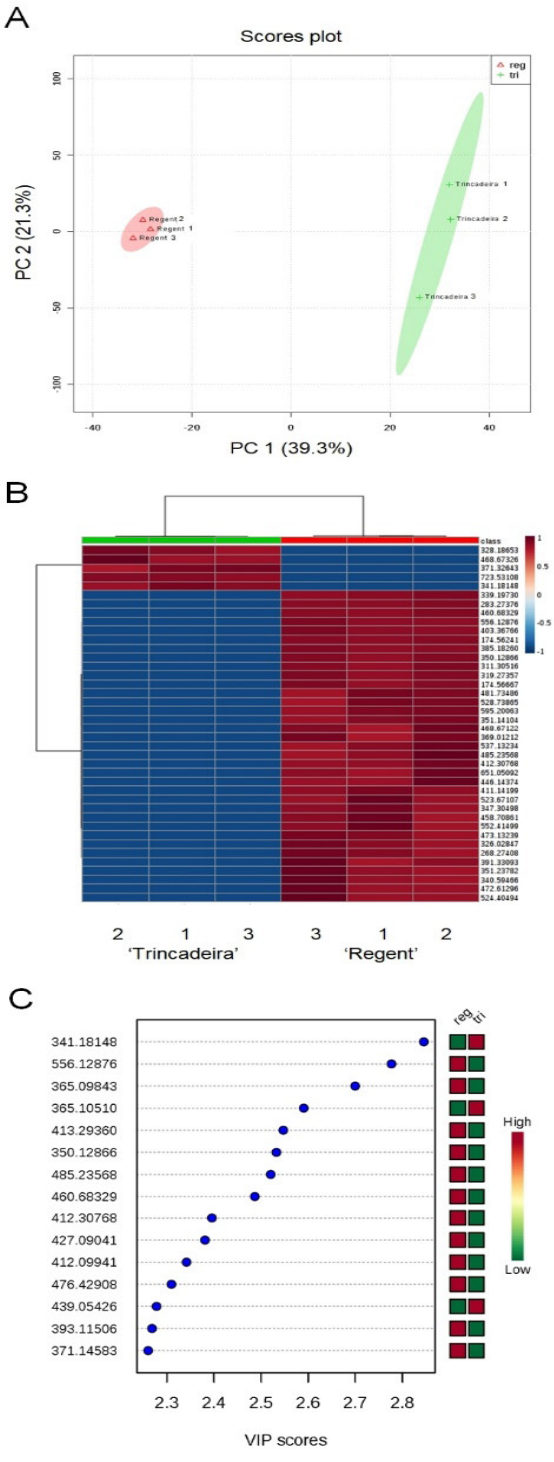

Figure 1 - Metabolome difference and discrimination between 'Trincadeira' and 'Regent'. (A) PCA scores with sample labels and 95% confidence regions shown. (B) Sample hierarchical clustering and heatmap using the top 50 most significant (t-test p-values) MS peaks. (C) Top discriminative peaks in a PLS-DA classification model, shown by decreasing scores of Variable Importance in Projection (VIP) over the first component.

The search for candidate compounds among the peaks that clearly discriminate the two cultivars was performed in the MassTRIX database. For *V. vinifera* 'Regent', one of these compounds was identified as caffeic acid 3-glucoside. This result corroborated the previous finding using NMR (Figueiredo *et al*., 2008), which reported the presence of higher amounts of caffeic acid in 'Regent' when compared to 'Trincadeira'. Two other discriminatory compounds for 'Regent' were oleic acid (18:1) and its methyl ester form. In *Arabidopsis thaliana*, oleic acid induces the activation of defence responses mediated by jasmonic acid (JA) and represses the salicylic acid (SA) signalling pathway (reviewed in Lim *et al*., 2017). Indeed, these signalling pathways are highly relevant for grapevine resistance, with the activation of the JA signalling pathway and the interaction between JA and SA being tailored in the defence response against *P. viticola* (Guerreiro *et al*., 2016). Another compound only identified in 'Regent', also associated with lipid signalling, was palmitoleic acid (16:1). This fatty acid also plays a likely role in plant defence against fungal pathogens (reviewed in Lim *et al*., 2017).

CONCLUSIONS

Grapevine breeding approaches offer forward-looking perspectives for an environmental friendly and sustainable viticulture. The discovery of biomarkers will allow a quick and accurate identification of the plantlets that inherited the resistant characteristic soon after germination. Using an untargeted metabolomics approach, based on ultra-high resolution and high-mass accuracy mass spectrometry, we compared two *V. vinifera* cultivars with different degrees of resistance towards the downy mildew. The Portuguese 'Trincadeira' is susceptible to this disease, whereas the interspecific hybrid 'Regent' contains a high degree of resistance. We were able to clearly discriminate both cultivars (without pathogen infection) and identify the most discriminatory compounds. Additionally, we identified features that are exclusive to one or the other cultivar. With the present work, we were able to show the potential of the metabolomics based on ultra-high resolution and ultra-high mass accuracy (FT-ICR). Our work will contribute, not only to grapevine variety discrimination, but also a deeper identification of

compounds that participate in the grapevine resistance mechanisms. Moreover, this approach may also contribute for the development of efficient biomarker assays, based on resistance-associated metabolites, to help future breeding programs and introgression line analysis.

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