

Subtilisin-like proteases and lipid signalling events play an important role in grapevine resistance to downy mildew: a systems biology approach

Uma abordagem de biologia de sistemas revela a importância de proteases serínicas e sinalização por lípidos na resistência da videira ao míldio

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ABSTRACT

The reduction of pesticide use to control pests and diseases is one of modern agriculture's demands. In the grapevine-*Plasmopara viticola* pathosystem, repeated preventive applications of fungicides occur during the vegetative period. The identification of specific mechanisms or molecules with bioactive fungicide properties may lead to new disease control strategies. Following an omics-based system biology approach we were able to identify both subtilisin-like proteases and lipid associated signalling events as key players on grapevine resistance mechanisms. Efforts are being conducted to characterize their role aiming at the definition of alternative control measures towards a sustainable viticulture.

Keywords: grapevine, downy mildew, subtilases, jasmonic acid, immunity

RESUMO

Uma das prioridades para a sustentabilidade da agricultura é a redução da aplicação de pesticidas para o controlo de pragas e doenças. Atualmente é utilizada a aplicação preventiva repetida de fungicidas durante a época de cultivo para controlo do míldio da videira. A identificação dos mecanismos de resistência ou de moléculas com atividade biofungicida é de extrema importância para a definição de novas estratégias de controlo da doença. Utilizando uma abordagem de biologia de sistemas baseada em "Ómicas" (transcritómica, proteómica e metabolómica) foi possível identificar proteases de tipo subtilisina e eventos de sinalização associados a lípidos como mecanismos chave na resposta de resistência da videira ao míldio (*Plasmopara viticola*). Uma caracterização mais aprofundada destes mecanismos é essencial para a definição de medidas de controlo de doenças em prol de uma viticultura sustentável.

Palavras-chave: videira, míldio, subtilases, ácido jasmónico, resposta imunitária

ASSESSING GRAPEVINE-PLASMOPARA VITICOLA INTERACTION BY A SYSTEMS BIOLOGY APPROACH

Grapevine (*Vitis vinifera* L.) is the most widely cultivated and economically important fruit crop in the world, with deep ties to human culture for more than 5000 years. Today the wine industry plays a

key role in many countries' economy, with a global market size of over 29 billion euro (OIV, 2017). In 2016, Portugal reached the 11th position in world wine production with 700 M€ revenue (OIV, 2017). Most of grapevine cultivars used for winegrowing are often affected by fungal and fungal-like pathogens causing severe harvest losses (Gessler *et al.*, 2011). One of the most threatening diseases is the

downy mildew caused by the obligate biotrophic oomycete *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni. This pathogen was introduced in Europe in the 19th century becoming one of the most devastating diseases for today's viticulture. European viticulture employs a large amount of agrochemicals to control fungal diseases and viticulture practices account for 65% of all fungicides used in agriculture (Eurostat report 2007, <http://ec.europa.eu/eurostat>). In the absence of treatments and favorable conditions, *P. viticola* may lead to losses up to 75% in one season (Buonassisi *et al.*, 2017). Even though nearly all *V. vinifera* cultivars are susceptible to *P. viticola*, American grapevine species exhibit natural host resistance against this pathogen and have been used in breeding programs for resistance to grapevine pathogens resulting on *V. vinifera* varieties such as 'Regent' and 'Solaris' (Vitis International Variety Catalogue 2011; <http://www.vivc.de>). However, the defenses of these bred hybrids are being overcome by new *P. viticola* resistance-breaking isolates in Europe (Peressotti *et al.*, 2010). Thus, for a sustainable viticulture it is imperative to get a deeper knowledge on the grapevine resistance mechanisms against *P. viticola* aiming

at defining new friendly approaches to control the disease and to identify resistance candidates for increase of resistance durability after trait introgression in breeding programs.

By combining several 'omic' studies (transcriptome, proteome and metabolome) in a system biology approach (Figure 1) our group has contributed to a deeper knowledge on the early host responses to *P. viticola* attack. Using as a study model two grapevine cultivars, 'Regent' a resistant hybrid and 'Trincadeira', a Portuguese traditional variety highly susceptible to *P. viticola*, we have identified mechanisms and players that may play key roles on the establishment of the incompatible interaction.

The resistant cultivar 'Regent' presents higher accumulation of proteins and metabolites related to primary and secondary metabolism and biosynthetic machinery that may pay for the fitness cost of a constitutive resistance (Figueiredo *et al.*, 2008, 2017). Moreover several genes associated with plant immunity are constitutively expressed, as the case of those encoding subtilisin-like proteases.

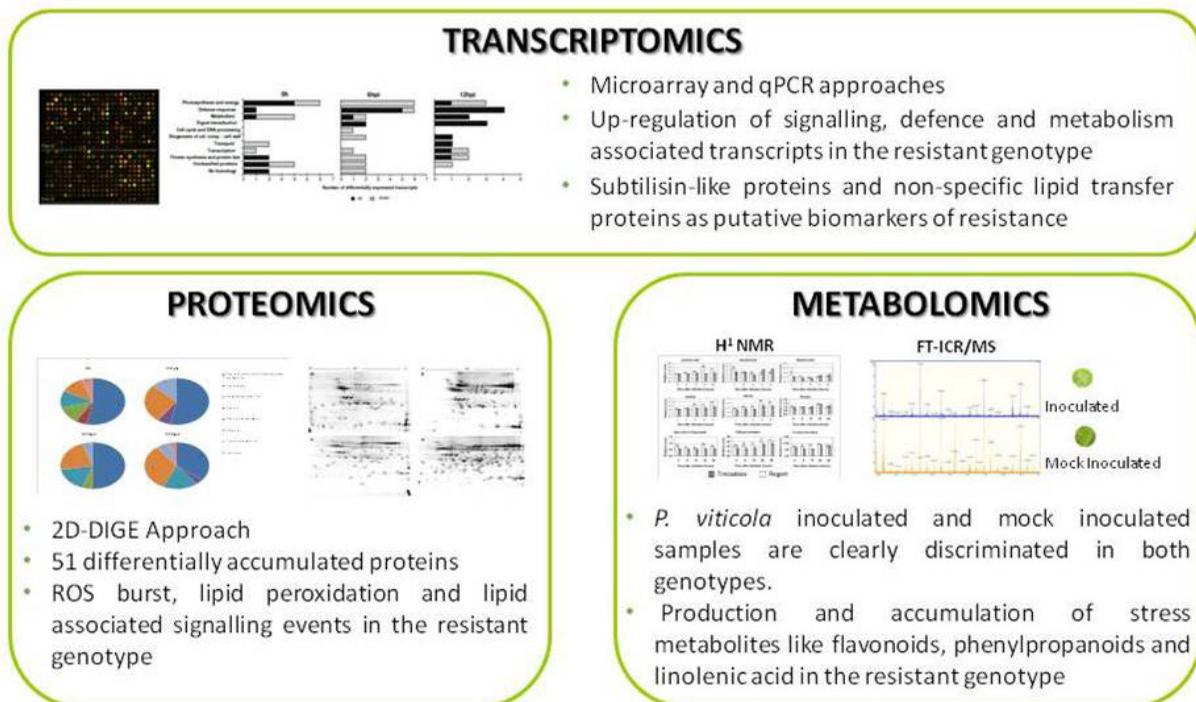


Figure 1 - A systems biology approach based on transcriptomic, proteomic and metabolomic analysis was used to characterize the differences between two grapevine genotypes 'Regent'(resistant) and 'Trincadeira' (susceptible) response to *Plasmopara viticola*.

In the resistant genotype, as soon as 6 hours post inoculation (hpi) a broad reprogramming of the transcriptome occurs with major changes associated to the stress response and signalling events, such as subtilisin-like proteins, IQ calmodulin-binding region- Apoptosis regulator Bcl-2 protein, cyclophilin, leucine-rich repeat (LRR) receptor like proteins, PR10 proteins and CBL-interacting serine/threonine (Figueiredo *et al.*, 2012; Monteiro *et al.*, 2013). Also, both the precursor of jasmonates in the octadecanoid cascade linolenic acid (C18:3) as well as several transcripts and proteins related to lipid signalling events are more accumulated in 'Regent' (Ali *et al.*, 2012; Figueiredo *et al.*, 2012, 2015, 2017; Guerreiro *et al.*, 2016). At the metabolome level the response of the resistant genotype is also characterized by accumulation of phenolic metabolites such as trans-caftaric acid (caffeoyl derivative), trans-fertaric acid (feruloyl derivative) and quercetin-3-O-glucoside (Ali *et al.*, 2012).

In summary, our results point out to two key mechanisms differentiating 'Regent' and 'Trincadeira', one is related to the involvement of serine proteases of the subtilisin family in pathogen recognition and immunity activation and the other is associated to lipid signalling events, particularly to the involvement of jasmonic acid (JA) in grapevine resistance against a biotrophic pathogen as *P. viticola* (Figure 1).

LIPID SIGNALLING AND JASMONIC ACID AS KEY PLAYERS

The signalling pathways associated to grapevine and *P. viticola* interaction are still poorly understood. In plant defense against pathogens, phytohormones such as jasmonates and salicylic acid (SA) have received considerable attention (reviewed in Berens *et al.*, 2017). It is generally assumed that SA is involved in the activation of defense responses against biotrophic and hemi-biotrophic pathogens, as well as the establishment of systemic acquired resistance, whereas inducible defense against leaf-chewing insects and necrotrophic microbes is mediated by jasmonic acid (JA)-dependent signalling (reviewed in Glazebrook, 2005; Antico *et al.*, 2012). These generalities are disputed in grapevine, as JA signalling has been implicated in resistance against biotrophs, such as powdery

and downy mildew (Hamiduzzaman *et al.*, 2005; Belhadj *et al.*, 2006; Trouvelot *et al.*, 2008; Antico *et al.*, 2012; Figueiredo *et al.*, 2015; Guerreiro *et al.*, 2016).

The importance of lipids in signalling processes, during the interaction between grapevine and *P. viticola*, has been highlighted in our recent studies (Ali *et al.*, 2012; Figueiredo *et al.*, 2015, 2017; Guerreiro *et al.*, 2016). We have shown that *Vitis vinifera* 'Regent' presents an early up-regulation of enzymes involved in JA biosynthesis and a later up-regulation for JA signalling enzymes (Guerreiro *et al.*, 2016), together with a higher accumulation of both JA, JA-ILE and SA in the first hours of interaction (Guerreiro *et al.*, 2016). These results provide strong evidences that JA may participate, in resistant cultivar, as a signalling molecule in defense response against *P. viticola* acting synergistically with SA. We have also shown both a higher increase in H₂O₂ accumulation and lipid peroxidation in the resistant genotype (Figueiredo *et al.*, 2017). Non-specific lipid transfer proteins as well as plastid lipid associated proteins (also named fibrillins) were also accumulated in 'Regent' suggesting lipid transport. Fibrillins were shown to act as supports for building lipid droplets that contain free fatty acids, carotenoids, phytols, quinones, and other lipophilic compounds and some of them could be damaged due photooxidative conditions. In stressed plants, an association between fibrillins levels and JA production was observed. The storage of JA precursors, as C18:3, would allow a rapid JA production after oxidative stress. Our results suggest that at the first hours after grapevine inoculation with *P. viticola* lipid associated signalling events occur in the resistant genotype, and that both JA and SA participate in defense response against this biotrophic pathogen. A study by Polesani *et al.* (2010) also corroborates our results on this pathosystem by showing an increase of jasmonates and their biosynthetic enzymes in the resistant grapevine specie *V. riparia* after inoculation with *P. viticola*.

GRAPEVINE SUBTILISIN-LIKE PROTEASES AND *P. VITICOLA* RESISTANCE

Subtilisin-like proteins or subtilases are gaining particular attention in the context of plant-pathogen interactions. Subtilases are serine proteases

that are synthesized as inactive proteins, suffer glycosylation during protein maturation and are secreted to the plant extracellular matrix (ECM) where they accumulate and presumably recognize and process substrates (Bykova *et al.*, 2006; Chichkova *et al.*, 2004; Cedzich *et al.*, 2009, Figueiredo *et al.*, 2017). Plant ECM is where the first host-pathogen interaction and recognition events take place. Thus accumulation of subtilases in the ECM rise the possibility of an important involvement during pathogenesis. In grapevine, the first clues highlighting subtilase participation in defence mechanisms were reported by Figueiredo and co-workers (2008), when comparing resistant ('Regent') and susceptible ('Trincadeira') genotypes prior and post-inoculation with *Plasmopara viticola*. A subtilisin-like protease (XM_010660203.1), identified as a cucumisin was constitutively expressed in the resistant genotype and increased its expression after *P. viticola* inoculation (Figueiredo *et al.*, 2008, 2012; Monteiro *et al.*, 2013). Also in this pathosystem, Gindro *et al.* (2012) have shown that by treating plants with serine protease inhibitors they became more sensitive to *P. viticola*. Recent studies revealed that grapevine subtilases are encoded by a multigenic family comprising 87 genes that encode 109 proteins (Figueiredo *et al.*, 2016, 2017a). We have analysed the expression modulation of 14 grapevine subtilases during *P. viticola* inoculation (Figueiredo *et al.*, 2016). The selected grapevine subtilases shared sequence similarity with Arabidopsis SBT3.3 and tomato P69, already described as participating in plant immunity events (reviewed in Figueiredo *et al.*, 2017b) and were located near *P. viticola* resistance associated loci (*Rpv*). It is clear that in the susceptible genotype there is a delay in the induction of all the subtilase genes studied. In the resistant genotype subtilases, in particular the subtilase *VvSBT4.19* (XM_010660203.1), are highly expressed at 6hpi, suggesting an involvement in the activation of defence responses.

THE LINK BETWEEN SUBTILASE AND PHYTOHORMONE SIGNALLING IN GRAPEVINE RESPONSE TO *P. VITICOLA*

The first clue into a link between subtilases and phytohormones signaling (SA and JA pathways) was shown by the study of Salzman *et al.* (2005)

where a subtilase increased its expression after *Sorghum bicolor* elicitation with methyl jasmonate (MeJA), SA, and aminocyclopropane carboxylic (ACC). It was observed a SA- and MeJA-mediated induction of putative homologs of genes composing the entire octadecanoid pathway for JA synthesis (Salzman *et al.*, 2005).

Moreover, despite little being known about plant subtilases' substrates, the substrates known are a prosystemin (Schaller and Ryan, 1994) and the leucine-rich repeat protein (LRP) (Tornero *et al.*, 1996) a leucine-rich repeat (LRR). Systemin is generated after prosystemin cleavage by subtilases and plays a fundamental role in response to herbivore damage or mechanical wounding through the systemic signalling pathway activation of plant defence genes. Systemin appears to be involved in the activation of the octadecanoid pathway for jasmonic acid biosynthesis, particularly by the activation of a lipase in receptor cell membranes resulting in the release of linolenic acid, the production of jasmonic acid, and the activation of proteinase inhibitor genes (Bergey *et al.*, 1996). Moreover, it was later shown that systemin is perceived at the cell surface by a leucine-rich repeat receptor-like kinase that induces the JA synthesis pathway at the site of wounding as a prerequisite for systemic defence gene induction (Howe and Schaller, 2008; Ryan, 2000; Wasternack *et al.*, 2006).

These results open new insights into the involvement of subtilases in the control of signalling pathways associated to lipids. Further work must be conducted to validate this hypothesis, and particularly in the grapevine-*P. viticola* pathosystem, were both subtilases and JA seem to be key players in the establishment of an incompatible interaction.

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