

PATHOGEN-RELATED SPECIFICITY OF SUBTILASE VVISBT4.19 X1 IN THE *VITIS VINIFERA* DEFENCE RESPONSE

ESPECIFICIDADE PATOGENICA DA SUBTILASE VVISBT4.19 X1 NA RESPOSTA DE DEFESA DA *VITIS VINIFERA*

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SUMMARY

Grapevine, *Vitis vinifera* L., is one of the most cultivated fruit plants worldwide with high economic value. Powdery mildew and gray mold diseases, caused by *Erysiphe necator* and *Botrytis cinerea*, respectively, are within the most devastating diseases, which are controlled by using several fungicide applications over a single growing season. A more sustainable and environmentally friendly alternative for pest control is associated to the development of breeding programs, in which American and Asian *Vitis* species, presenting natural resistance characteristics, are crossed with *V. vinifera* varieties that are susceptible to diseases caused by fungal or oomycete pathogens. As a result, new grapevine varieties that combine the good berry quality with a high degree of resistance to grapevine pathogens are obtained. One example is the *Vitis vinifera* cv 'Regent' that acquired high tolerance degree against *E. necator* and *Plasmopara viticola*. To ensure durable resistance introgression in breeding programs, a full understanding of grapevine defence mechanisms is crucial. Previous studies on grapevine-*P. viticola* pathosystem have suggested the participation of serine proteases in the establishment of the interaction between both organisms, which is the case of VviSBT4.19 X1. The gene expression of this subtilase increases up to 300-fold 6 hours after 'Regent' inoculation with *P. viticola*. Nowadays, no information is available about the participation of subtilases in grapevine response to *E. necator* and *B. cinerea* infection. In the present study, the gene expression profile of VviSBT4.19 X1 in the first hours of 'Regent' inoculation with *E. necator* and *B. cinerea* was analysed to understand its response towards different pathogenic agents.

RESUMO

A videira, *Vitis vinifera* L., é uma das plantas de fruto mais cultivadas em todo o mundo, possuindo um elevado valor económico. As doenças do oídio e da podridão cinzenta, causadas pelo *Erysiphe necator* e pela *Botrytis cinerea*, respetivamente, estão entre as doenças mais devastadoras da videira, cujo controlo é feito através de diversas aplicações de fungicidas ao longo de cada ciclo vegetativo. Uma alternativa mais sustentável para o controlo de pragas está relacionada com os programas de melhoramento, em que espécies de *Vitis* americanas e asiáticas, que possuem características intrínsecas de resistência, são cruzadas com videiras suscetíveis a doenças causadas por fungos ou oomicetes. Como resultado, são desenvolvidas novas variedades que combinam as características que definem a boa qualidade da uva com as que conferem um elevado grau de resistência aos patógenos. Um exemplo é a *V. vinifera* cv 'Regent' que adquiriu um elevado grau de tolerância contra o *E. necator* e o *Plasmopara viticola*. Para garantir uma introgressão duradoura da resistência nestes programas, é essencial compreender os mecanismos de defesa da videira. Estudos anteriores realizados na interação videira-*P. viticola* sugeriram a participação das subtilases no estabelecimento desta interação, como é o caso da VviSBT4.19 X1. Verificou-se que esta subtilase é constitutivamente mais expressa em génotipos de videira tolerantes e que a sua expressão genética aumenta até 300 vezes 6 horas após a infeção do *P. viticola* na cultivar 'Regent'. Até ao momento, não há informações disponíveis sobre a participação das subtilases na resposta de defesa da videira à infeção pelo *E. necator* e *B. cinerea*. Neste estudo foi analisado o perfil de expressão genética da VviSBT4.19 X1 nas primeiras horas após inoculação da cultivar 'Regent' com *E. necator* e *B. cinerea* de forma a compreender a resposta deste gene a diferentes agentes patogénicos.

Key words: Subtilisin-like proteases, grapevine, downy and powdery mildews, gray mold.

Palavras-chave: Proteases do tipo subtilisina, videira, míldio, oídio, podridão cinzenta.

INTRODUCTION

Vitis vinifera L. is a domesticated *Vitis* species as a result of natural and human influences for thousands of years leading to the development of about 5000 different grapevine varieties worldwide (This *et al.*, 2006). In Portugal, there are about 250 different grapevine varieties, placing Portugal in the third position in the world for the number of grapevine varieties (Martins, 2018). In 2018, grapevine was one of the most cultivated crops with over 7.4kHa of plantation and 292 million of hectolitres (mhL) of wine produced (excluding juice and must) (OIV, 2019).

The domesticated grapevines widely used for fruit and wine production do not present a natural resistance to several fungal and oomycete driven diseases. Within grapevine diseases, powdery mildew, caused by the biotrophic obligate oomycete *Erysiphe necator* (Schwein.) is one of the most destructive ones. This oomycete depends on genus *Vitis* to growth and development, and for infection it needs warm temperatures (23 °C to 30 °C) and relative humidity of approximately 85% (Gadoury *et al.*, 2012). To penetrate the leaf, *E. necator* needs to cause a wound on the adaxial face of the leaf, so the conidium attaches to the tissue cells allowing the formation of a primary germ tube that differentiates into a specialized infectious structure, the appressorium. This causes a mechanical pressure on the leaf surface which results in the penetration of the pathogen in the host cells. After invasion, *E. necator* develops an haustoria that penetrates the cell wall of the host for pathogen feeding. After haustoria establishment, a secondary hypha spreads within the leaf tissue and asexual reproductive bodies (conidiophores and conidia) emerge from them. Disease symptoms appear as a white-greyish powder on the surface of the infected tissue mostly leaves and stems. The *E. necator* infection causes a declining in the sugar content and acidity of the berries leading to a reduction of fruit quality (Armijo *et al.*, 2016).

Infection caused by necrotrophic fungi also leads to several yield losses. These pathogens take advantage of existing wounds to develop their infection structures. One of the most important grapevine diseases caused by a necrotrophic fungus is the “gray mold”, whose causal agent is the *Botrytis cinerea* Pers. Fr. This fungus is not specific to the genus *Vitis* and it is able to live as a parasite in green tissues and as a saprophyte in dead tissues. Fluctuations in temperature and humidity, like a rapid decrease in humidity with a rise in temperature in the early morning, cause a drying of the conidiophores with a consequent release of conidia into air currents (Jarvis,

1962). In the host, when the conidia recognize the surface nutrients, this fungus develops an appressorium that disrupt the cuticle through a penetration peg. To help in plant cell penetration, appressorium also secretes several lytic enzymes to cross cuticle and outer epithelial wall. This primary lesion leads to an oxidative burst that helps the fungus to kill and degrade the host tissue. The most typical symptoms on leaves and soft fruits are the appearance of grey masses of conidia as well as the collapse and water-soaking of parenchyma tissues. “Gray mold” disease appears along all the growing season, but most commonly occurs near the harvest time, affecting complete berry clusters and thus becoming one of the most important pathogens that affects table grapes and wine production (Dean *et al.*, 2012; Armijo *et al.*, 2016).

The current strategy to control these diseases is the extensive use of fungicides leading to severe environmental impacts, namely through soil and groundwater contamination. A sustainable strategy to fight these diseases is the development of new grapevine varieties through breeding programs. In these programs, the natural resistant traits found in American and Asian *Vitis* species are introgressed into the genome of natural susceptible *V. vinifera* cultivars through plant crossing. The new varieties will combine good berry quality with a high degree of tolerance to grapevine pathogens (Gessler *et al.*, 2011). One successful example is the *V. vinifera* cv ‘Regent’, a plant breeding resulting from the cross between *V. vinifera ssp vinifera* cv. ‘Diana’ and the interspecific hybrid ‘Chambourcin’ (created by Professor Gerhardt Alleweldt, in 1967, at the Geilweilerhof Institute for Grape Breeding, Germany). ‘Regent’ presents high tolerance degree to the main pathogens that affect European viticulture, such as *Plasmopara viticola* (Resistance Locus RPV3.1) and *E. necator* (Resistance Loci Ren3 and Ren9) (Maul and Töpfer, 2015).

To ensure durable resistance introgression in breeding programs, a full understanding of grapevine defence mechanisms is crucial, particularly the response towards different pathogens. Previous studies on *grapevine-P. viticola* interaction suggested the participation of serine proteases in the establishment of the incompatible interaction (Figueiredo *et al.*, 2016). Subtilases are serine proteases involved in several plant biological functions (Figueiredo *et al.*, 2018). Although plant subtilases participate in several biological processes, including mobilization of storage proteins during seed germination to the initiation of cell death and senescence, they have been increasingly associated with plant defence against several pathogens, including biotrophs, hemibiotrophs, necrotrophs, insects and nematodes

(Figueiredo *et al.*, 2018). Interestingly, in the same plant, different subtilases can be induced as a response to different pathogens (Figueiredo *et al.*, 2018). Specific subtilases have already been described as associated with a particular defence mechanism and pathosystem (Figueiredo *et al.*, 2018). For example, in cotton, a subtilase called GsSBT1 was related to the induction of plant resistance against *Verticillium dahliae*, a necrotrophic fungus (Duan *et al.*, 2016). In tomato, there are several evidences of the role of P69B subtilase in response to *P. infestans* and *P. syringae* (Zhao *et al.*, 2003; Tian *et al.*, 2004; Tian, 2005). Also, in potato, several subtilase genes have been shown to increase their expression after *P. infestans* infection (Norero *et al.*, 2016). In grapevine, little is known about the role of subtilases in plant response to environmental stimuli.

Previous results showed that the grapevine subtilase *VviSBT4.19 XI* is constitutively over-expressed in several grapevine genotypes tolerant to *P. viticola*, like 'Regent', and other *Vitis* species, increasing its expression up to 300-fold 6 hours after *P. viticola* infection in 'Regent' cultivar (Figueiredo *et al.*, 2016). At this time point, in grapevine-*P. viticola* interaction, recognition of pathogen effectors by plant membrane receptors may occur triggering an immune response. The involvement of this protease in the grapevine defence response to this oomycete was suggested. In grapevine-*E. necator* and grapevine-*B. cinerea* interactions there is no information regarding subtilase, particularly *VviSBT4.19 XI*, involvement in the plant response to pathogen inoculation. Hence, in this study, the expression of the *VviSBT4.19 XI* subtilase in the response of *V. vinifera* cv 'Regent' to *E. necator* and *B. cinerea* pathogens was analysed in order to understand this subtilase specificity.

MATERIAL AND METHODS

Plant material

The obtained *Vitis vinifera* cv. Regent, bred by multiple introgressions from resistant wild genotypes (Welter *et al.*, 2017), was used in this study. Young fully expanded leaves, the 2nd to 3rd from the shoot apex, from field plants were collected in the Portuguese Grapevine Germplasm Bank (Veloso *et al.*, 2010). Portuguese Ampelographic Collection (PRT051) is property of National Institute of Agrarian and Veterinary Research (INIAV), a governmental institute. It is located at 40 Km of north of Lisbon, in Quinta da Almoíña, Dois Portos (9°11'19''W; 39°02'31''N; 75 m above sea level). This collection was established in 1988, and was

replicated to a new place in 2013 and 2014. All accessions are grafted on SO4 rootstock and each accession is represented by seven plants. Vine pruning system is royat double cordon, leaving only four spurs in each arm. These spurs are yearly pruned leaving only eight to nine buds in each arm and a total of 16 to 18 buds in each plant, depending upon the vigour of the plant. All agronomic treatments and canopy maintenance in the field are applied as in commercial vineyards depending on the annual weather. For this study, leaves from *Vitis vinifera* cv 'Regent' plants were harvested and washed in a bleach solution (5.5% (v/v) sodium hypochlorite) during 1 min and 30 s, and then rinsed three times in distilled water. Leaves were air dried and placed in 0.8% (w/v) agar plates with the adaxial face up for *Erysiphe necator* inoculation and with the abaxial face up for the *Botrytis cinerea* inoculation. The data presented was the result of a preliminary assay. In this assay, 12 complete leaves, collected from the seven field plants, were used per treatment. Each biological replicate is represented by four leaves, from different plates.

Erysiphe necator inoculation assay

For inoculation, field grapevine leaves with symptoms of powdery mildew were collected, and *Erysiphe necator* spores were brushed directly to the leaves in the agar plates (average value of 8483 spores/mm² of leaf). Control samples without inoculation were also made. After inoculation, plates were kept in a climate chamber under a 16/8 h (light/dark) at 25 °C and with a relative humidity ranging between 40 and 95%. Leaves were collected at 6 and 24 hours post inoculation (hpi), immediately frozen in liquid nitrogen and stored at -80 °C. Three independent biological replicates were obtained.

Botrytis cinerea inoculation assay

Botrytis cinerea was cultured in Petri dishes on potato dextrose agar (PDA) medium (Merck, Germany) at 20 °C for 8 days. Conidial suspension was obtained by flooding the fungal culture with sterile distilled water and rubbing the mycelium. The conidial suspension was adjusted with sterile distilled water to 2.5×10⁵ conidia/mL. Inoculum solution concentration was confirmed using a Neubauer counting chamber. For inoculation, grapevine leaves were dipped in the conidia solution and placed on the agar plates. Negative controls using water were also made. The plates were kept in a climate chamber under a 16/8h (light/dark) with temperature and relative humidity ranging between 22 °C - 25 °C and 60 - 90%, respectively. Samples were collected at 6 and 24 hpi, immediately frozen in liquid nitrogen, and stored at

-80 °C. Three independent biological replicates were obtained.

RNA extraction and cDNA synthesis

Total RNA was isolated from frozen leaves using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. Residual genomic DNA (gDNA) was digested with DNase I (On-Column Dnase I Digestion Set, Sigma-Aldrich, USA). RNA integrity was verified by agarose gel electrophoresis (1.2% (w/v) agarose in TAE buffer). RNA purity and concentration were measured at 260/280 nm in a spectrophotometer (Nanodrop-1000, Thermo Fisher Scientific, USA). The presence of gDNA in RNA samples was assessed by qPCR analysis (Vandesompele *et al.*, 2002). Complementary DNA (cDNA) was synthesized from

2 µg of total RNA using RevertAid® H Minus Reverse Transcriptase (Thermo Fisher Scientific, USA), anchored with Oligo(dT)₂₃ primer (Thermo Fisher Scientific, USA), according to the manufacturer's instructions.

Quantitative real-time PCR (qPCR)

Quantitative real time PCR (qPCR) experiments were carried out using Maxima™ SYBR Green qPCR Master Mix (2x) kit (Thermo Fisher Scientific, USA) in a StepOne™ Real-Time PCR system (Applied Biosystems, USA). A final concentration of 2.5 mM MgCl₂ and 0.2 µM of each primer were used in 20 µL volume reactions, together with 4 µL of cDNA (diluted 1:40) as template. Primer sequences and reaction details are provided in Table I.

Table I

Target and reference gene oligonucleotide sequences employed in the current study
Sequências oligonucleotídicas dos genes de interesse e de referência usadas no presente estudo

Identifier/ NCBI Accession Number	Primer sequence	Amplicon length (bp)	Amplification efficiency (E)	T _a (°C)	T _m (°C)
Reference gene					
<i>EF1a</i>	F:GAACTGGGTGCTTGATAGGC	164	1.99	60	79.5
XM_002284888.3	R:ACCAAATATCCGGAGTAAAAGA				
<i>GADPH</i>	F:TCAAGGTCAAGGACTCTAACACC	226	1.98	60	80.5
XM_002263109.3	R:CCAACAACGAACATAGGAGCA				
<i>SAND</i>	F:CAACATCCTTTACCCATTGACAGA	76	1.94	60	78.6
XM_002285134.3	R:GCATTTGATCCACTTGCAGATAAG				
Target gene					
<i>VviSBT4.19 XI</i>	F:AATCCTGGTGTCTTGTGG	73	2.04	58	72.3
XM_010660203.2	R:ATTAGGTAATAATGTTGTGCTTG				

Thermal cycling for all genes started with a denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing (Table 1) for 30 s. For each set of reactions, a negative control without cDNA template was included. Three biological and two technical replicates were analysed for each treatment. Dissociation curves were used to analyse non-specific PCR products (data not shown). Gene expression

(fold change) was calculated using Hellemans method (Hellemans *et al.*, 2007). The reference genes used for the normalization were Elongation factor 1-alpha (*EF1a*) (Pessina, 2016) and Glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) (Welter *et al.*, 2017) for the *V. vinifera-E. necator* interaction and *EF1a* (Gruau *et al.*, 2015) and SAND family protein (*SAND*) (Nanni *et al.*, 2014) for the *V. vinifera-B. cinerea* interaction. Statistical significance ($p < 0.05$)

of gene expression was determined by the Mann–Whitney U test using IBM® SPSS® Statistics version 23.0 software (SPSS Inc., USA).

RESULTS AND DISCUSSION

In *Vitis vinifera* cv ‘Regent’ plants inoculated with *Plasmopara viticola*, a subtilase, *VviSBT4.19 XI*, potentially involved in the establishment of an effective defence response against pathogens, was identified (Figueiredo *et al.*, 2016). In the present work, the gene expression of *VviSBT4.19 XI* subtilase in ‘Regent’ after inoculation with the biotrophic pathogen *Erysiphe necator* and with the necrotrophic fungus *Botrytis cinerea* was accessed. The aim is to understand if *VviSBT4.19 XI* is associated to host-pathogen specificity or if it is part of a broader defence mechanism against pathogens with different infection strategies.

V. vinifera cv ‘Regent’ leaves were inoculated either with *E. necator* or *B. cinerea* and harvested at 6 and 24 hpi. In ‘Regent’ inoculated with *E. necator*, no

significant difference in gene expression between inoculated and non-inoculated leaves after 6 hpi (1.86 ± 0.91 fold) was observed, while a slight down-regulation at 24 hpi (0.55 ± 0.06 fold) was detected (Figure 1). After inoculation of ‘Regent’ leaves with *B. cinerea*, no significant differences in *VviSBT4.19 XI* gene expression were also observed, in both analysed time-points (6 hpi: 0.84 ± 0.12 fold; 12 hpi: 1.09 ± 0.09 fold) – Figure 1. These results show that the infection of ‘Regent’ by these two pathogens does not appear to induce a significant variation in the level of expression of the *VviSBT4.19 XI* gene. As such, it seems that this particular subtilase did not have a relevant role in the defence response of ‘Regent’ to *E. necator* and *B. cinerea*, at least in the first hours after inoculation. However, when compared to our previous results on ‘Regent’ inoculated with *P. viticola*, in which *VviSBT4.19 XI* presented a significant expression increase at both 6 and 24 hpi, our combined results may suggest some specificity of this subtilase towards *P. viticola*.

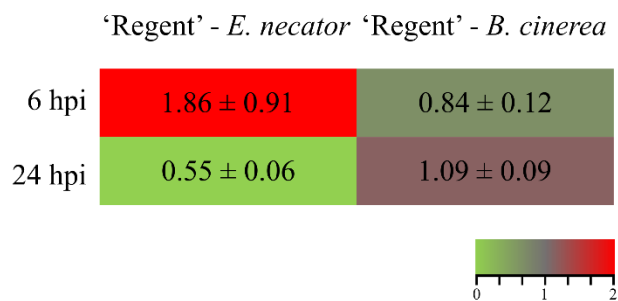


Figure 1. Gene expression profile of *VviSBT4.19 XI* in *Vitis vinifera* cv. ‘Regent’ leaves at 6 and 24 hours after inoculation (hpi) with *Erysiphe necator* and *Botrytis cinerea*.

Perfil de expressão genética de VviSBT4.19 XI em folhas de Vitis vinifera cv. 'Regent' às 6 e 24 horas após a inoculação (hpi) com Erysiphe necator e Botrytis cinerea.

Specificity of host–pathogen associations can be explained to a large extent by major genes of hosts and pathogens, as in the gene-for-gene model of plant–pathogen compatibility (Jones and Dangl, 2006). In fact, several studies have pointed out that some subtilases, particularly the tomato P69, are induced by a specific pathogen (Vera and Conejero, 1988; Vera *et al.*, 1989; Tornero *et al.*, 1996a). P69 is responsible for the cleavage of a leucine-rich repeat protein (LRP) associated with the extracellular matrix, contributing for the plant immunity establishment (Tornero *et al.*, 1996b).

Few studies have been conducted in serine proteases role in grapevine–pathogen interaction. Transcriptome

characterization studies have shown that several serine proteases are modulated in response to *P. viticola* (Figueiredo *et al.*, 2012), *E. necator* (Weng *et al.*, 2014) and *B. cinerea* (Dadakova *et al.*, 2015). However, their role still remains uncovered. In grapevine–*P. viticola* interaction, studies have highlighted a specificity between *P. viticola* secretome and plant serine proteases. Gindro *et al.* (2012) have hypothesized that *P. viticola* inhibits the endogenous subtilases of susceptible varieties, thereby inhibiting the plant’s normal defence reaction, whereas resistant or immune varieties may possess endogenous subtilases that are not recognized because of slight structural modifications of the

protein patterns of these cultivars (Gindro *et al.*, 2012).

A full understanding of serine proteases role in grapevine-pathogen interactions is crucial. These results provide the first clues suggesting that host-pathogen specificity may occur in the case of *VviSBT4.19 XI*.

CONCLUSIONS

In the present work, the gene expression of *VviSBT4.19 XI* in 'Regent'-*E. necator* and 'Regent'-*B. cinerea* interactions was analysed and no significant up-regulation of gene expression was observed in both pathosystems. These results lead us to hypothesize a host-pathogen specificity for *VviSBT4.19 XI*. However, until now, it has not been proved that, within a plant genotype, a subtilase participates in the defence response exclusively against a specific pathogen. Genes like *VviSBT4.19 XI*, which are only activated in response to a specific pathogen, are strong resistance biomarkers that can be used in the screening of new grapevine crossing lines in breeding programs. Further work is being developed to gain more information into *VviSBT4.19 XI* specificity, namely full time-course assays with the three pathogens.

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