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Revisiting *Vitis vinifera* subtilase gene family: links to grapevine resistance against *Plasmopara viticola*

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Declaração

De acordo com o disposto no artigo n.º 19 do Regulamento de Estudos de Pós–Graduação da Universidade de Lisboa, Despacho n.º 2950/2015, publicado no Diário da República, 2.ª série — N.º 57 — 23 de março de 2015, esclarece-se ser da minha responsabilidade a execução das experiências que estiverem na base dos resultados apresentados (exceto quando referido em contrário), assim como a interpretação e discussão dos mesmos.

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Joana Frazão Figueiredo

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Resumo

Proteases do tipo subtilisina, também conhecidos por subtilases, são uma família de proteínas muito diversa e a segunda maior dentro das peptidases de serina, e que existe nos mais variados organismos. Nas plantas, as subtilases são especialmente abundantes, como por exemplo, em *Oryza sativa* (arroz) são conhecidos 63 genes, em *Arabidopsis thaliana* são conhecidos 56 genes e em *Lycopersicum esculentum* (tomate) estão presentes pelo menos 15 genes. Estes proteases funcionam como enzimas secretores e são direcionados para a retículo endoplasmático migrando depois para a membrana plasmática da célula. A maioria dos subtilases são sintetizados como pré-pro-proteínas precursoras inativas, apresentando um péptido sinal, um pró-domínio (domínio inibidor I9), um domínio subtilase (domínio peptidase S8) e um domínio associado a proteases (PA, *protease-associated*) localizado no interior do domínio S8. Curiosamente, alguns subtilases podem ter apenas um ou dois destes domínios ou até domínios adicionais. Os subtilases apresentam uma tríade catalítica extremamente conservada localizada dentro do domínio S8 peptidase, constituída por um resíduo de aminoácido de aspartato (Asp), um de histidina (His) e um de serina (Ser), podendo alguns subtilases apresentar também um outro resíduo catalítico conservado de asparagina (Asn) dentro do mesmo domínio. Normalmente, os subtilases de plantas apresentam uma estrutura monomérica, apesar de vários estudos sugerirem que muitos deles possam sofrer uma homo-dimerização mediada pelo domínio associado a proteases (PA) de modo a serem ativados. Uma outra característica destes proteases é a aparente independência de cálcio (Ca^{2+}), contrariamente ao que é conhecido para outros proteases. Em plantas, os subtilases estão envolvidos nas mais diversas funções biológicas, desde a mobilização de proteínas de armazenamento durante a germinação de sementes, até à iniciação de programas de senescência e morte celular. Evidências recentes realçaram também a participação de subtilases na resposta a estímulos ambientais bióticos e abióticos, bem como nas interações simbióticas das plantas com outros organismos. Em 1987 foi demonstrado pela primeira vez o envolvimento de subtilases na resposta de defesa em plantas através da identificação da acumulação de um subtilase, o P69, nas folhas de tomate após infeção com o viroide *citrus exocortis*. Mais recentemente, estudos em *Arabidopsis thaliana* identificaram o gene SBT3.3 cuja expressão aumenta muito rapidamente durante a ativação da resposta imune inata, seguida da ativação de genes de resposta ao ácido salicílico (SA). Outros estudos demonstraram também que os subtilases são glicosilados e secretados para a matriz extracelular da planta. Uma vez que é nesta matriz que acontece a primeira interação hospedeiro-patogénio, o respetivo reconhecimento e consequente sinalização, a acumulação de subtilases neste espaço extracelular pode ter um

papel importante durante a patogenicidade. Recentemente foi relatada a expressão constitutiva de um subtilase numa videira resistente e um aumento da expressão desta proteína após inoculação com o oomycete *Plasmopara viticol*a (Berk. et Curt.) Berl. et de Toni, o agente causador do míldeo em videira (*Vitis vinifera*). Este subtilase apresenta elevada homologia com o P69 de tomate. Apesar de recentemente várias hipóteses serem colocadas relativamente à presença e importância das subtilases em *Vitis vinifera*, estas proteínas ainda não foram associadas com a resposta imunitária da videira, particularmente contra o *Plasmopara viticola*. Uma vez que as videiras cultivadas (*Vitis vinifera* L.) são atualmente a mais importante planta de fruto cultivada em todo o mundo e altamente suscetível a várias doenças incluindo o míldio causado pelo *P. viticola*, a importância das subtilases na resistência deve ser explorada.

Em 2014, a família de subtilases em *Vitis vinifera* foi preliminarmente caracterizada. Simultaneamente, uma nova anotação do genoma de videira foi publicada, havendo genes que deixaram de ter anotação e outros que mudaram de nome. Assim, o principal objetivo deste projeto foi realizar uma re-caracterização genómica da família de subtilases de videira, tomando como referência a re-anotação do genoma de videira que ocorreu em 2014, e associar alguns desses genes com a resposta de defesa da *Vitis vinifera* contra o *Plasmopara viticola*. Para cumprir este objetivo, foram realizadas pesquisas em bases de dados considerando os três domínios conservados característicos de subtilases (domínios peptidase S8, PA e inibidor I9), que conduziram à identificação de 85 genes de subtilases em videira, desigualmente distribuídos ao longo de 15 dos 19 cromossomas da videira. Verificou-se que estes genes codificam 97 possíveis proteínas (resultantes de eventos de *splicing* alternativo). Estes subtilases foram organizadas em 6 grupos de acordo com a semelhança entre a sequência das proteínas, resultante de uma análise filogenética. Uma análise a nível da localização subcelular demonstrou que a maioria dos subtilases de videira estão localizados no apoplasto, na parede celular ou na região extracelular. A comparação dos subtilases de videira com subtilases de *Arabidopsis thaliana* e *Solanum lycopersicum* demonstrou que, uma elevada percentagem dos mesmos, partilham grande semelhança de sequência com os subtilases SBT3.3 e P69. Estes proteases já tinham sido anteriormente relacionados com a resposta de defesa da *Arabidopsis* e do tomate, respetivamente, contra estímulos ambientais bióticos. Para além disso, foi demonstrado neste estudo que alguns genes de subtilases em videira estão localizados perto de genes associados à resistência da *Vitis vinifera* contra o *Plasmopara viticola*.

O segundo objetivo deste projeto foi realizar estudos de expressão de genes de forma a elucidar a participação de subtilases de videira anteriormente selecionadas na resistência da *Vitis vinifera* contra o *Plasmopara viticola*. Esta expressão foi analisada em duas cultivares de *V.*

vinifera (Regent e Trincadeira) após inoculação com o oomycete *Plasmopara viticola.* Para além disso, o padrão de expressão constitutivo destes subtilases foi analisado em várias espécies selvagens de *Vitis* e cultivares de *Vitis vinifera* que apresentam vários graus de resistência quando infetadas com o *P. viticola*. Os resultados sugeriram que, sob ataque do patogénio, as cultivares resistentes têm uma rápida resposta através do aumento da expressão de alguns subtilases. Este rápido aumento pode estar relacionado com o estabelecimento imediato de uma estratégia defensiva contra o patogénio invasor. Por outro lado, a cultivar suscetível demonstrou um atraso no aumento da expressão de subtilases, que pode estar relacionado com a sua tentativa de iniciar uma estratégia defensiva, mas que não é rápida nem robusta o suficiente para prevenir a invasão e o crescimento do patogénio. Ao nível constitutivo, em várias espécies e cultivares de videira não foi observado um padrão de expressão de subtilases. Ambos os resultados sugerem que existe uma expressão diferencial de certos subtilases nas espécies resistentes, mas esta só acontece após estimulação da planta com o ataque do patogénio, o que suporta a hipótese de que alguns subtilases de videira podem ter um papel importante na resposta de defesa contra o *Plasmopara viticola*.

Em último lugar, dois subtilases de videira foram selecionados com base nas suas características e nos seus perfis de expressão e foram clonados com o intuito de expressar a respetiva proteína recombinante.

Do que se sabe até agora, este estudo é o primeiro a realizar uma caracterização em larga escala de subtilases de videira associados à resposta de imunidade contra o patogénio responsável pelo míldeo. Estudos futuros serão realizados com o intuito de caracterizar as proteínas recombinantes, elucidar respetivas estruturas e identificar possíveis substratos, a fim de estabelecer estes subtilases como candidatos para a introgressão de genes em programas de melhoramento.

Palavras-chave*:* Subtilases, *Vitis vinifera*, *Plasmopara viticola*, imunidade, expressão de genes

Summary

Subtilisin-like proteases, also known as subtilases, are a very diverse family of serine peptidases present in many organisms. In plants, subtilases are especially abundant with, e.g., 63 known genes in *Oryza sativa*, 56 genes in *Arabidopsis thaliana* and 15 in *Lycopersicon esculentum*. These proteins act as secretory enzymes and are targeted to the endoplasmic reticulum migrating to the cell plasma membrane. The majority of plant subtilases are synthesized as an inactive pre-pro-protein precursor formed by a signal peptide, a pro-domain (I9 inhibitor domain), a subtilase (S8 peptidase) domain, and a protease-associated (PA) domain located within the subtilase domain. Moreover, some of them may have only one or two of these domains or even additional domains. Subtilases present a highly conserved catalytic triad within the S8 peptidase domain, constituted by aspartate (Asp), histidine (His) and serine (Ser) amino acid residues, and some subtilases may have also a conserved catalytic asparagine (Asn) residue. Concerning protein structure, plant subtilases are generally monomeric, although several studies have indicated that many subtilases suffer a homo-dimerization mediated by PA domain in order to become activated. In plants, subtilases are involved in many biological functions from the mobilization of storage proteins during seed germination to the initiation of cell death and senescence programs. Recent evidences have also highlighted the participation of subtilases in response to biotic and abiotic environment stimulus and in symbiotic interactions of plants with other organisms. In 1987 was demonstrated for the first time the involvement of subtilisin-like proteases in plant defence response with the identification of subtilase P69 accumulation in tomato leaves after the infection with citrus exocortis viroid. More recently, studies in *A. thaliana* identified the SBT3.3 gene which expression rapidly increases during the activation of innate immunity preceding the activation of salicylic acid (SA) responsive genes. Studies have also shown that subtilases are glycosylated and secreted to the plant extracellular matrix (ECM). Since ECM is where the first host-pathogen interactions, recognition and signalling events take place, the accumulation of subtilases in this cellular location may account for an important role during pathogenesis.

Recently, was reported a constitutively expression of a subtilisin-like protein, sharing high sequence similarity with the tomato subtilases P69C, in a resistant grapevine and an increase of protein expression after inoculation with the oomycete *Plasmopara viticola.* Besides these clues of the presence and importance of the subtilases in *V. vinifera*, these proteins were not yet associated with grapevine immune responses particularly concerning *P. viticola* resistance. As cultivated grapevine (*Vitis vinifera* L.) is currently the most important fruit plant cultivated

worldwide and highly susceptible to several disease including downy mildew, caused by *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni, the importance of subtilases in resistance should be further investigated.

In 2014, the subtilase family in *Vitis vinifera* was preliminarily characterized but at the same time a new annotation of the grapevine genome was published. Thus, the main purpose of this project was perform a genome-wide update of the grapevine subtilase gene family and associate some subtilase genes with the defence response of the *V. vinifera* against the *P. viticola*, taking as reference the grapevine genome reannotation that occurred in 2014. For that, several database searches were performed considering the three domains (S8, PA and I9) leading to the identification of 85 grapevine subtilase genes, unevenly distributed among 15 of the 19 grapevine chromosomes. From these genes, it was predicted to obtain 97 subtilase proteins, which were organized into 6 groups accordingly with their similarity. An analysis at subcellular location level, showed that the majority of the grapevine subtilases were located in apoplast, cell wall or extracellular region. Comparison of the grapevine subtilases with *Arabidopsis thaliana* and *Solanum lycopersicum* subtilases showed that a high percentage of them shared high sequence similarity with SBT3.3 and P69C subtilases. These proteases have been already related to the defence response of *Arabidopsis* and tomato, respectively, against biotic environment stimulus. Moreover, it was demonstrated in this study that some grapevine subtilases genes were located near of locus associated to *Vitis vinifera* resistance against *Plasmopara viticola*.

The second goal of this project was performed gene expression studies elucidating the role of selected grapevine subtilases in the grapevine resistance against *P. viticola*. Subtilase gene expression was studied in two *Vitis vinifera* cultivars (Regent and Trincadeira) after *P. viticola* inoculation and the constitutive expression pattern of the subtilases was studied in several grapevine species/ cultivars showing varying degrees of resistance towards *P. viticola*. The results suggested that under pathogen attack, resistant grapevine cultivar have an early response increasing the expression of some subtilases. This early increase of subtilase expression may be related to the establishment of a defence strategy against the invading pathogen. On the other hand, the susceptible grapevine cultivar showed a delay of the subtilase expression increase, which may be related to an attempt by the susceptible cultivar to initiate a defence strategy that was not fast or robust enough to prevent pathogen growth. At a constitutive level, in several grapevine species/ cultivars, it was not observed a pattern of subtilases expression. Both results suggest that there was a differential expression of certain

subtilases in resistant species but only after stimulation with the pathogen attack, which supports the hypothesis that some grapevine subtilases may have a role in defence response against *P. viticola*.

Finally, two subtilases were selected, based on their characteristics and on expression profiles, and cloned for recombinant protein expression.

Up to our knowledge, this study is the first to preformed a large scale characterization of grapevine subtilases associated to immune responses against the downy mildew pathogen. Further studies in order to characterize the recombinant proteins, to access their structure and substrates should be done in order to establish these subtilases as candidates for introgression in breeding programs.

Keywords: Subtilases, *Vitis vinifera*, *Plasmopara viticola*, immunity, gene expression

Abbreviations

AICc	Akaike Information Criteria
Asp	Aspartate
BI	Bayesian Inference
bp	Base pair
$CaCl_2$ ($2H_2O$)	Calcium chloride dihydrate
cDNA	Complementary DNA
Cq	Quantification cycle
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
ECM	Extracellula matrix
EDTA	Ethylenediaminetetraacetic acid
EF1α	Elongation factor 1-alpha
ER	Endoplasmic reticulum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	Genomic DNA
His	Histidine
hpi	Hours post inoculation
HR	Hypersensitive response
KAC	Potassium acetate
LB	Luria-Bertani
LRR-RLK	Leucine-rich repeat receptor-like kinase
$MgCl_2$ ($4H_2O$)	Magnesium chloride tetrahydrate
ML	Maximum likelihood
MOPS	3-(N-Morpholino)propanesulfonic acid
mRNA	Messenger RNA
Mw	Molecular weight
N_2	Nitrogen
PA	Protease-associated
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PCR	Polymerase chain reaction
PCs	Proprotein convertases

pI	Isoelectric point
PR	Pathogenesis-related
PRR	Pattern recognition receptors
qPCR	Quantitative real time PCR
QTL	Quantitative trait locus
$RbCl_2$	Rubidium chloride
RNA	Ribonucleic acid
RPV	'*Resistance Plasmopara viticola*'
SA	Salicylic acid
SAND	SAND family protein
SDD1	Stomatal density and distribution 1
Ser	Serine
SOB	Super optimal broth
TBE	Tris-borate-EDTA
UBQ	Ubiquitin-conjugating enzyme

Units

°C	Celsius degrees
g	Earth's gravitational acceleration
kDa	kilodalton
Mg	Milligram
mL	Millilitre
mM	Millimolar
Ng	Nanogram
nm	Nanometre
Ta	Annealing temperature
Tm	Melting temperature
µL	Microlitre
µM	Micromolar

1 | Introduction

1.1 Subtilisin-like proteases: classification and basic features

Subtilisin-like proteases (also known as subtilases) are a very diverse and the second largest family of serine peptidases present in archaea, bacteria, eukarya, fungi and yeast (Siezen et al., 1991). They belong to the S8 family within the SB clan of serine proteases, according to the classification of the peptidase database MEROPS (Rawlings et al., 2014; http://merops.sanger.ac.uk).

In mammals, the subtilase homologs are the proprotein convertases (PCs), responsible for the formation of peptide hormones, growth factors, neuropeptides, and receptor proteins from inactive pro-proteins by limited proteolysis at highly specific sites (Barr, 1991; Schaller and Ryan, 1994; Seidah et al., 1999, 1994). Beyond the highly specific maturation of peptide hormones and processing of protein precursors, by the kexin in *Saccharomyces cerevisiae* and AtSBT1.1 in *Arabidopsis thaliana* (Srivastava et al., 2008), subtilases can have also a non-selective degradation function in general protein turnover, like the subtilisin Carlsberg from *Bacillus licheniformis* (Rose et al., 2010). There are several examples of degradative subtilases from the cucumisin from *Cucumis melo* that cleaves a broad variety of peptide and protein substrates (Kaneda and Tominaga, 1975; Uchikoba et al., 1995; Yamagata et al., 1994), to macluralisin from *Maclura pomifera* for which similar characteristics have been reported (Rudenskaya et al., 1995).

Unlike mammals on which only nine subtilases have been identify, subtilases from plants are especially abundant, with 63 known genes in *Oryza sativa* (Tripathi and Sowdhamini, 2006), 56 genes in *Arabidopsis thaliana* (Rautengarten et al., 2005), 15 genes in *Lycopersicon esculentum* genome (Meichtry et al., 1999), 23 genes in the moss *Physcomitrella patens*, 90 genes in *Populus trichocarpa* (Schaller et al., 2012) and 80 genes in *Vitis vinifera* genome (Cao et al., 2014). In plants, subtilases act as secretory enzymes and are targeted to the endoplasmic reticulum (ER) (Siezen and Leunissen, 1997) migrating to the cell plasma membrane, e.g, subtilisin-like serine protease SDD1 from *Arabidopsis thaliana* that is located at the plasma membrane, mediate cell-to-cell signalling and controls stomatal distribution and density during leaf development (von Groll, 2002).

1.2 Structure and biochemistry of plant subtilases

The majority of the subtilases from plants are synthesized as an inactive pre-pro-protein precursor. Their structure usually presents a signal peptide, a pro-domain (also known as I9 inhibitor domain), a subtilase domain (also known as S8 peptidase domain) and a protease-associated (PA) domain located within the subtilase domain (Figure 1), although some of them may have only one or even additional domains (Antão and Malcata, 2005; Dodson and Wlodawer, 1998; Siezen et al., 2007; Siezen and Leunissen, 1997; Vartapetian et al., 2011). The presence of a highly conserved catalytic triad within the S8 peptidase domain, composed by aspartate (Asp), histidine (His) and serine (Ser) amino acid residues (Dodson and Wlodawer, 1998), is characteristic of the subtilase family (Figure 1Figure *1*). Additionally, certain subtilases may also have a conserved catalytic asparagine (Asn) residue in the same S8 peptidase domain (Dodson and Wlodawer, 1998; Jordá et al., 1999; Siezen and Leunissen, 1997). Contrary to other organisms, PA domain in plants is found within the S8 peptidase domain. The PA domains is an insertion of 120–160 amino acids between the His and Ser active site residues that cause a displacement of the reactive Ser from the catalytic triad to the C-terminal (Siezen and Leunissen, 1997), (Figure 1Figure *1*). Most plant subtilases also contain a fibronectin (Fn) III-like domain, required for the activity of some of these enzymes, but dispensable in others (Rawlings and Salvesen, 2013).

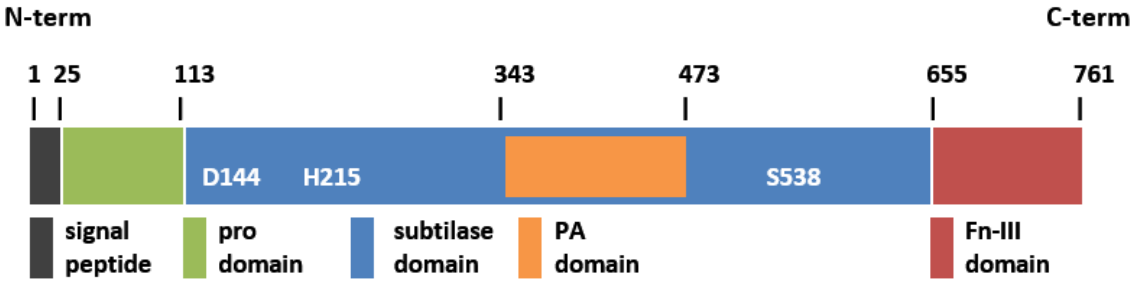


Figure 1 – Example of a subtilase domain architecture showing the four characteristics domains (adapted from Rose et al 2010). This in particular is the architectural domain of SlSBt3, a subtilase from tomato (*Solanum lycopersicum*). The main domains are represented, as well as the 3 amino acid residues [aspartate (D), histidine (H) and serine (S)] in the subtilase domain.

As consequence from the pre-pro-protein structure, the active enzyme maturation from its inactive precursor requires the removal of the signal peptide, that is responsible for targeting the protease for secretion (Cedzich et al., 2009; Feliciangeli et al., 2006; Nour et al., 2003), and

of the pro-domain. In plants, this process occurs late in the ER or in the early Golgi, either as an intramolecular autocatalytic reaction, or as result of the interaction with a secondary peptidase (Bergeron et al., 2000; Cedzich et al., 2009; Chichkova et al., 2010). The I9 inhibitor domain (also known as pro-domain) works as an auto-inhibitory domain maintaining the inactive state of the zymogen and preventing the access of the substrate to the active site. It also works as an intramolecular chaperone that is required transiently to assist in folding of the catalytic domain (Baker et al., 1993; Bryan, 2002; Huang et al., 1997; Li and Inouye, 1994; Zhu et al., 1989). After cleavage, the pro-domain remains non-covalently bound, acting as a specific inhibitor of proteolytic activity (Anderson et al., 2002; Nour et al., 2003; Steiner, 1998), and thus protecting proteins involved in the secretory pathway from nonspecific proteolytic degradation (Yamagata et al., 1994). Hence, the cleavage of the N-terminal of the subtilase is a prerequisite for the protease to acquire its functional conformation and perform its function at the action site. Once the N-terminal inhibitory domain is removed, the activity of the subtilase is therefore stimulated (Bergeron et al., 2000).

Concerning protein structure, plant subtilases generally present a monomeric structure (Figure 2), although several studies have indicated that many subtilases suffer a homo-dimerization mediated by PA domain in order to activate it (Rose et al., 2010; Siezen and Leunissen, 1997). Without this activation process subtilases cannot perform their function (Ottmann et al., 2009).

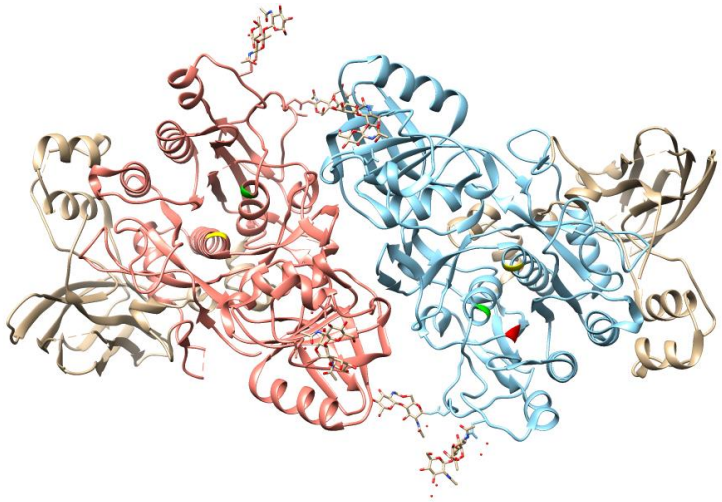


Figure 2 – Dimeric structure of the subtilase SISBt3 (PDB ID 3I6S) from *S. lycopersicum*, highlighting the two S8 peptidase domains (coral and blue) and the three catalytic residues (aspartate [D144] in red, histidine [H215] in green and serine [S538] in yellow). This image was prepared with the UCSF Chimera package [Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311), (Pettersen et al., 2004)].

The PA domain also interacts with the I9 inhibitor domain, leading to the cleavage of the N-terminal and allowing the access of the substrates to the catalytic site, stimulating the subtilase activity (Bergeron et al., 2000). The PA domain has been also implicated in protein-protein interactions and substrate recognition (Rautengarten et al., 2005; Schaller et al., 2012). Studies in soybean have shown that PA domain plays an important role in substrate selection, namely in substrate length determination (Rautengarten et al., 2005; Tan-Wilson and Wilson, 2012).

Another feature of subtilases is the apparent Ca^{2+}-independence of most of them, contrarily to what was expected by modelling studies of the first subtilases described such as the bacterial protease subtilisin BPN' (Alexander et al., 2001; Siezen and Leunissen, 1997). In 2009, Ottmann and co-workers demonstrated that the thermostability and activity of the subtilase SISBT3 from *Solanum lycopersicum* were not influenced by the addition of Ca^{2+} or chelating agents. Instead, in SISBT3, another subtilase from tomato, a positively charged site chain of Lys498 mimics the calcium ion bound function. This region, including the stabilizing lysine residue, is highly conserved in all the plant subtilases studied so far (Rose et al., 2010).

1.3 Subtilase participation in plant-specific developmental processes

In plants, subtilases are involved in many biological functions from the mobilization of storage proteins during seed germination to the initiation of cell death and senescence programs (Schaller et al., 2012). There are several examples of the involvement of subtilases in plant development, as the *Arabidopsis thaliana* AtSBT1.7 that participates in seed germination (Rautengarten et al., 2008), LIM9 from *Lilium longiflorum* that is involved in microspore development (Riggs and Horsch, 1995; Taylor et al., 1997), the papaya CpSUB1 that is involved in fruit ripening (Othman and Nuraziyan, 2010), the ARA12 in *Arabidopsis* and SCS1 in soybean that participate in seed coat development (Batchelor et al., 2000). Moreover, previous studies in *Arabidopsis thaliana* have shown the participation of the subtilases XSP1 and AIR1 in xylem differentiation (Zhao et al., 2000) and lateral root formation (Neuteboom et al., 1999). In 2001, Tanaka and co-workers have suggested that ALE1 protease is necessary for cuticle formation and epidermal differentiation during embryo development in *A. thaliana*. Also in *A. thaliana*, von Groll and co-workers have elucidated the role of the SDD1 (stomatal density and distribution 1) protease, a apoplast-secreted protein that acts as a processing protease in the generation of a signal responsible for regulation of stomata distribution and density during leaf development

(von Groll, 2002). Apparently, the SDD1 is involved in cell-to-cell communication through the processing of the ligand for the TMM (Too Many Mouths), a member of the large leucine-rich repeat receptor-like kinase (LRR-RLK).

Besides the cellular functions already mentioned, plant subtilases may also contribute to the cell wall dynamics, either directly by cleavage of structural protein, or indirectly by the regulation of cell wall remodelling enzymes (Schaller et al., 2012).

1.4 Subtilases in environment stimulus and pathogen attack responses

It is evident the importance and the participation of subtilases in a lot of plant development functions. Recent evidences have also highlighted the participation of subtilases in response to biotic and abiotic environment stimulus (Chichkova et al., 2004; Liu et al., 2007; Tian, 2005; Tian and Kamoun, 2005). One example is the participation in the response to abiotic environment of the subtilase AtSBT6.1 from *A. thaliana*, the ortholog of mammalian site-1-protease (S1P). This protease is involved in the unfolded protein response through the cleavage of an ER-resident type II membrane protein (bZIP28). The bZIP28 protein moves to Golgi where unfolded proteins accumulate, cleavage releases the bZIP domain that translocates to the nucleus in order to activate gene expression related to the stress response (Che et al., 2010; Liu et al., 2007; Liu and Howell, 2010a, 2010b). Additional substrates of AtSBT6.1 include the precursor proteins of a peptide growth factor (Rapid Alkalinization Factor 13) and pectin methylesterases (Srivastava et al., 2009; Wolf et al., 2009). In recent years, subtilases were shown to be involved in symbiotic interactions of plants with other organisms, such as the mutualistic interactions of plant roots with fungi resulting in as arbuscular mycorrhiza or with nitrogen-fixing Rhizobia as nodule symbiosis (Takeda et al., 2007).

The first evidence of the involvement of plant subtilisin-like proteases in plant-pathogen interactions was reported by Granell and co-workers (1987) in tomato plants. They identified the accumulation of the subtilase P69 in tomato leaves after infection with citrus exocortis viroid (CEV) (Granell et al., 1987). Two years later, Christ and Mösinger (1989) and Fischer and co-workers (1989) associated the same subtilase with the response of tomato leaves to *Phytophothora infestans* (Christ and Mösinger, 1989; Fischer et al., 1989). The P69 subtilase was characterized as an alkaline proteinase located in the vacuole and intercellular spaces of leaf parenchyma cells (Vera et al., 1989; Vera and Conejero, 1988). Tornero and co-workers (1996)

cloned for the first time a P69 subtilase from tomato and revealed the presence of at least six closely related genes in tomato (Jordá et al., 2000, 1999; Meichtry et al., 1999; Tornero et al., 1997). Two of them, P69A and P69D, are constitutively expressed (Jordá et al., 1999), P69E and P69F have a specific developmental expression pattern (Jordá et al., 2000). P69B and P69C were shown to behave as pathogenesis-related (PR) genes being induced by pathogen infection and salicylic acid (Jordá et al., 1999; Tornero et al., 1997). P69 was also the first plant subtilase for which protein substrates were identified: the systemin, a travelling peptide hormone mediating signalling processes during wound response in plants (Schaller and Ryan, 1994), and the leucine-rich repeat (LRP) protein (Tornero et al., 1996a), an extracellular matrix associated leucine-rich repeat (LRR) protein that mediates molecular recognition and/or protein interaction processes (Kobe and Deisenhofer, 1995). However, the consequences of these substrates processing events for plant pathogen interaction, remains unknown.

More recently, studies in *A. thaliana* identified the SBT3.3 gene as encoding a serine protease homologue to the P69C subtilase from tomato (Ramírez et al., 2013). Like P69C in tomato, SBT3.3 can specifically process an extracellular LRP containing-protein, suggesting the involvement of the SBT3.3 on the LRR-containing proteins' cleavage, including pattern recognition receptors (PRR) as PRR-type receptors and activation of plasma membrane receptors and downstream signalling processes (Ramírez et al., 2013). Thus, like the tomato P69C, *A. thaliana* SBT3.3 may be linked to pathogen recognition. Ramirez and co-workers (2013) have shown that the expression of SBT3.3 rapidly increases during the activation of innate immunity preceding the activation of salicylic acid (SA) responsive genes, responding very rapidly to H_2O_2, a common ROS species generated very early during pathogen-associated molecular pattern (PAMP) recognition by PRR leading to activation of innate immune responses. Despite the SBT3.3 substrate is not yet identified, this subtilase may have activity on the extracellular domain (ectodomain) of a larger protein that works like a receptor located in the plasma membrane. Thus, as consequence of the proteolytic shedding of the ectodomain, the receptor could become activated and initiate a downstream immune signalling process. After that, a positive feedback loop circuit would maintain the SBT3.3 expression in a level sufficient to keep cells in a sustained sensitized mode (Ramírez et al., 2013). This expression pattern would consequently be the basis to explain the memory-based characteristics of priming and induced resistance (Ramírez et al., 2013).

Studies have also shown that subtilases are glycosylated and secreted to the plant extracellular matrix (ECM) where they accumulate and presumably exert their biochemical

functions by recognition and processing pericellular substrates (Siezen and Leunissen, 1997; Taylor et al., 1997; Tornero et al., 1997, 1996a, 1996b; Yamagata et al., 1994). Considering that ECM is where the first host-pathogen interaction, recognition and signalling events take place (Dixon and Lamb, 1990), the accumulation of subtilases in plant ECM may account for an important role during pathogenesis. This seems to be the case for grapevine infection with the mildew causing agent, *Plasmopara viticola*, in which some signalling events might occur in ECM. The first clues were given by Figueiredo and co-workers (2008) when comparing resistant and susceptible genotypes prior and post-inoculation with *P. viticola.* The results showed constitutively expression of a subtilisin-like protein, sharing sequence similarity with the tomato P69C, in the resistant genotype and an increase of protein expression after inoculation with *P. viticola* (Figueiredo et al., 2012, 2008; Monteiro et al., 2013).

1.5 Study model: the interaction between *Vitis vinifera* and *Plasmopara viticola*

Grapevine (*Vitis vinifera* L.) is currently the most important fruit plant cultivated worldwide due to its economic importance in the wine industry. In Portugal this industry accounts over 890 million euro per year of exports (Bettini, 2014). Cultivated grapevine is highly susceptible to downy mildew disease which is caused by the obligate oomycete *Plasmopara viticola* (Berk. et Curt.) Berl. et de Toni. *P. viticola* requires the genus *Vitis* to complete its life cycle once it cannot survive outside its host except as oospores (Yu et al., 2012). This oomycete, under optimal conditions, such as high humidity and warm temperatures, can spread rapidly over large areas within a very short period of time (Müller and Sleumer, 1934). For infection, upon contact with water, *P. viticola* release several flagellate zoospores that swarm within the water film on the lower surface of the leaf. On susceptible hosts, the zoospores are targeted to the stomata, where they shed their flagella, attach and encyst (Kiefer et al., 2002). Then, a germ tube is formed that reaches into the substomatal cavity, where it dilates into a substomatal vesicle. From this vesicle a primary hypha emerges developing a mycelium that spreads within the leaf tissue, extending mainly into the intercellular spaces of the spongy parenchyma and forming haustoria that penetrate into the cell wall of the host (Unger et al., 2007). The *P. viticola* infection interfere with the normal regulation of the stomata guard cells resulting in water loss (Allègre et al., 2007), cause tissue damage and reduces the functional green area of the leaf as well as assimilation rates by the leaf remainder (Moriondo et al., 2005). In resistant species, the infection progress is slowed down, inhibited, or completely stopped (Yu et al., 2012).

The attack by this pathogen leads to heavy crop losses which represent a cost of several million euro each year. The current strategy for downy mildew disease control is the massive use of pesticides in each growing season. However, as for the crops, excessive use of pesticides is highly prejudicial to human health. So, the search for alternative methods to control grapevine downy mildew is crucial (reviewed in Gessler et al., 2011).

1.6 Grapevine resistance to *Plasmopara viticola*

Contrary to *V. vinifera* cultivars that have no known natural resistance to downy mildew infection (Cadle-Davidson, 2008; Staudt and Kassemeyer, 2015), sources of resistance against the mildews were identified in a range of American wild species, like *V. labrusca*, *V. riparia* and *V. rupestris* (Alleweldt and Possingham, 1988; Eibach et al., 2010; Wan et al., 2015). With this in mind, grape breeders tried to combine the resistance traits from the American wild species with the quality of *V. vinifera* cultivars, resulting in new cultivars with high-quality features and considerable mildew resistance characteristics (Bundessortenamt, 2008). Resistance mechanisms elucidated so far vary from physical barriers such as hairs and stomatal closure, accumulation of phenolic antimicrobial compounds, increase of peroxidase activity, accumulation of pathogenesis-related proteins (PRs) and hypersensitive response (HR) (Allègre et al., 2007; Greenberg and Yao, 2004; Kortekamp, 2006; Kortekamp et al., 1998; Kortekamp and Zyprian, 2003).

Also, until now, quantitative trait locus (QTL) for resistance to downy mildew have been reported, e.g., *Rpv3* (Bellin et al., 2009; Welter et al., 2007), *Rpv8* (Blasi et al., 2011) and *Rpv11* (Bellin et al., 2009; Fischer et al., 2004; Schwander et al., 2011) (Supplementary Table 1). With the grapevine genome sequencing and physical mapping (Jaillon et al., 2007; Moroldo et al., 2008; Velasco et al., 2007), the distribution pattern of candidate genes for disease resistance across the whole genome of *V. vinifera* has been recently elucidated.

1.7 Participation of subtilases in grapevine resistance to *Plasmopara viticola*

The first clues regarding subtilase participation in grapevine - *P. viticola* interaction were reported by Figueiredo and co-workers (2008), when comparing resistant and susceptible genotypes prior and post-inoculation with this pathogen. A subtilisin-like protein, sharing

sequence similarity with the tomato P69C, was constitutively expressed in resistant genotype and increased its expression after *P. viticola* inoculation (Figueiredo et al., 2012, 2008; Monteiro et al., 2013). Studies on the grapevine- *P. viticola* interaction with serine protease inhibitors, shown that after the treatment with the inhibitor, an immune cultivar becomes resistant, a resistant cultivar reaches the level of a susceptible one, and a susceptible cultivar becomes even more sensitive (Gindro et al., 2012). Therefore, after treatment with a serine protease inhibitor, the infection rate could rise due to the inhibition of the proteases involved either in the regulation of stomatal density (Berger and Altmann, 2000) or in plant defence against pathogens (Oh et al., 2008; van der Hoorn and Jones, 2004; van der Hoorn, 2008). These results point to a possible involvement of subtilases given what is known about their cellular functions.

In grapevine - *P. viticola* interaction, inhibition of phytaspases, a subgroup of plant subtilases, could partially inhibit the overall activation of programmed cell death (PCD) and thereby change the level of susceptibility of resistant cultivars to the pathogen (Gindro et al., 2012). The secretome of *P. viticola* is specific, but it could also be tailored to the host plant to a certain extent. Therefore, under natural conditions, the secretome of *P. viticola* could inhibit the endogenous subtilases of susceptible varieties, thereby inhibiting the plant's normal defence reaction. By contrast, it is possible to hypothesize that resistant or immune varieties possess endogenous subtilases that are not recognized by the secretome of *P. viticola* due to slight structural modifications of the protein patterns of these cultivars. In this case, plant defence mechanisms would continue to operate, with fatal consequences for the pathogen and restricting its development.

In 2014, the subtilase family was preliminarily characterized by Cao and co-workers (2014). These authors identified 80 subtilase genes in *Vitis vinifera* that were divided into 8 groups based on their phylogenetic relationships. Besides these clues of the presence and importance of the subtilases in *V. vinifera*, these proteins were not yet associated with grapevine immune responses particularly concerning *P. viticola* resistance.

1.8 Aims

The main purpose of this project is perform a genome-wide update of the grapevine subtilase gene family and associate some subtilase genes with the defence response of the *V. vinifera* against the *P. viticola*, taking as reference the grapevine genome reannotation that occurred in 2014.

The second goal of this project was to perform gene expression studies elucidating the role of selected grapevine subtilases in the grapevine resistance against *P. viticola*. Subtilase gene expression will be studied in two *Vitis vinifera* cultivars (Regent and Trincadeira) after *P. viticola* inoculation and the constitutive expression pattern of the subtilases will be studied in several vine species and grapevine cultivars showing different degrees of resistance towards *P. viticola*.

Finally, based on the most promising results of qPCR, selected subtilases were cloned for further recombinant protein expression.

2 | Materials and Methods

2.1 Updating grapevine subtilase gene family

2.1.1 Identification and retrieval of Grapevine Subtilase gene sequences

Grapevine subtilase gene family has been previously characterized (Cao et al., 2014). As the grapevine genome annotation has been recently updated (2014), new database searches were performed to identify and select the members of the subtilase gene family in *Vitis vinifera*. The conserved domains of subtilases, i.e., PA (PF02225), S8 Peptidase (PF00082) and I9 Inhibitor (PF05922) were used as query for blast searches at NCBI (http://www.ncbi.nlm.nih.gov/) and MEROPS (Rawlings et al., 2014, https://merops.sanger.ac.uk/) databases. MEROPS database was also used to identify S8 peptidase domain containing-proteins in *Vitis vinifera*. The retrieved sequences were compared with the sequences obtained from the NCBI database. The results of both databases were manually curated. A new search using the NCBI BLAST tool (Manual and Altschul, 1990, https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed using the selected subtilase sequences as query in order to retrieve both mRNA and protein sequence identifiers.

2.1.2 Chromosomal location

The subtilase genes were mapped in *V. vinifera* chromosomes with the Map Viewer tool from NCBI (http://www.ncbi.nlm.nih.gov/mapview/).

2.1.3 Protein Sequence Alignment and Phylogenetic Analysis

Protein sequences were obtained from the NCBI Database using the protein sequence identifiers (prefix XP), for each of the subtilase genes (Supplementary Table *2*). The protein sequences were initially aligned using the MAFFT software with the L-INS-i option (version 7, Katoh and Standley, 2013, http://mafft.cbrc.jp/alignment/software/) and gaps were manually checked and edited in BioEdit v. 7.2.5 (Hall, 1999).

A preliminary maximum likelihood (ML) phylogenetic analysis was performed with RAxML-HPC v.8, on CIPRES Science Gateway (Miller et al., 2010, https://www.phylo.org), with the following parameters: protein substitution model PROTCAT; protein substitution model + BLOSUM62; bootstrap 1000 iterations with rapid bootstrap analysis (-f a). No outgroups were selected.

The best amino acid substitution model (WAG +G) was selected with ProtTest3.4 (Darriba et al., 2011) under the corrected Akaike Information Criteria (AICc), removing gamma-invariable mixture (+I+G) models from the analysis.

Dataset matrixes consisting of 97 aligned protein sequences were converted from Fasta to Nexus format with Concatenator 1.1.0 (Pina-Martins and Paulo, 2008).

A Bayesian Inference (BI) phylogenetic tree was generated by MrBayes 3.2.1 (Ronquist et al., 2012), outgroups were set according to the basal proteins obtained from RAxML and amino acid substitution model, WAG+G, with four rate categories for the gamma distribution. The Metropolis-coupled Markov chain Monte Carlo analysis was carried out with four chains. The posterior probabilities for each node were generated from 10^8 generations, sampling at every 1000^{th} iteration. The burn-in was set to the first 10% trees, and the remaining trees were used to generate a consensus tree by the 50% majority rule.

ML and BI trees were viewed on FIGTree (http://tree.bio.ed.ac.uk/software/figtree/) and edited on Inkscape (Harrington, B. et al., 2004; http://www.inkscape.org/).

2.1.4 Sequence properties

Molecular weight (Mw) and theoretical isoelectric point (pI) were predicted using the Protparam tool from ExPASy (Gasteiger et al., 2005; http://web.expasy.org/protparam/). Signal peptide prediction of subtilase proteins was performed using the MemPype software (Pierleoni et al., 2011; http://mu2py.biocomp.unibo.it/mempype). Subcellular location of proteins was predicted using Blast2GO (version 3.3, Conesa et al., 2005; https://www.blast2go.com/), TargetP (Emanuelsson et al., 2000; http://www.cbs.dtu.dk/services/TargetP/), PredoTar (Small et al., 2004; https://urgi.versailles.inra.fr/predotar/predotar.html) and LocTree3 (Goldberg et al., 2014, https://rostlab.org/services/loctree3/). Putative function was predicted using the Blast2GO tool. The presence of the PA, S8 peptidase and I9 inhibitor domains was confirmed using the Pfam tool (Finn et al., 2016; http://pfam.xfam.org/). All the molecular predictions were manually curated and compiled.

2.1.5 Selection of subtilase sequences involved in grapevine immunity

Previous studies in plants associated some subtilases with the defence response to pathogen attack, like the subtilase SBT3.3 in *A. thaliana* (Ramírez et al., 2013), the P69 in *S.*

lycopersicum (Jordá et al., 1999; Tornero et al., 1996a) and cucumisin in grapevine (Figueiredo et al., 2012, 2008). The subtilase genes from *V. vinifera* were blasted against the *A. thaliana* genome (using the TAIR database, https://www.arabidopsis.org/) and the tomato genome (using the SolGenomics database, https://solgenomics.net/) to retrieve the grapevine sequences presenting higher homology to *A. thaliana* SBT3.3 and tomato P69 genes. The Solgenomics results were corroborated in NCBI BLAST tool, restricting to *Solanum lycopersicum* organism, and was assumed the NCBI accession for further studies. Moreover, subtilase sequences with a chromosomal location near 'Resistance to *Plasmopara viticola*' (RPV) locus (Bellin et al., 2009; Blasi et al., 2011; Fischer et al., 2004; Schwander et al., 2011; Welter et al., 2007) on grapevine genome were also selected for further studies. Grapevine subtilase genes were selected for analysis by qPCR (Real-time polymerase chain reaction).

2.1.6 Biochemical predictions of grapevine subtilases possibly involved in immunity

Multiple alignment of the grapevine subtilases putatively involved in immunity was performed in DNASTAR software (version 13, Burland, 1999, http://www.dnastar.com/) and prediction of protein glycosylation was done using the NetNGlyc online server (version 1.0, Ramneek and Brunak, 2001). The presence of signal peptides was searched using SignalP server (version 4.1, Petersen et al., 2011), automatically run on all sequences analysed with NetNGlyc.

2.1.7 Prediction of protein–protein interaction network for the subtilases putatively involved in grapevine immunity

The protein interaction network of the selected subtilases was obtained (STRING, version 10.0, http://string-db.org/). The gene accessions for all the interacting proteins were queried at the NCBI database. The GO terms for all the interacting proteins were also obtained.

2.2 Expression analysis by qPCR

2.2.1 Plant Material for inoculation experiments

Two *Vitis vinifera* cultivars were selected to access subtilase expression during interaction with *P. viticola.* The cultivar Regent, bread by multiple introgressions from resistant wild genotypes (Welter et al., 2007), presenting a high degree of resistance to downy and powdery

mildews (Anonymous, 2000), and Trincadeira, a Portuguese traditional grapevine cultivar widely used for quality wine production and highly sensitive to *Plasmopara viticola* (Figueiredo et al., 2008). Plant material was already available, grown and inoculated as described in Figueiredo et al. (2012). The third to fifth fully expanded leaves beneath the shoot apex were harvested at 0, 6, 12 and 24 hpi (hours post inoculation), immediately frozen in liquid nitrogen and stored at −80 °C. For each genotype and condition (inoculated and mock inoculated), three independent biological replicates were collected, being each biological replicate a pool of three leaves from three different plants.

2.2.2 Plant material for species comparison

In order to access if subtilases are constitutively expressed, young leaves from several *Vitis* species and *Vitis vinifera* cultivars (Table 1), showing different degrees of resistance towards *P. viticola*, were harvested from five different plants, at the Portuguese Grapevine Germplasm Bank at INIA - Estação Vitivinícola Nacional (Dois Portos), immediately frozen in liquid nitrogen and stored at -80 °C.

Table 1 - *Vitis* species and *Vitis vinifera* cultivars used in qPCR analysis of subtilase expression; R (resistant), T (tolerant), S (susceptible)

Species	Type of Accession	Origin	Response to downy mildew
V. labrusca	Wild species	America	R
V. rupestris	Wild species	Southern and Western America	R
V. rotundifolia	Wild species	America	R
V. riparia	Wild species	North America	R
V. sylvestris	Wild species	America	T
V. candicans	Wild species	Southern America	R
***V. vinifera* cultivars**			
Trincadeira	Cultivated grapevine	South Europe	S
Regent	Complex hybrid	Breeding	T

2.2.3 RNA extraction and cDNA synthesis

Total RNA was isolated from frozen leaves with the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA), according to manufacturer's instructions. Residual genomic DNA was digested with DNase I (On-Column DNase I Digestion Set, Sigma-Aldrich, USA). RNA purity and concentration were measured at 260/280 nm using a spectrophotometer (NanoDrop-1000, Thermo Scientific) while RNA integrity was verified by agarose gel electrophoresis (1.2% agarose in TBE buffer). Genomic DNA (gDNA) contamination was checked by qPCR analysis of a target on the crude RNA (Vandesompele et al. 2002). Complementary DNA (cDNA) was synthesized from 2.5 µg of total RNA using RevertAid®H Minus Reverse Transcriptase (Fermentas, Ontario, Canada) anchored with Oligo$(dT)_{23}$ primer (Fermentas, Ontario, Canada), according to manufacturer's instructions.

2.2.4 Primer Design

Grapevine subtilases specific primers were designed with Primer Express software version 3.0 (Applied Biosystems, Sourceforge, USA) using the following parameters: amplicon length between 75 and 200 bp; size: 20 ± 2 bp; melting temperature (Tm): 58 ± 2 °C; GC content ± 50 % (Table 2).

The reference genes used for the normalization of the target genes results were the previously described in Monteiro et al. (2013).

2.2.5 Quantitative Real time PCR

Quantitative RT-PCR (qPCR) experiments were carried out using Maxima™ SYBR Green qPCR Master Mix (2×) kit (Fermentas, Ontario, Canada) in a StepOne™ Real-Time PCR system (Applied Biosystems, Sourceforge, USA). A final concentration of 2.5 mM $MgCl_2$ and 0.2 µM of each primer were used in 25 µL volume reactions, together with 4 µL of cDNA as template. The amplification efficiency of each candidate/target gene was determined using a pool representing all cDNA samples. The pool was used to generate a five-point standard curve based on a ten-fold dilution series. Each standard curve was amplified in two independent qPCR runs and each dilution was run in duplicate. Amplification efficiency (E) was calculated from the slope of the standard curve ($E = 10^{(-1/a)}$), where *a* is the slope of the linear regression model (y=a log(x)+ b) fitted over log-transformed data of the input cDNA concentration (y) plotted against quantification cycle (Cq) values (x).

Thermal cycling for all genes started with a denaturation step at 95 °C for 10 minutes followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing for 30 seconds (annealing temperatures are indicated in (Table 2). Each set of reactions included a control without cDNA template. Dissociation curves were used to analyse non-specific PCR products. Three biological replicates and two technical replicates were used for each sample. Gene expression (fold change) was calculated by the Hellemans et al. method* (2007) for both compatible ('Trincadeira' versus mock inoculated control samples) and incompatible interactions ('Regent' versus mock inoculated control samples). Statistical significance ($p < 0.05$) of gene expression between the two genotypes was determined by the Mann–Whitney U test using IBM® SPSS® Statistics version 23.0 software (SPSS Inc., USA).

* The Hellemans et al. method was also applied by me in the qPCR analysis of the gene expression results of 'Guerreiro, A., **Figueiredo, J.**, Silva, M. S., & Figueiredo, A. (2016). Linking Jasmonic Acid to Grapevine Resistance against the Biotrophic Oomycete *Plasmopara viticola*. *Frontiers in Plant Science*, *7*: 565.'

Table 2 - Reference genes and target gene primer sequences, amplicon length, amplification efficiency, annealing and melting temperature are represented.

Adopted Identifier/ Accession Number	Primer sequence	Amplicon length (bp)	Amplification efficiency (E)	Ta (°C)	Tm (°C)
Reference genes (Monteiro et al. 2013)					
EF1α (elongation factor 1-alpha) *XM_002284888.2*	F: GAACTGGGTGCTTGATAGGC R: ACCAAAATATCCGGAGTAAAAGA	164	1.89	60	79.16
GAPDH (glyceraldehyde-3-phosphate dehydrogenase) *XM_002263109.3*	F: TCAAGGTCAAGGACTCTAACACC R: CCAACAACGAACATAGGAGCA	226	1.99	60	80.95
UBQ (ubiquitin-conjugating enzyme) *XM_002284161.3*	F: GAGGGTCGTCAGGATTTGGA R: GCCCTGCACTTACCATCTTTAAG	75	1.95	60	78.86
SAND (SAND family protein) *XM_002285134.2*	F: CAACATCCTTTACCCATTGACAGA R: GCATTTGATCCACTTGCAGATAAG	76	1.93	60	79.16
Target genes					
VtSBT1922 *XM_010663620.1*	F: CCATTATACACGACTCCCTT R: TAACCGTCGCCACCAAACA	88	1.96	58	77.48
VtSBT3195 *XM_002273159.3*	F: CAAGCCCCATTAGCACAC R: TTAGAATCAAGATCAAAGAAG	87	1.92	56	72.95
VtSBT4101 *XM_002284065.3*	F: CCAGTCCCTACAGTTTATC R: ACACGCCGGAGTAGTTCTT	120	1.96	58	83.45
VtSBT4152 *XM_003634104.2*	F: GGCGTTCCCATTGCTTGAT R: TTCCCTCGTCTTTGATTATTC	111	1.96	58	76.59
VtSBT4153 *XM_003634105.2*	F: CCTCCCAATGGAAAAATCTG R: GGCTCATGCTATACAACAAG	170	2.01	58	77.93
VtSBT5381 *XM_002275345.2*	F: GCCGGAGGGTGGAGTTTTT R: CATGCGTTCTTGCTGTTTTGA	100	1.95	58	80.34
VtSBT5410 *XM_002275374.2*	F: GGACGGCCTGCAACAACAA R: ATGGCCCTCTTCATCAATAG	86	1.90	58	79.28
VtSBT5429 *XM_002275393.2*	F: TTGCATAAGGGGTCAGGGTT R: CATTTCGCAGGTGGAGGTG	134	-	60	-
VtSBT5471 *XM_002275435.2*	F: TGACGGAGGAAGAAGTGAGA R: GGGTGAATGCGTTGTTAGTA	95	2.04	58	76.13
VtSBT7502 *XM_010659200.1*	F: CAGCGAGTTTTAGTGATGAAG R: GGGGTATGGAAGGAAGAGT	172	1.96	58	79.58
VtSBT7672 *XM_010649370.1*	F: GGGATATGGCCTGAGTCTGA R: CAACGCGCACCGATTATTTT	134	2.03	60	79.44
VtSBT7899 *XM_002277863.3*	F: GTCCAACCTCACACTACC R: GTTTTCCCATACCCTCGTC	160	-	58	-

VtSBT8450 *XM_002278414.3*	F: AAGGTGTACAAAGTGGCTAAA R: CCTGGAAATGGAAAGATGTT	102	1.89	58	75.39
VtSBT8505 *XM_010660203.1*	F: AATCCTGGTGTTCTTGTGG R: ATTAGGTAAAATGTTGTGCTTG	73	2.05	58	72.11

2.3 Cloning of the immunity-related grapevine subtilases

2.3.1 Primer Design

Based on the qPCR results, candidate genes were selected for cloning. Grapevine specific primers were designed with Primer Express software version 3.0 and restriction sites for *EcoRI* and *XhoI* were added (Table 3).

Table 3 – Primer sequences, amplicon length and annealing temperature for VtSBT7502 and VtSBT8505 subtilases. The forward and reverse primers contained *EcoRI* and *XhoI* restriction sites (underlined), respectively.

Adopted identifier	Primer sequence	Amplicon length (bp)	Ta (°C)
VtSBT7502	F: CCGGAATTCGAAGTAGCTGCATGACAAC R: CCGCTCGAGTGAGAACTAATAAGGCAAGAA	1972	56
VtSBT8505	F: CCGGAATTCATGTGCATAGCTTACCTTCTA R: CACCGCTCGAGGTGCTTGCCGCATCATTTA	2307	56

2.3.2 Gene amplification

VtSBT7502 and VtSBT8505 cDNA's were amplified from a cDNA sample of the *Vitis vinifera* cv Regent by polymerase chain reaction (PCR) using the forward and reverse gene-specific primers and 0.02 U/μL of Phusion® High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, Massachusetts, EUA). The 50 μL PCR reactions contained 0.5 μM of each forward and reverse primers, 2 mM dNTPs and 1 μL of cDNA sample. The PCR were performed on a thermal cycler (Thermal Cycler 2720, Applied Biosystem), using the following conditions: initial denaturation at 98 °C for 30 seconds; 35 cycles of 98 °C for 10 seconds, annealing for 30 seconds (see Table 3 for annealing temperature (Ta) of each gene), and 72 °C for 2.5 minutes; final extension at 72 °C for 10 minutes.

The PCR products were separated by agarose gel electrophoresis (1.2% agarose in TBE) to confirm the amplified gene sizes. The corresponding band for each gene was excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. Each purified gene was quantified at 260/280 nm using a spectrophotometer.

2.3.3 Purification of the Bacterial Expression Vector

The prokaryotic expression vector pET28a (+) (Novagen) (Figure 3) was used for cloning the selected subtilases. The vector was extracted from *Escherichia coli* DH5α cells (Novagen), stored in glycerol stock. Briefly, cells were plated in LB (Luria-Bertani) medium supplemented with 50 µg/mL of kanamycin and incubated at 37 °C overnight. Five bacterial colonies were inoculated into liquid LB medium supplemented with 50 µg/mL of kanamycin and incubated overnight at 37 °C, 200 rpm. The vector was extracted from bacteria with the NZYMiniprep Kit (NZYTech, Lisbon, Portugal), according to manufacturer's instructions. The purified vector concentration was measured at 260/280 nm using a spectrophotometer.

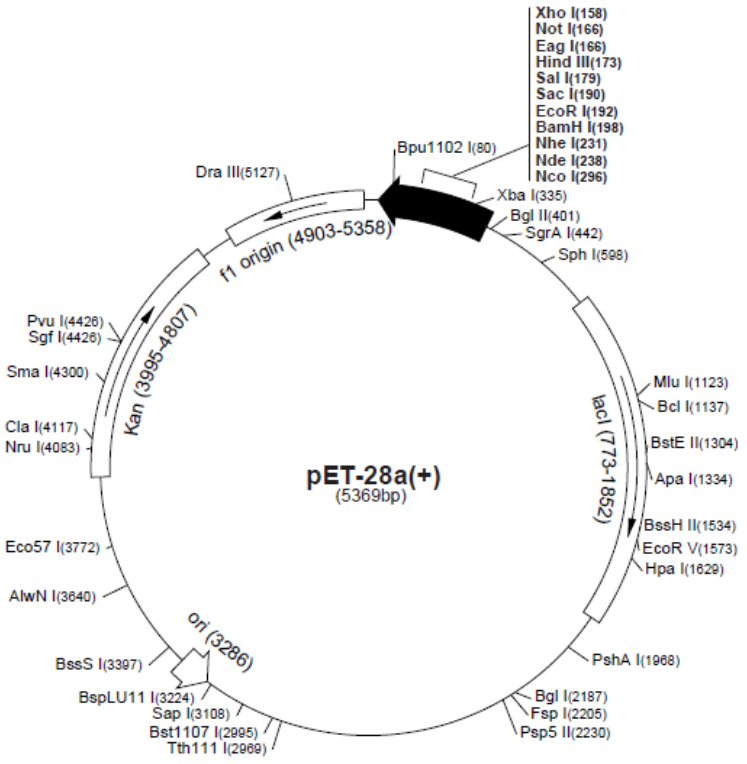


Figure 3 - Representation of prokaryotic expression vector pET28a (+) (Novagen).

2.3.4 Plasmid and cDNA digestion with restriction enzymes and ligation

Subtilase genes and pET28a (+) expression vector were digested with *EcoRI* and *XhoI* restriction enzymes (Thermo Scientific, Waltham, Massachusetts, EUA), according to manufacturer's instructions, at 37 °C for 3 hours. The enzymes were inactivated at 80 °C for 20 minutes. The digestion products were purified with the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions, and the DNA concentration was measured at 260/280 nm using a spectrophotometer. The efficiency of the digestion for pET28a (+) expression vector was confirmed by agarose gel electrophoresis (1.2% agarose in TBE).

For the construction of the plasmids containing each amplified gene (see Equation 1), subtilase genes were cloned into the pET28a (+) vector using the T4 DNA Ligase enzyme (Thermo Scientific, Waltham, Massachusetts, EUA), according to manufacturer's instructions. The ligation was performed at 22 °C during 4 hours. The obtained constructs were named after the plasmid and the respective subtilase: pET28a – VtSBT7502 and pET28a – VtSBT8505.

$$ng\ insert = \frac{bp\ insert}{bp\ vector} \times \frac{2}{1} \times ng\ vector$$

Equation 1 - Calculation formula for vector-insert proportion.

2.3.5 Preparation of chemically competent *E. coli* cells

E. coli One Shot TOP10 (Novagen) was submitted to a protocol for competence induction and used as host for amplification of recombinant plasmids. Cells were plated in SOB (Super Optimal Broth) medium at 37 °C overnight. One colony was inoculated in 225 mL of SOB medium and grown for 2 hours at 37 °C, 250 rpm until the OD_{600nm} reached 0.7. Cells were kept on ice for 10 minutes and centrifuged at 1400 *g* for 5 minutes. The supernatant was discarded and the pellet re-suspended in RF1 buffer (100 mM $RbCl_2$, 50 mM $MgCl_2$ ($4H_2O$), 30 mM KAC, 10 mM $CaCl_2$ ($2H_2O$), 15 % glycerol). Cells were kept on ice for 15 minutes and centrifuged at 1400 *g* for 5 minutes. The supernatant was discarded and the pellet re-suspended in RF2 buffer (100 mM MOPS, 10 mM $RbCl_2$, 75 mM $CaCl_2$-$2H_2O$, 15 % glycerol). Cells were divided in 200 μL aliquots and frozen in liquid N_2.

2.3.6 *E. coli* One Shot TOP10 transformation

E. coli One Shot TOP10 were transformed with the pET28a – VtSBT7502 and pET28a – VtSBT8505 constructs. For each transformation, cells were thawed one ice and 10 μl of ligation product was added. Cells were incubated on ice for 30 minutes, submitted to a heat-shock for 45 seconds at 42 °C, without shaking, and re-incubated on ice for 2 minutes. LB culture medium (800 μL) was added and cells were incubated at 37 °C, 250 rpm for 45 minutes. Cells were centrifuged at 7000 *g* for 4 minutes (room temperature) and the supernatant was discarded. The cell pellet was re-suspended in a minimum volume of culture medium and plated in LB agar medium supplemented with 50 μg/mL of kanamycin for growth at 37 °C overnight.

2.3.7 Colony PCR and plasmid purification

Gene cloning was confirmed by colony PCR using the cell colonies grown in the agar plate (Figure 4). The PCR conditions used for each construct were the same as described above for the selected genes (see in 'Gene amplification'). The colony PCR products were analysed by agarose gel electrophoresis (1.2% agarose in TBE) for confirmation of the positive colonies. The positive transformants were inoculated in LB medium supplemented with 50 μg/mL of kanamycin and incubated at 37 °C, 200 rpm, overnight.

The constructs were extracted from bacteria using the NZYMiniprep Kit, according to manufacturer's instructions. The purified products were quantified at 260/280 nm using a spectrophotometer. To further confirm the presence of the pET28a (+) expression vector and the genes, the purified plasmids were digested using *EcoRI* and *XhoI* restriction enzymes, as described above (see in 'Plasmid and cDNA digestion with restriction enzymes and ligation'), with subsequent analysis by agarose gel electrophoresis (1.2% agarose in TBE) and also analysed by gene sequencing using the T7 promotor and T7 terminator primers (StabVida Company (Caparica, Portugal).

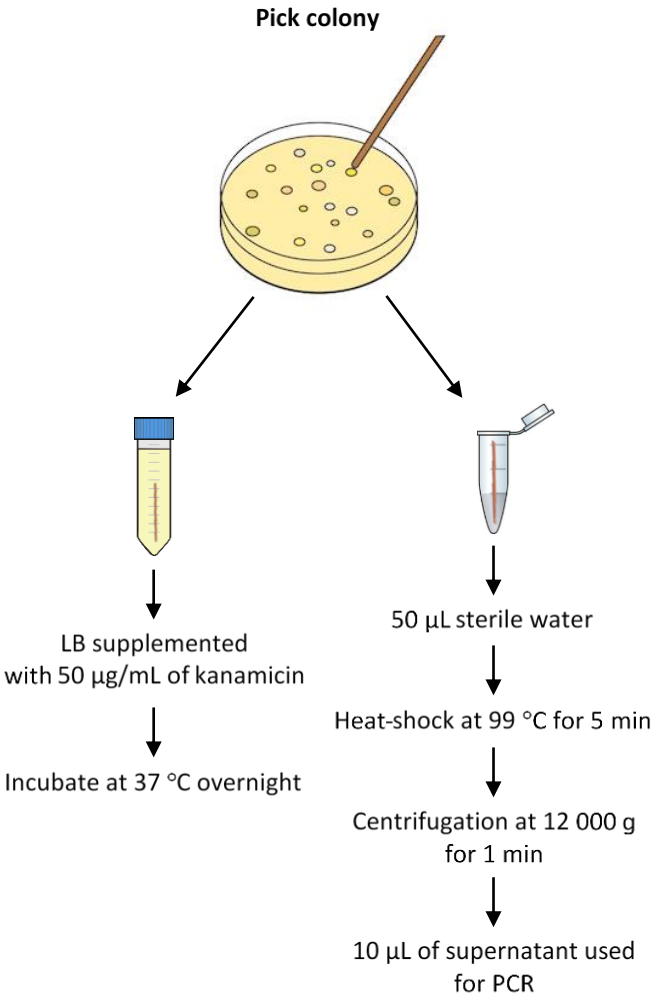


Figure 4 – Colony PCR scheme.

3 | Results and Discussion

3.1 Characterization of the subtilase gene family in *V. vinifera*

3.1.1 Identification of grapevine subtilase genes

Subtilisin-like protease (also known as subtilase) family in plants comprise a group of enzymes involved in several important cellular functions, such as storage proteins' mobilization during seed germination, initiation of cell death and senescence programs (Schaller et al., 2012), and response to biotic and abiotic environmental stimuli (Chichkova et al., 2004; Liu et al., 2007; Tian, 2005; Tian and Kamoun, 2005). This family was already characterized in some plant species like *Populus trichocarpa*, *Arabidopsis thaliana* and *Lycopersicum esculentum* (Meichtry et al., 1999; Rautengarten et al., 2005; Schaller et al., 2012). but in *Vitis vinifera* this protein group is not fully described.

The first characterization of the grapevine subtilase family was made by Cao and co-workers in 2014, where 80 subtilase genes were identified (Cao et al., 2014). These authors restricted their search only to the subtilase conserved PA domain, although subtilases are usually characterized by three conserved domains (PA, S8 peptidase and I9 inhibitor). With the grapevine genome reannotation in December of 2014 (Vitulo et al., 2014), nine of the previously identified genes were completely removed from the databases and eight were replaced by other genes. Hence, this work aimed at the re-characterization of the grapevine subtilase family, taking into account the *V. vinifera* genome reannotation and performing a broader search using the three conserved domains of subtilases.

With this major goal, a thorough search was performed in NCBI and MEROPS databases, using the subtilase PA, S8 peptidase and I9 inhibitor domains as query. As result, 85 grapevine subtilase genes were identified, from which, as a consequence of alternative splicing, it was predicted to obtain 97 subtilase proteins (Supplementary Table 2). This search resulted in the introduction of 17 new subtilase genes and the reannotation of other eight from the subtilase genes previously identified by Cao and co-workers (Cao et al., 2014). The number of genes members in *V. vinifera* subtilase family is similar to the one from *Populus trichocarpa* (90 subtilase genes; Schaller et al., 2012) and higher than those reported in other plant species, like *Arabidopsis* or tomato, which were detected 56 and 15 subtilase genes, respectively (Meichtry et al., 1999; Rautengarten et al., 2005).

The 85 identified genes identified were mapped in *V. vinifera* chromosomes. These genes were unevenly distributed among 15 of the 19 grapevine chromosomes (Figure 5),

comparatively to the 14 chromosomes identified by Cao and co-workers, which was result of the identification of a subtilase gene in chromosome 4. In the others chromosomes, the gene distribution was identical to described by the authors (Cao et al., 2014). No subtilase genes were detected on chromosomes 1, 5, 14 and 17, and the specific location of eight of the 85 subtilase genes was still unknown. The majority of the subtilase genes were present on chromosomes 6, 13 and 16, with nine genes in chromosome 6 and ten in chromosomes 13 and 16 (Figure 5).

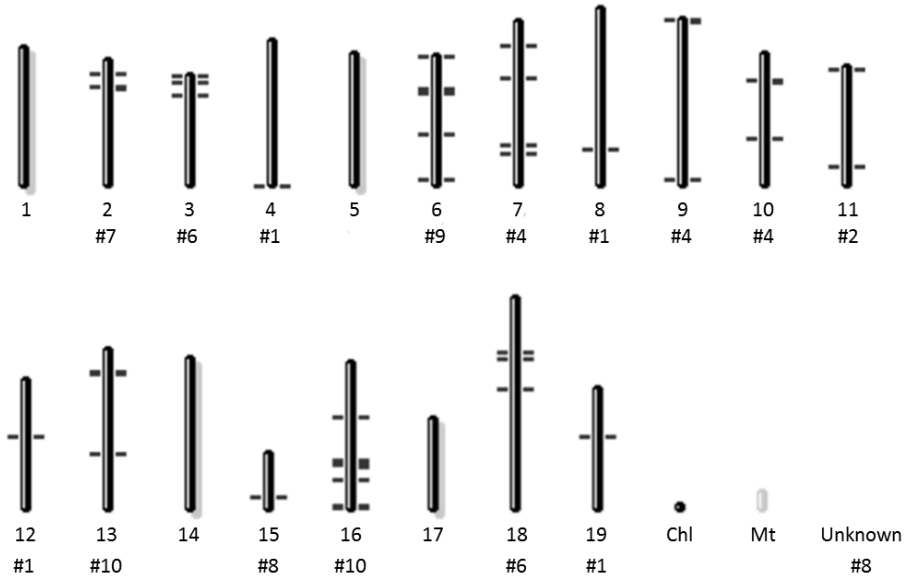


Figure 5 - Prediction of the subtilase genes' location in the grapevine chromosomes. In each illustrated chromosome the number of subtilase genes detected (#number) is shown. Chl and Mt are chloroplast and mitochondrial DNA, respectively.

3.1.2 Phylogenetic analysis of grape subtilases

A phylogenetic analysis of the 97 grapevine subtilase proteins was carried out. This analysis may evidence some relationships between subtilase proteins at functional level or subcellular location. First, a multiple alignment of the 97 putatively grapevine subtilases was performed using the MAFFT software. The phylogenetic tree was built based on maximum likelihood method (Figure 6). It was possible to divide of the subtilases into 6 groups: VvSBT1 (including all the cucumisin protein sequences), VvSBT2 (comprising all the sequences with xylem serine proteinase annotation), VvSBT3 (with all the sequences showing similarity with *A. thaliana* SBT3.3/SBT3.5 sequences), VvSBT4 (including all the sequences showing similarity to *A. thaliana* SBT5.3/SBT5.4), VvSBT5 (containing all the sequences with subtilase annotation) and

VvSBT6 (an outgroup considering all the subtilase presences presenting the fibronectin (Fn) III-like domain). The protein subtilases in this phylogenetic tree are grouped in the same form as the analysis made by Cao and co-workers considering the genomic sequences of grapevine subtilases presenting a PA domain (Cao et al., 2014). However, the authors have divided the subtilases into 8 groups. The present results suggest a division in only 6 groups taking in considering the high posterior probability for the branches. Rautengarten and co-workers have equally performed a phylogenetic analysis of the predicted 56 AtSBT full-length subtilase sequences in *A. thaliana* that showed a division of the subtilases into 6 groups (Rautengarten et al., 2005).

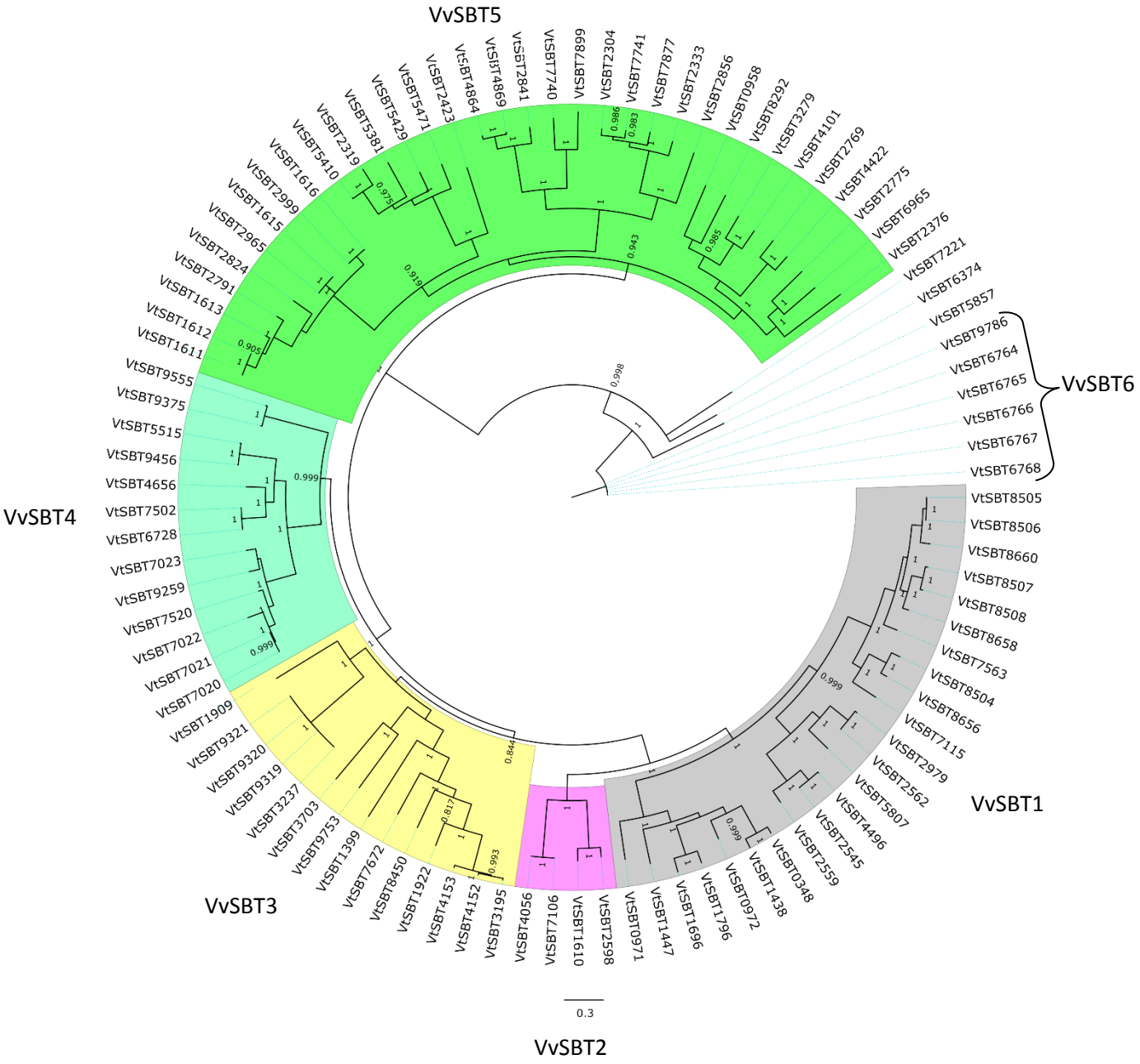


Figure 6 - Maximum likelihood-estimated phylogeny for grapevine subtilase proteins. The six groups are shown (VvSBT1 – VvSBT6). The numbers above branches show posterior probability values (values below 0.7 were not shown).

3.1.3 Grapevine subtilase proteins: properties prediction

With the 85 members of the subtilase gene family identified, it was performed a preliminary characterization of some properties of the corresponding 97 subtilase proteins. This analysis includes molecular weight (Mw) and isoelectric point (pI) determination

(Supplementary Table 2), identification of the conserved domains (Supplementary Table 3), signal peptide prediction, and subcellular location and functionality prediction, through the Gene Ontology (GO) terms (Supplementary Table 4). The results showed that *Vitis vinifera* subtilase proteins have a wide range of molecular weights, between 19 kDa to 164 kDa, slightly higher than the already described for other plant serine proteases (between 19 kDa to 110 kDa, but the majority lies between 60 and 80 kDa (Antão and Malcata, 2005)). Of the 97 grapevine subtilases, the majority (60 %) present a theoretical molecular weight between 80 and 90 kDa, and only 26 % between 60 and 80 kDa.

Relatively to the isoelectric point, grapevine subtilases present a theoretical pI between 4.69 and 9.57. This pI range is comparable to other subtilase proteins, like, for example the STB3.3 (AT1G32960.1) from *A. thaliana* and P69C (CAA76726) from tomato with a theoretical pI of 6.27 and 5.27, respectively (predicted from protein sequence with Compute pI/Mw tool from ExPASy, http://web.expasy.org/compute_pi/) or the CpSUB1 from papaya with a pI of 8.97 (Othman and Nuraziyan, 2010).

The presence of the major domains in grapevine subtilases (S8 peptidase, PA and I9 inhibitor domains) was searched in the 97 proteins using the Pfam tool, (Supplementary Table 2). The S8 peptidase domain (PF00082) is present in all the 97 proteins, and 7 subtilase sequences present a domain duplication. The PA domain (PF02225) was detected once in 46 proteins and twice in 3 subtilases. This domain was however not detected in 48 of the analysed proteins. The I9 inhibitor domain (PF05922) was predicted in almost all subtilases, being absent in 7 proteins and duplicated in 6 proteins. Of the 97 subtilase proteins, only 6 showed a predicted presence of an additional domain, the fibronectin (Fn) III-like domain (PF06280) (Supplementary Table 3). This domain of unknown function, present in bacterial and plant peptidases, belonging to MEROPS peptidase family S8 (subfamily S8A subtilisin, clan SB). It is located at the C-terminal, adjacent to the S8 peptidase domain, and can be found in conjunction with the PA domain and, additionally in Gram-positive bacteria, with the surface protein anchor domain (InterPro Database, https://www.ebi.ac.uk/interpro/). Besides the unknown function, several plant subtilases required this domain for their activity, but for others it is dispensable (Rawlings and Salvesen, 2013).

The presence of subtilases with domain repeats can be a result of the evolution and a way to improve the subtilase features and its functions. Gene duplication and mutation processes in biological evolution have been largely recognized since the 1930s (Bridges, 1936; Brown and Doolittle, 1995; Zhang, 2003). Gene duplication may result in domain repeats in protein

structure. These repeats have a rich variety of functional properties involving protein-protein interactions as well as binding to other molecules like DNA or RNA. Furthermore, long tandem of repeats can play an important role in the folding of three dimensional structures of multi-domain proteins. Structural studies in proteomics have shown that the abundance of domain repeats in organisms of higher complexity is highly correlated with domain families involved in complex-assembly, cell-adhesion and signalling processes (Han et al. 2007).

Observing the domain prediction results, not all grapevine subtilases have simultaneously the three domains. Despite I9 inhibitor, S8 peptidase and PA domains are conserved in plant subtilases, the presence of all of them simultaneously is not a requisite. Moreover, it is yet to be confirmed if the presence or not of this set of domains simultaneously has some effects in the subtilases' functions. An example of the non-existence of the three conserved domains simultaneously is the P69C subtilase from tomato. So far, the predicted primary structure of this subtilase revealed the presence of the signal peptide, the I9 inhibitor domain and the S8 peptidase domain (Tornero et al., 1997). Contrarily, the first predictions of the SBT3.3 subtilase structure from *A. thaliana* showed the presence of the three conserved domains and also a fibronectin (Fn) III-like domain (Rose et al., 2010).

A prediction of the signal peptide presence in the subtilase proteins was also performed, using the MemPype software. Only 60 subtilases presented a possible signal peptide (Supplementary Table 4), with a length between 17 and 44 amino acid residues. The size of the signal peptide in grapevine subtilases is similar to the previously described for other subtilases such as tomato P69C and *A. thaliana* SBT3.3 with a signal peptide of 22 and 25 amino acid residues, respectively.

Predictions of the subcellular localization of a gene product can provide additional information for its functional involvement. Different subcellular localizations of plant subtilases have been found to correlate with their different physiological functions (Cao et al., 2014). For example, the CpSUB1 subtilase from papaya which is secreted to extracellular space, where it plays a role in the early stage of fruit development and ripening by degrading cell wall matrix (Othman and Nuraziyan, 2010). Or the rice subtilase RSP1 which is only present in the reproductive organ and absent in leaves, roots, embryos or rice panicles (Yoshida and Kuboyama, 2001). This suggests that the role for each plant subtilase is related to its location event in spite of analogous structural features (Othman and Nuraziyan, 2010). A prediction of the subcellular location of grapevine subtilases, together with their possible functions, was performed using the Blast2GO software. This tool allows functional annotation of sequence data

that is a primary requirement for the utilization of functional genomics approaches in plant research. This annotation allows the categorization of genes in functional classes, which can be very useful to understand the physiological meaning of large amounts of genes and to assess functional differences between subgroups of sequences (Conesa and Götz, 2008). A first analysis of the Blast2GO results, revealed that the majority of the grapevine subtilases were involved in metabolic processes, in processes associated to plant development and in stimulus response (Figure 7 and Supplementary Table 4). Concerning the subcellular location of grapevine subtilases, results suggested that the majority of these proteases were located in cell membrane, extracellular matrix (ECM) and in the apoplast (Figure 7 and Supplementary Table 4). Considering that the first host-pathogen interaction, recognition and signalling events take place in the ECM (Dixon and Lamb, 1990), the accumulation of subtilases in this site may account for an important role during pathogenesis.

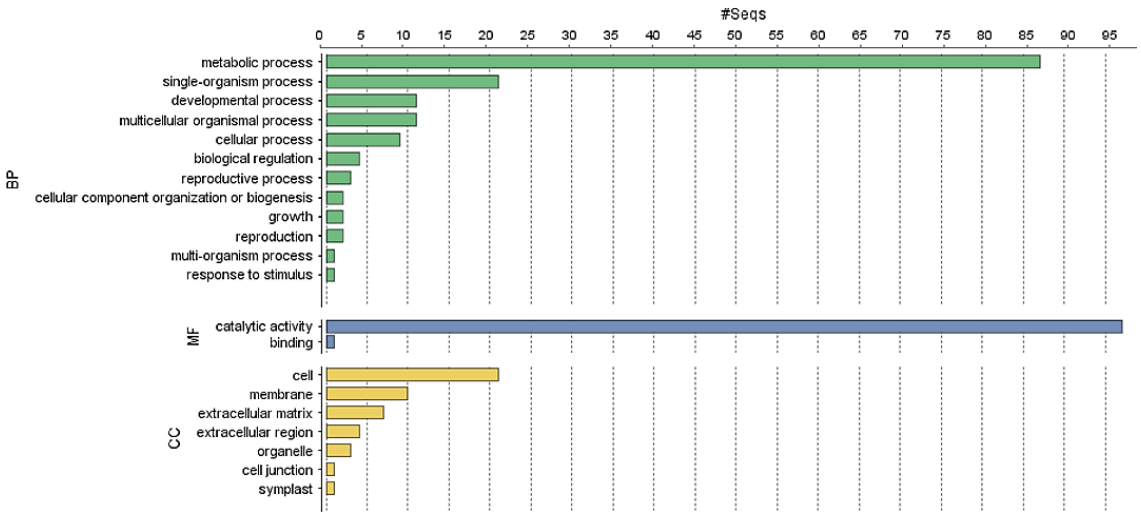


Figure 7 - Gene Ontology results of a level 2 analysis for grapevine subtilases; BP – biological process; MF – molecular function; CC – cellular component

3.1.4 Putative subtilases involved in grapevine immunity

Previous studies of *V. vinifera-P. viticola* interaction in resistant and susceptible genotypes shown a constitutive expression of a subtilisin-like protease, similarly to P69 from tomato, in a *P. viticola*-resistant species. This result suggested a possible involvement of the subtilases in the defence response to pathogen attack in grapevine (Figueiredo et al., 2012, 2008; Monteiro et al., 2013). So far, as already mentioned, the subtilases SBT3.3 from *A. thaliana* and P69 from

tomato are the most studied and associated to defence responses to pathogen attack (Jordá et al., 1999; Ramírez et al., 2013; Tornero et al., 1997). Like in *A. thaliana* and *S. lycopersicum*, the subtilases in *V. vinifera* might as well have a role in immunity caused by pathogen attack. Grapevine subtilases putatively involved in the defence against *Plasmopara viticola* were selected from the 97 proteins identified as members of the subtilase family. Search was based on: a) homology with *A. thaliana* and tomato subtilases involved in immunity; b) chromosome location near to the *resistance to Plasmopara viticola* locus and c) expression data published on the same pathosystem.

The search for homology between grapevine subtilases and the subtilases of *A. thaliana* and tomato was performed blasting the 97 subtilase genes sequences against the *A. thaliana* (using the TAIR database) and the tomato (using the SolGenomics database) genomes. Tomato P69 subtilases present at least six closely related genes (P69A to P69F; Jordá et al., 2000, 1999; Meichtry et al., 1999; Tornero et al., 1997), but only P69B and P69C were shown to behave as pathogenesis-related (PR) genes being induced by pathogen infection and salicylic acid (Jordá et al., 1999; Tornero et al., 1997). In this study, only grapevine subtilases presenting homology with P69B or P69C from tomato were considered. In *A. thaliana*, the only subtilase described as being involved in defence mechanisms is SBT3.3. This subtilase gene is embedded in a genomic cluster encompassing three additional subtilases (i.e. *SBT3.5, SBT3.4 and SBT3.2*) (Ramírez et al., 2013). In this study, grapevine subtilases presenting high homology with *Arabidopsis* SBT3.2, SBT3.3, SBT3.4 and SBT3.5 were selected for expression studies. Once the *A. thaliana* is the plant model, the majority of subtilases in other plants acquired the nomenclature of *A. thaliana* subtilases, so in tomato it is possible found subtilases with SBT3.3 name, which represent tomato proteases with high homology with SBT3.3 from *A. thaliana*, so grapevine subtilases presenting high homology with *S. lycopersicum* P69, SBT3.3, SBT3.4 and SBT3.5 were selected. The obtained results showed that, from the initial 97 grapevine subtilases, 54 have a sequence similarity with P69C (CAA76726.1), SBT3.3 (XP_010318060.1) and SBT3.5 (XP_004235142.1; XP_004233282.1 for isoform X2) from tomato, and 11 subtilases have a sequence similarity with SBT3.3 (AT1G32960.1) and SBT3.5 (AT1G32940.1) from *A. thaliana* (Table 4). Within the 54 subtilases homolog with the proteins from *S. lycopersicum*, 24 of them are homologous with SBT3.5, 23 with P69C and 7 with SBT3.3, presenting a sequence identity between 39 - 77 %, 43 – 59 % and 44 – 59 %, respectively. When compared to *A. thaliana* SBT3.3, 6 of the 11 grapevine subtilases detected show high sequence similarity (sequence identity between 66 and 78 %) with SBT3.5, and 5 with SBT3.3 with a sequence identity above 57 %, (Table 4). These subtilases presenting a high sequence similarity with the previously described subtilases involved in defence might point

out to a comparable function in grapevine. Hence, putative subtilases involved in defence mechanisms in grapevine were selected for further studies. These were chosen with the following criteria: sequence identity higher than 50% with SBT3.3 or P69C. With this prerequisite, six grapevine subtilases with SBT3.3 homology (five of them with SBT3.3 homology in both *A. thaliana* e *S. lycopersicum*) and four with P69C homology from *S. lycopersicum* (marked with an asterisk in Table 4) were selected.

Table 4 - Grapevine subtilase proteins with sequence similarity to SBT3.3, SBT3.5 and P69C from *Arabidopsis thaliana* and *Solanum lycopersicum*, their sequence identity (in %) and E-value; Asterisk (*) indicate subtilases selected for further studies.

Adopted identifier	*S. lycopersicum*	NCBI ID	Identity (%)	E-value	*A. thaliana*	TAIR ID	Identity (%)	E-value
VtSBT0348	SBT3.5	XP_004233282.1	41	e-154				
VtSBT0958	P69C	CAA76726.1	44	e-146				
VtSBT0972	SBT3.5	XP_004233282.1	42	e-159				
VtSBT1399	SBT3.3	XP_010318060.1	44	0	SBT3.5	AT1G32940.1	58	e-157
VtSBT1438	SBT3.5	XP_004233282.1	42	e-151				
VtSBT1610	SBT3.5	XP_004233282.1	39	e-154				
VtSBT1611	P69C	CAA76726.1	43	e-156				
VtSBT1612	P69C	CAA76726.1	43	e-156				
VtSBT1613	P69C	CAA76726.1	44	e-156				
VtSBT1615	P69C	CAA76726.1	46	e-174				
VtSBT1616	P69C	CAA76726.1	46	e-160				
VtSBT1696	SBT3.5	XP_004233282.1	42	e-164				
VtSBT1796	SBT3.5	XP_004233282.1	41	e-160				
VtSBT1909	SBT3.5	XP_004233282.1	46	0				
VtSBT1922*	SBT3.3	XP_010318060.1	59	0	SBT3.3	AT1G32960.1	78	0
VtSBT2376	P69C	CAA76726.1	46	e-171				
VtSBT2423	P69C	CAA76726.1	45	e-163				
VtSBT2598	SBT3.5	XP_004233282.1	41	e-167				
VtSBT2769	P69C	CAA76726.1	46	e-166				
VtSBT2775	P69C	CAA76726.1	43	e-154				
VtSBT2791	P69C	CAA76726.1	44	e-159				
VtSBT2824	P69C	CAA76726.1	44	e-176				
VtSBT2965	P69C	CAA76726.1	46	e-173				
VtSBT2999	P69C	CAA76726.1	46	e-159				
VtSBT3195*	SBT3.3	XP_010318060.1	59	0	SBT3.3	AT1G32940.1	70	0
VtSBT3237	SBT3.5	XP_004233282.1	75	0	SBT3.5	AT1G32940.1	57	e-148
VtSBT3279	P69C	CAA76726.1	47	e-168				
VtSBT3703	SBT3.5	XP_004233282.1	44	e-176				
VtSBT4101	P69C	CAA76726.1	45	e-162				

VtSBT4152*	SBT3.3	XP_010318060.1	59	0	SBT3.3	AT1G32960.1	69	0
VtSBT4153*	SBT3.3	XP_010318060.1	59	0	SBT3.3	AT1G32960.1	70	0
VtSBT4422	P69C	CAA76726.1	46	e-171				
VtSBT5381*	P69C	CAA76726.1	59	0				
VtSBT5410*	P69C	CAA76726.1	59	0				
VtSBT5429*	P69C	CAA76726.1	57	0				
VtSBT5471*	P69C	CAA76726.1	59	0				
VtSBT5857	SBT3.5	XP_004235142.1	43	0				
VtSBT6764	SBT3.5	XP_004235142.1	41	0				
VtSBT6765	SBT3.5	XP_004235142.1	41	0				
VtSBT6766	SBT3.5	XP_004235142.1	41	0				
VtSBT6767	SBT3.5	XP_004235142.1	41	0				
VtSBT6768	SBT3.5	XP_004235142.1	41	0				
VtSBT6965	P69C	CAA76726.1	44	e-146				
VtSBT7115	SBT3.5	XP_004233282.1	44	e-156				
VtSBT7221	SBT3.5	XP_004235142.1	66	0	-	-		
VtSBT7672*	SBT3.3	XP_010318060.1	50	0	SBT3.5	AT1G32940.1	58	e-161
VtSBT8292	P69C	CAA76726.1	46	e-158				
VtSBT8450*	SBT3.3	XP_010318060.1	53	0	SBT3.3	AT1G32960.1	66	0
VtSBT8507	SBT3.5	XP_004233282.1	41	e-157				
VtSBT9319	SBT3.5	XP_004233282.1	76	0	SBT3.5	AT1G32940.1	57	e-146
VtSBT9320	SBT3.5	XP_004233282.1	77	0	SBT3.5	AT1G32940.1	57	e-146
VtSBT9321	SBT3.5	XP_004233282.1	75	0	SBT3.5	AT1G32940.1	57	e-146
VtSBT9753	SBT3.5	XP_004233282.1	43	e-177				
VtSBT9786	SBT3.5	XP_004235142.1	41	0				

The second search for grapevine subtilases putatively involved in the defence against *P. viticola* was based on previous studies that reported the association of some chromosomal locus with the resistance of the *Vitis vinifera* against *P. viticola*, named '*Resistance to Plasmopara viticola* – RPV' (Supplementary Table 1). Those RPVs are a result of the introgressions of genes as consequence of the cross between American wild species with resistance traits and *V. vinifera* cultivars (Bundessortenamt, 2008). Chromosomal location of the previously identified grapevine subtilase genes (not considering the subtilase genes with unknown specific location) was compared with the location of the known RPV's in *V. vinifera* chromosomes. This analysis revealed three subtilase genes located near RPV's. The LOC100266245 (adopted identifier: VtSBT7899) and LOC100242388 (adopted identifier: VtSBT4101) genes located at 16.7 Mb and 15.7 Mb, respectively, were near the *Rpv9*, found at 16.6 Mb in chromosome 7. The LOC100252770 (adopted identifier for one of their transcripts: VtSBT7502) was located at 10.2 Mb between two RPV's, *Rpv1* situated at 10.3 Mb and *Rpv13* placed at 10.0 Mb in chromosome 12 (Table 5). Like the previously selected subtilases based on sequence similarity with SBT3.3

and P69C from *A. thaliana and S. lycopersicum*, respectively, these three subtilase genes were also chosen for further studies (marked with an asterisk in the Table 5).

Table 5 – Comparison between chromosomal location of grapevine subtilase genes and chromosomal location of '*Resistance to Plasmopara viticola* – RPV' sites; Asterisk (*) indicate subtilases selected for further studies.

Adopted identifier	Gene ID	Chromosome	Position (Mb)	RPV	Position in the Chromosome (Mb)
VtSBT3237	LOC100246533	4	23.8	*Rpv4*	4.7
VtSBT9319					
VtSBT9320					
VtSBT9321					
VtSBT7899*	LOC100266245	7	16.7	*Rpv7* *Rpv9*	11.4 16.6
VtSBT4101*	LOC100242388		15.7		
VtSBT2376	LOC100245280		3.5		
VtSBT2423	LOC100265217		7.5		
VtSBT7740	LOC100254813	9	21.9	*Rpv5* *Rpv10*	4.0 3.7
VtSBT2769	LOC100251409		0.8		
VtSBT2775	LOC100852502		0.3		
VtSBT4422	LOC100251577		0.8		
VtSBT6728	LOC100252770	12	10.2	*Rpv1* *Rpv6* *Rpv13*	10.3 20.4 10.0
VtSBT7502*					
VtSBT9753	LOC100257482	18	12.9	*Rpv3*	24.9 26.9
VtSBT2841	LOC100259061		8.7		
VtSBT2856	LOC100248833		8.6		
VtSBT3279	LOC100257444		7.9		
VtSBT4864	LOC100248944		8.7		
VtSBT4869	LOC100243797		8.6		

So, based on the sequence similarity analysis and the chromosomal proximity to RPV locus, the selected subtilases with a putative involvement in defence were 13 in total. Another subtilisin-like protein was added to this list, identified in the first studies that correlated *V. vinifera* subtilase genes with defence response against pathogen attack (Figueiredo et al., 2012, 2008; Monteiro et al., 2013). This subtilase (adopted identifier: VtSBT8505) was constitutively expressed in the resistant genotype and increased its expression after *P. viticola* inoculation, and could have a role in grapevine immunity. These 14 subtilases were selected for a thorough analysis and studies related with *Vitis vinifera-Plasmopara viticola* interaction (Table 6).

Table 6 – Possible subtilases associated to grapevine immunity and selected for further studies.

Adopted identifier	Chosen by
VtSBT1922	Sequence similarity with SBT3.3
VtSBT3195	
VtSBT4104	Located near *Rpv9*
VtSBT4152	Sequence similarity with SBT3.3
VtSBT4153	Sequence similarity with SBT3.3
VtSBT5381	Sequence similarity with P69C
VtSBT5410	
VtSBT5429	
VtSBT5471	
VtSBT7502	Located near *Rpv1* and *Rpv13*
VtSBT7672	Sequence similarity with SBT3.3
VtSBT7899	Located near *Rpv9*
VtSBT8450	Sequence similarity with SBT3.3
VtSBT8505	Expression studies in *Vitis vinifera-P. viticola* pathosystem (Figueiredo et al. 2008, 2012)

3.2 Analysis of subtilases putatively involved in *V. vinifera* immunity

3.2.1 Biochemical characterization of grapevine subtilases possibly involved in immunity

The fourteen selected grapevine subtilases potentially linked to *V. vinifera* immunity were further analysed. A prediction of glycosylated sites was performed, a search for the main subtilase domains was carried out, the protein–protein interaction network (interactome) was investigated and an expression analysis by qPCR was done during grapevine interaction with the oomycete *P. viticola*.

Previous studies have shown that plant subtilases are glycosylated and this post-translational modification regulates their activity (Bykova et al., 2006; Cedzich et al., 2009). Glycosylated subtilases are then secreted to the plant extracellular matrix (ECM) where they accumulate and presumably exert their biochemical functions (Siezen and Leunissen, 1997;

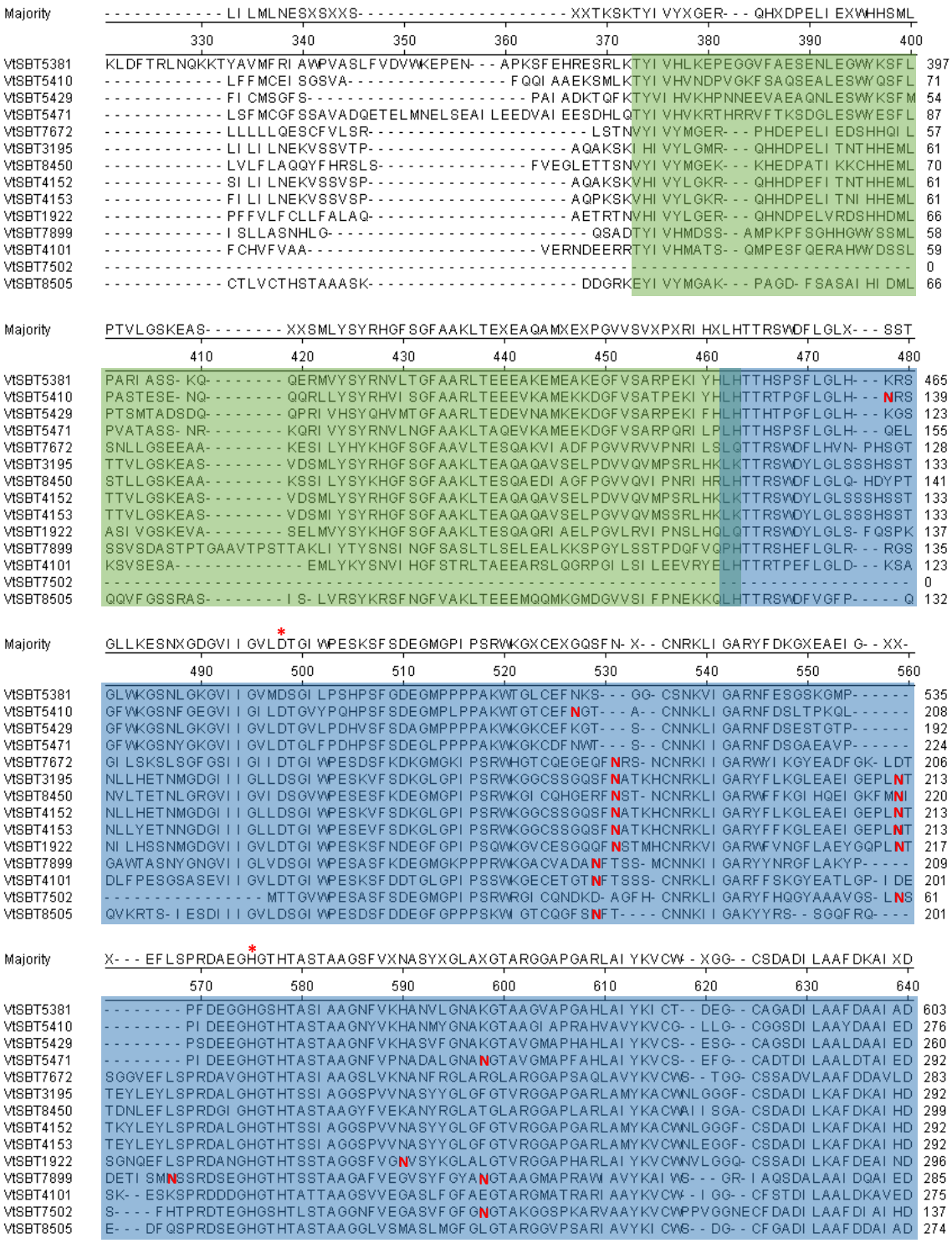
Taylor et al., 1997; Tornero et al., 1997, 1996a, 1996b; Yamagata et al., 1994). Since the ECM is where the first host-pathogen interaction, recognition and signalling events take place (Dixon and Lamb, 1990), the accumulation of subtilases in plant ECM may account for an important role during pathogenesis. Thus, it was performed a prediction of protein glycosylation in the 14 grapevine subtilases. The most important protein glycosylation form is *N*-linked, formed by the covalent attachment of asparagine-linked carbohydrates to the protein (Bykova et al., 2006; Ramneek and Brunak, 2001). Protein *N*-glycosylation was previously described in subtilases P69B from tomato, predicted by NetNGlyc server and further confirmed by mass spectrometry (Bykova et al., 2006). Hence, it was searched for protein *N*-glycosylation in the 14 grapevine subtilases using the NetNGlyc online server. This tool searches for the signature N-X-S/T. Sites in which X is a proline are highly unlikely to be glycosylated, presumably due to conformational constraints, and were not considered in this results. SignalP server is automatically run on all sequences analysed with NetNGlyc, to identify possible signal peptides, since proteins without signal peptides are unlikely to be *N*-glycosylated. From the fourteen protein sequences analysed, only two may not contain a signal peptide (VtSBT5381 - subtilisin-like protease SDD1 and VtSBT7502 - subtilisin-like protease SBT5.3 isoform X2, Table 7). This result was confirmed with the previously signal peptide prediction made in MemPype software. Proteins without signal peptides are unlikely to be exposed to the *N*-glycosylation machinery and thus may not be glycosylated *in vivo*, even though they contain potential motifs. The remaining twelve proteins seem to contain a signal peptide and *N*-glycosylation was predicted in several Asp residues (Table 7). Despite the subtilases VtSBT5381 and VtSBT7502 have not signal peptide, all of the 14 grapevine subtilases proteins were predicted to be located in apoplast, plant-type cell wall or extracellular region. So, in some way, these subtilases seem to pass into the secretory pathway and are sent to the cell membrane zone.

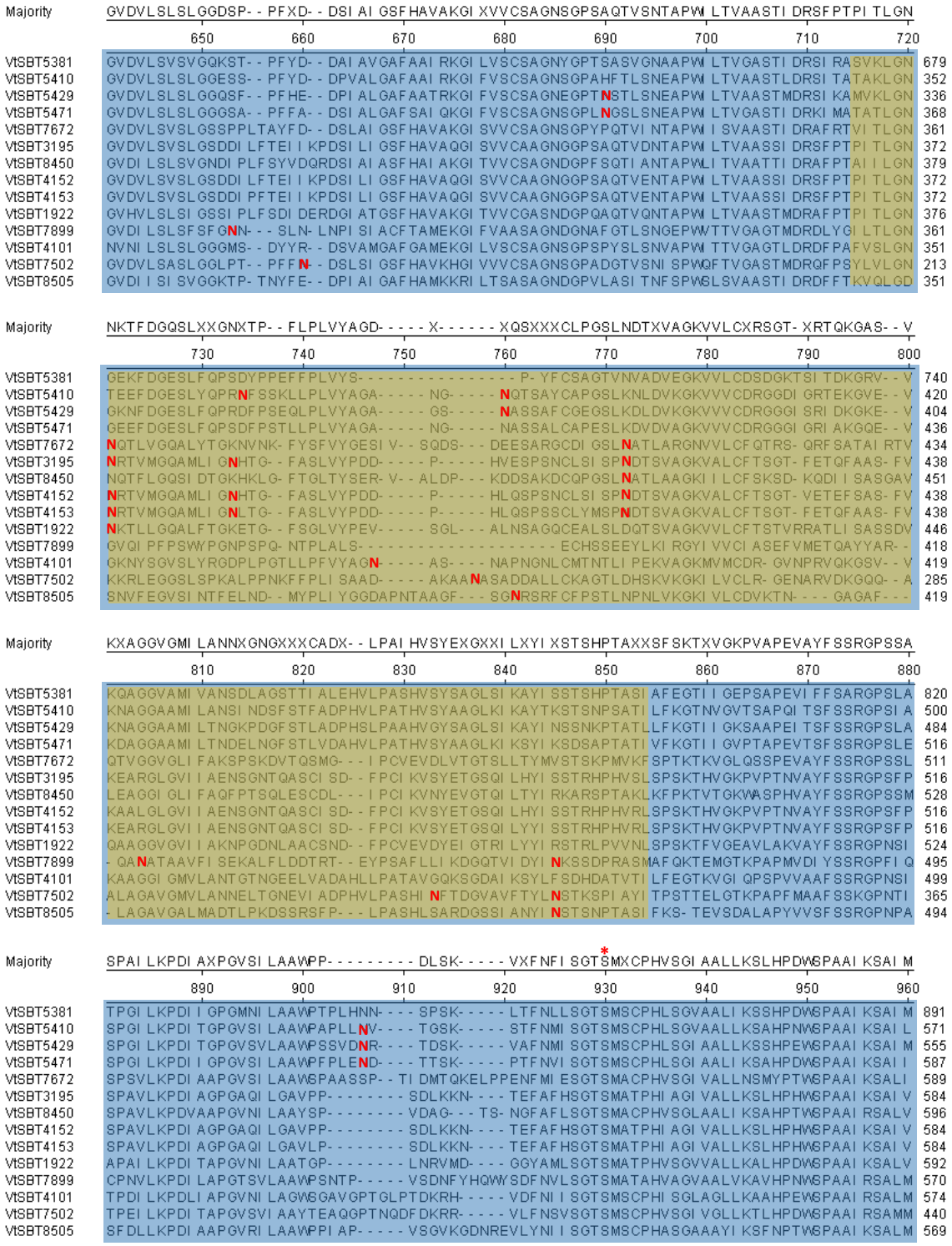
Table 7 – Signal peptide, *N*-glycosylation and subcellular location prediction for the subtilases putatively involved in grapevine immunity. Asterisk (*) indicates sequences that may not contain a signal peptide; proteins without signal peptides are unlikely to be exposed to the *N*-glycosylation machinery and thus may not be glycosylated *in vivo*, even though they contain potential motifs.

Adopted identifier and predicted name	**Signal Peptide prediction, MemPype Software (position)**	**Signal Peptide prediction, SignalP 4.1 Server (position)**	***N*-glycosylation prediction (Asn-X-Ser/Thr), NetNGlyc 1.0 Server (position and sequence)**	**Subcellular location prediction, Blast2GO**
VtSBT5381 subtilisin-like protease SDD1	NO	NO	YES * (15 **N**GT) (259 **N**CS) (314 **N**VS) (951 **N**DT)	Apoplast; Plant-type cell wall
VtSBT5410 subtilisin-like protease SDD1	YES (1-31)	YES (1-31)	YES (137 **N**RS) (186 **N**GT) (366 **N**FS) (382 **N**QT) (526 **N**VT) (644 **N**CS)	Apoplast; Plant-type cell wall
VtSBT5429 subtilisin-like protease	YES (1-20)	YES (1-20)	YES (306 **N**ST) (510 **N**RT) (615 **N**DT)	Apoplast; Plant-type cell wall
VtSBT5471 uncharacterized protein LOC100242816	YES (1-31)	YES (1-31)	YES (254 **N**GT) (338 **N**GS) (398 **N**AS) (542 **N**DT) (656 **N**RT) (699 **N**SS) (1053 **N**TT) (1196 **N**ST)	Apoplast; Plant-type cell wall
VtSBT7672 subtilisin-like protease SBT3.5	YES (1-27)	YES (1-27)	YES (179 **N**RS) (362 **N**QT) (407 **N**AT) (651 **N**TT)	Extracellular region
VtSBT3195 subtilisin-like protease SBT3.5	YES (1-33)	YES (1-27)	YES (184 **N**AT) (212 **N**TT) (373 **N**RT) (385 **N**HT) (412 **N**DT) (646 **N**NS) (682 **N**ST) (689 **N**VT) (697 **N**ST)	Extracellular region
VtSBT8450 subtilisin-like protease SBT3.3	YES (1-39)	YES (1-35)	YES (192 **N**ST) (219 **N**IT) (424 **N**AT) (642 **N**IS) (734 **N**LT)	Extracellular region

VtSBT4152 subtilisin-like protease SBT3.5	YES (1-33)	YES (1-27)	YES (184 **N**AT) (212 **N**TT) (373 **N**RT) (385 **N**HT) (412 **N**DT) (646 **N**NS) (682 **N**ST) (689 **N**VT) (697 **N**ST)	Apoplast; Plant-type cell wall
VtSBT4153 subtilisin-like protease SBT3.5	YES (1-30)	YES (1-27)	YES (184 **N**AT) (212 **N**TT) (373 **N**RT) (385 **N**LT) (412 **N**DT) (646 **N**NS) (682 **N**ST) (689 **N**VT) (697 **N**ST) (722 **N**ST)	Extracellular region
VtSBT1922 LOW QUALITY PROTEIN: subtilisin-like protease SBT3.5	YES (1-36)	YES (1-36)	YES (188 **N**ST) (216 **N**TS) (247 **N**VS) (377 **N**KT) (925 **N**TT) (956 **N**AS) (1086 **N**VT)	Extracellular region
VtSBT7899 subtilisin-like protease	YES (1-23)	YES (1-23)	YES (184 **N**FT) (216 **N**SS) (247 **N**GT) (298 **N**NS) (421 **N**AT) (460 **N**KS) (588 **N**DT) (614 **N**AT) (687 **N**YT) (719 **N**LT)	Apoplast; Plant-type cell wall
VtSBT4101 subtilisin-like protease	YES (1-24)	YES (1-24)	YES (172 **N**FT) (378 **N**AS) (633 **N**YS)	Apoplast; Plant-type cell wall
VtSBT7502 subtilisin-like protease SBT5.3 isoform X2	NO	NO	YES * (60 **N**SS) (95 **N**GT) (155 **N**DS) (245 **N**AS) (318 **N**FT) (330 **N**ST) (455 **N**AS) (500 **N**QT)	Apoplast; Plant-type cell wall
VtBST8505 cucumisin isoform X1	YES (1-35)	YES (1-29)	YES (180 **N**FT) (387 **N**RS) (460 **N**ST) (640 **N**GT) (666 **N**RT)	Extracellular region

A multiple alignment of the fourteen subtilases was made in DNASTAR software to highlight the three conserved domains (I9 inhibitor, S8 peptidase and PA), the location of the three catalytic residues (aspartate (Asp), histidine (His) and serine (Ser)) and the glycosylated sites (Figure 8).




Majority GVDVLSLSLGGDSP--PFYD--DSIAIGSFHAVAKGIXVVCSAGNSGPSAQTVSNTAPWLTVAASTIDRSFPTPITLGN
650 660 670 680 690 700 710 720
VtSBT5381 GVDVLSVSVGQKST--PFYD--DAIAVGAFAAIRKGILVSCSAGNYGPTSASVGNAAPWLTVGASTIDRSIRASVKLGN 679
VtSBT5410 GVDVLSLSLGGESS--PFYD--DPVALGAFAAIRKGIFVSCSAGNSGPAHFTLSNEAPWLTVAASTLDRSITATAKLGN 352
VtSBT5429 GVDVLSLSLGGQSF--PFHE--DPIALGAFAATRKGIFVSCSAGNEGPTNSTLSNEAPWLTVAASTMDRSIKAMVKLGN 336
VtSBT5471 GVDVLSLSLGGGSA--PFFA--DSIALGAFSAIQKGIFVSCSAGNSGPLNGSLSNEAPWLTVGASTIDRKIMATATLGN 368
VtSBT7672 GVDVLSVSLGSSPPLTAYFD--DSLAIGSFHAVAKGISVVCSAGNSGPYPQTVINTAPWISVAASTIDRAFRTVITLGN 361
VtSBT3195 GVDVLSVSLGSDDILFTEIIKPDSILIGSFHAVAQGISVVCAAGNGGPSAQTVDNTAPWLTVAASSIDRSFPTPITLGN 372
VtSBT8450 GVDILSLSVGNDIPLFSYVDQRDSIAIASFHAIAKGITVVCSAGNDGPFSQTIANTAPWLITVAATTIDRAFPTAIILGN 379
VtSBT4152 GVDVLSVSLGSDDILFTEIIKPDSILIGSFHAVAQGISVVCAAGNGGPSAQTVENTAPWLTVAASSIDRSFPTPITLGN 372
VtSBT4153 GVDVLSVSLGSDDIPFTEIIKPDSILIGSFHAVAQGISVVCAAGNGGPSAQTVENTAPWLTVAASSIDRSFPTPITLGN 372
VtSBT1922 GVHVLSLSIGSSIPLFSDIDERDGIATGSFHAVAKGITVVCGASNDGPQAQTVQNTAPWLTVAASTMDRAFPTPITLGN 376
VtSBT7899 GVDILSLSFSFGNN---SLN-LNPISIACFTAMEKGIFVAASAGNDGNAFGTLSNGEPWVTTVGAGTMDRDLYGILTLGN 361
VtSBT4101 NVNILSLSLGGGMS--DYYR--DSVAMGAFGAMEKGILVSCSAGNSGPSYSLSNVAPWITTVGAGTLDRDFPAFVSLGN 351
VtSBT7502 GVDVLSASLGGLPT--PFFN--DSLSIGSFHAVKHGIVVVCSAGNSGPADGTVSNISPWSQFTVGASTMDRQFPSYLVLGN 213
VtSBT8505 GVDIISISVGGKTP-TNYFE--DPIAIGAFHAMKKRILTSASAGNDGPVLASITNFSPWSLSVAASTIDRDFFTKVQLGD 351
Majority NKTFDGQSLXXGNXTP--FLPLVYAGD-----X------XQSXXXCLPGSLNDTXVAGKVVLCXRSGT-XRTQKGAS--V
730 740 750 760 770 780 790 800
VtSBT5381 GEKFDGESLFQPSDYPPEFFPLVYS----------P-YFCSAGTVNVADVEGKVVLCDSDGKTSITDKGRV--V 740
VtSBT5410 TEEFDGESLYQPRNFSSKLLPLVYAGA-----NG-----NQTSAYCAPGSLKNLDVKGKVVVCDRGGDIGRTEKGVE--V 420
VtSBT5429 GKNFDGESLFQPRDFPSEQLPLVYAGA-----GS-----NASSAFCGEGSLKDLDVKGKVVVCDRGGGISRIDKGKE--V 404
VtSBT5471 GEEFDGESLFQPSDFPSTLLPLVYAGA-----NG-----NASSALCAPESLKDVDVAGKVVVCDRGGGIGRIAKGQE--V 436
VtSBT7672 NQTLVGQALYTGKNVNK-FYSFVYGESIV--SQDS---DEESARGCDIGSLNATLARGNVVLCFQTRS-QRFSATAIRTV 434
VtSBT3195 NRTVMGQAMLIGNHTG--FASLVYPDD-----P-----HVESPSNCLSISPNDTSVAGKVALCFTSGT-FETQFAAS-FV 438
VtSBT8450 NQTFLGQSIDTGKHKLG-FTGLTYSER-V--ALDP---KDDSAKDCQPGSLNATLAAGKIICFSKSD-KQDIISASGAV 451
VtSBT4152 NRTVMGQAMLIGNHTG--FASLVYPDD-----P-----HLQSPSNCLSISPNDTSVAGKVALCFTSGT-VETEFSAS-FV 438
VtSBT4153 NRTVMGQAMLIGNLTG--FASLVYPDD-----P-----HLQSPSSCLYMSPNDTSVAGKVALCFTSGT-FETQFAAS-FV 438
VtSBT1922 NKTLLGQALFTGKETG--FSGLVYPEV-----SGL---ALNSAGQCEALSLDQTSVAGKVVLCFTSTVRRATLISASSDV 446
VtSBT7899 GVQIPFPSWYPGNPSPQ-NTPLALS----------ECHSSEEYLKIRGYIVVCIASEFVMETQAYYAR--
VtSBT4101 GKNYSGVSLYRGDPLPGTLLPFVYAGN-----AS---NAPNGNLCMTNTLIPEKVAGKMVMCDR-GVNPRVQKGSV--V 419
VtSBT7502 KKRLEGGSLSPKALPPNKFFPLISAAD-----AKAANASADDALLCKAGTLDHSKVKGKILVCLR-GENARVDKGQQ--A 285
VtSBT8505 SNVFEGVSINTFELND--MYPLIYGGDAPNTAAGF---SGNRSRFCFPSTLNPNLVKGKIVLCDVKTN----GAGAF---
Majority KXAGGVGMILANNXGNGXXXCADX--LPAIHVSYEXGXXILXYISXSTSHPTAXXSFSKTXVGKPVAPEVAYFSSRGPSSA
810 820 830 840 850 860 870 880
VtSBT5381 KQAGGVAMIVANSDLAGSTTIALEHVLPASHVSYSAGLSIKAYISSTSHPTASIAFEGTIIGEPSAPEVIFFSARGPSLA 820
VtSBT5410 KNAGGAAMILANSINDSFSTFADPHVLPATHVSYAAGLKIKAYTKSTSNPSATILFKGTNVGVTSAPQITSFSSRGPSIA 500
VtSBT5429 KNAGGAAMILTNGKPDGFSTLADPHSLPAAHVGYSAGLSIKAYINSSNKPTATLLFKGTIIGKSAAPEITSFSSRGPSLA 484
VtSBT5471 KDAGGAAMILTNDELNGFSTLVDAHVLPATHVSYAAGLKIKSYIKSDSAPTATIVFKGTIIGVPTAPEVTSFSSRGPSLE 516
VtSBT7672 QTVGGVGLIFAKSPSKDVTQSMG---IPCVEVDLVTGTSLLTYMVSTSKPMVKFSPTKTKVGLQSSPEVAYFSSRGPSSL 511
VtSBT3195 KEARGLGVIIAENSGNTQASCISD--FPCIKVSYETGSQILHYISSTRHPHVSLSPSKTHVGKPVPTNVAYFSSRGPSFP 516
VtSBT8450 LEAGGIGLIFAQFPTSQLESCDL---IPCIKVNYEVGTQILTYIRKARSPTAKLKFPKTVTGKWASPHVAYFSSRGPSSM 528
VtSBT4152 KAALGLGVIIAENSGNTQASCISD--FPCIKVSYETGSQILHYISSTRHPHVRLSPSKTHVGKPVPTNVAYFSSRGPSFP 516
VtSBT4153 KEARGLGVIIAENSGNTQASCISD--FPCIKVSYETGSQILYYISSTRHPHVRLSPSKTHVGKPVPTNVAYFSSRGPSFP 516
VtSBT1922 QAAGGVGVIIAKNPGDNLAACSND--FPCVEVDYEIGTRILYYIRSTRLPVVNLSPSKTFVGEAVLAKVAYFSSRGPNSI 524
VtSBT7899 -QANATAAVFISEKALFLDDTRT--EYPSAFLLIKDGQTVIDYINKSSDPRASMAFQKTEMGTKPAPMVDIYSSRGPFIQ 495
VtSBT4101 KAAGGIGMVLANTGTNGEELVADAHLLPATAVGQKSGDAIKSYLFSDHDATVTILFEGTKVGIQPSPVVAAFSSRGPNSI 499
VtSBT7502 ALAGAVGMVLANNELTGNEVIADPHVLPASHINFTDGVAVFTYLNSTKSPIAYITPSTTELGTKPAPFMAAFSSKGPNTI 365
VtSBT8505 -LAGAVGALMADTLPKDSSRSFP---LPASHLSARDGSSIANYINSTSNPTASIFKS-TEVSDALAPYVVSFSSRGPNPA 494
Majority SPAILKPDIAXPGVSILAAWPP---------DLSK-----VXFNFISGTSMXCPHVSGIAALLKSLHPDWSPAAIKSAIM
890 900 910 920 930 940 950 960
VtSBT5381 TPGILKPDIIGPGMNILAAWPTPLHNN----SPSK-----LTFNLLSGTSMSCPHLSGVAALIKSSHPDWSPAAIKSAIM 891
VtSBT5410 SPGILKPDITGPGVSILAAWPAPLLNV----TGSK-----STFNMISGTSMSCPHLSGVAALLKSAHPNWSPAAIKSAIL 571
VtSBT5429 SPGILKPDITGPGVSVLAAWPSSVDNR----TDSK-----VAFNMISGTSMSCPHLSGIAALLKSSHPEWSPAAIKSAIM 555
VtSBT5471 SPGILKPDIIGPGVSILAAWPFPLEND----TTSK-----PTFNVISGTSMSCPHLSGIAALIKSAHPDWSPAAIKSAII 587
VtSBT7672 SPSVLKPDIAAPGVSILAAWSPAASSP--TIDMTQKELPPENFMIESGTSMACPHVSGIVALLNSMYPTWSPAAIKSALI 589
VtSBT3195 SPAVLKPDIAGPGAQILGAVPP--------SDLKKN----TEFAFHSGTSMATPHIAGIVALLKSLHPHWSPAAIKSAIV 584
VtSBT8450 SPAVLKPDVAAPGVNILAAYSP--------VDAG---TS-NGFAFLSGTSMACPHVSGLAALIKSAHPTWSPAAIRSALV 596
VtSBT4152 SPAVLKPDIAGPGAQILGAVPP--------SDLKKN----TEFAFHSGTSMATPHIAGIVALLKSLHPHWSPAAIKSAIV 584
VtSBT4153 SPAVLKPDIAGPGAQILGAVLP--------SDLKKN----TEFAFHSGTSMATPHIAGIVALLKSLHPHWSPAAIKSAIV 584
VtSBT1922 APAILKPDITAPGVNILAATGP--------LNRVMD----GGYAMLSGTSMATPHVSGVVALLKALHPDWSPAAIKSALV 592
VtSBT7899 CPNVLKPDILAPGTSVLAAWPSNTP-----VSDNFYHQWWSDFNVLSGTSMATAHVAGVAALVKAVHPNWSPAAIRSALM 570
VtSBT4101 TPDILKPDLIAPGVNILAGWSGAVGPTGLPTDKRH-----VDFNIISGTSMSCPHISGLAGLLKAAHPEWSPAAIRSALM 574
VtSBT7502 TPEILKPDITAPGVSVIAAYTEAQGPTNQDFDKRR-----VLFNSVSGTSMSCPHVSGIVGLLKTLHPDWSPAAIRSAMM 440
VtSBT8505 SFDLLKPDIAAPGVRILAAWPPIAP-----VSGVKGDNREVLYNIISGTSMSCPHASGAAAYIKSFNPTWSPAAIKSALM 569

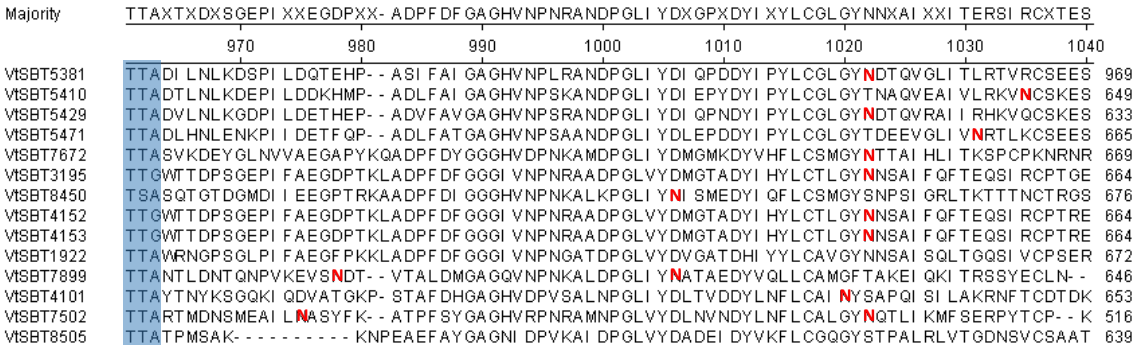


Figure 8 - Multiple alignment of fourteen subtilases putatively involved in grapevine immunity. Represented only the zone of major consensus between the proteins. Highlighted the three conserved domains (I9 inhibitor domain – green; S8 peptidase domain – blue; PA domain – yellow). Asterisk (*) represent the three catalytic residues (aspartate [D], histidine [H] and serine [S]). 'N' at red represent the *N*-glycosylated residues. For the proteins with domains duplication (VtSBT5381, VtSBT5471 and VtSBT1922) was only showed once the domains.

The protein–protein interaction network for the selected subtilases putatively involved in grapevine immunity was performed. By understanding the protein environment where these proteins can be involved, it can be possible obtain relevant information about their functions and the biological processes. For this analysis, the STRING database was used. This database aims to provide a critical assessment and integration of protein–protein interactions, including direct (physical) as well as indirect (functional) associations, from computational prediction, knowledge transfer between organisms, and interactions aggregated from other (primary) databases. The functional partnerships and interactions that occur between proteins are at the core of cellular processing and their systematic characterization helps to provide context in molecular systems biology (Szklarczyk et al., 2014). The protein sequences of the fourteen selected subtilases were used for the protein interaction search. STRING uses the gene identifiers associated with the proteins. Hence, for each of the 14 subtilases, we searched the gene identifier in UniProt Database (The UniProt Consortium 2015, http://www.uniprot.org/), (Table 8).

Table 8 - Correspondence between the adopted identifier for each subtilases and the subtilase gene identifier in UniProt Database

Adopted Identifier	Gene name
VtSBT1922	VIT_02s0025g02850.t01
VtSBT3195	VIT_16s0098g01160.t01
VtSBT4101	VIT_07s0129g00490.t01
VtSBT4152	VIT_16s0098g00970.t01
VtSBT4153	VIT_16s0098g00950.t01

VtSBT5381	VIT_02s0025g04820.t01
VtSBT5410	VIT_02s0025g04810.t01
VtSBT5429	VIT_02s0025g04800.t01
VtSBT5471	VIT_02s0025g04780.t01
VtSBT7502	VIT_12s0057g01450.t01
VtSBT7672	VIT_03s0038g00470.t01
VtSBT7899	VIT_07s0031g00500.t01
VtSBT8450	VIT_02s0025g02880.t01
VtSBT8505	VIT_13s0019g02490.t01

The top 50 proteins that interact with the 14 grapevine subtilases were analysed individually in UniProt in order to access the biological processes to which they are associated. Five of these proteins were predicted to interact with the selected 14 grapevine subtilases and are involved in biological processes associated to defence responses, namely fatty acid beta-oxidation, protein kinase activity and protein serine/threonine kinase activity (Table 9).

Table 9 - Information about the top five proteins that interact with the grapevine subtilases selected.

Gene name (gene identifier from STRING)	UniProt ID	Protein name	GO terms associated
VIT_10s0116g00330	D7TR79	Putative uncharacterized protein	**ER-associated ubiquitin-dependent protein catabolic process; positive regulation of RNA polymerase II transcriptional preinitiation complex assembly**; positive regulation of proteasomal protein catabolic process; nucleotide binding; ATP binding; TBP-class protein binding; proteasome-activating ATPase activity; proteasome regulatory particle, base subcomplex; nuclear proteasome complex; cytosolic proteasome complex
VIT_10s0116g00260	D7TR76	Putative uncharacterized protein	ER-associated ubiquitin-dependent protein catabolic process; **positive regulation of RNA polymerase II transcriptional preinitiation complex assembly;** positive regulation of proteasomal protein catabolic process; nucleotide binding; ATP binding; TBP-class protein binding; proteasome-activating ATPase activity; proteasome regulatory particle, base subcomplex; nuclear proteasome complex; cytosolic proteasome complex
VIT_05s0102g00260	F6HYR3	Putative uncharacterized protein	**negative regulation of chromatin silencing; positive regulation of transcription from RNA polymerase II promoter**; nucleotide binding; chromatin binding; ATP binding; ATPase activity; histone binding; nucleus

VIT_00s0125g00170	F6H2Q4	Putative uncharacterized protein	protein targeting to peroxisome; **fatty acid beta-oxidation**; peroxisome organization; protein import into peroxisome matrix; nucleotide binding; ATP binding; ATPase activity, coupled; peroxisome; peroxisomal membrane
VIT_00s0540g00020	F6HWU5	Putative uncharacterized protein	carbohydrate metabolic process; chitin catabolic process; protein phosphorylation; **defence response**; hydrolase activity, hydrolyzing *O*-glycosyl compounds; **chitinase activity; protein kinase activity; protein serine/threonine kinase activity**; ATP binding; plasma membrane; plasmodesma; integral component of membrane

One of the key processes in early plant defence signalling is enhanced lipid peroxidation and production of a vast array of oxylipins. Lipid peroxidation has been often linked to cell apoptosis, necrosis and programmed cell death; however, it is also linked to the synthesis of jasmonic acid (Walley et al., 2013) and recently, lipid peroxidation products, such as MDA or 4-HNE, have been described as signalling molecules in regulation of several transcription factors sensible to stress (reviewed in Ayala et al., 2014). Very recently, Guerreiro and co-workers (2016) showed that, after inoculation of grapevine species with *P. viticola*, an increase of lipid peroxidation was observed, suggesting an involvement of fatty acid oxidation in defence response to pathogen attack (Guerreiro et al., 2016).

Protein kinases are known to regulate the majority of cellular pathways, especially those involved in signal transduction (Dhanasekaran and Premkumar Reddy, 1998), and so far, it is known that the first host-pathogen interaction and recognition occurs in extracellular matrix where signalling events take place (Dixon and Lamb, 1990). Moreover, the SBT3.3 subtilase from *A. thaliana* has been associated to the activation of plasma membrane receptor and downstream signalling processes (Ramírez et al., 2013). This protein-protein interaction network result reinforces the idea that these fourteen subtilases may have some involvement in the grapevine immunity.

Based on the predictions obtained and in the results of Figueiredo and co-workers (2008, 2012) that associated a subtilase, similarly to P69 from tomato, with defence response of *Vitis vinifera* to *Plasmopara viticola* attack (Figueiredo et al., 2012, 2008; Monteiro et al., 2013), the expression of the fourteen grapevine subtilases was analysed in a pathosystem to understand the behaviour of these proteases when subjected to an environmental biotic stimulus.

Also, a re-analysis of these fourteen subtilases in the phylogenetic tree was performed to understand possible relationship between the proteins. Subtilases within the same group may

have the same response to *P. viticola* attack once it was suspected that have similar function. The subtilases VtSBT3195, VtSBT4152, VtSBT4153, VtSBT1922, VtSBT8450 and VtSBT7672 are located in the VvSBT3 group where all the sequences showed similarity with *A. thaliana* SBT3.3/SBT3.5. The subtilases VtSBT5410, VtSBT5381, VtSBT5429, VtSBT5471, VtSBT7899 and VtSBT4101 were included in VvSBT5 group containing all the sequences with subtilase annotation. The subtilase VtSBT8505 was within the VvSBT1 group referent to cucumisin proteins and the subtilase VtSBT7502 was included in VvSBT4 of proteins showing similarity to *A. thaliana* SBT5.3/SBT5.4 proteins. With the expression profile analysis of these grapevine subtilases in a pathosystem can be possible associated some of these groups with a defence response.

3.2.2 Subtilase expression profiles in *V. vinifera-P. viticola* pathosystem

The first gene expression studies in a grapevine-*P. viticola* pathosystem were carried out by Figueiredo and co-workers (2008) by comparing resistant and susceptible genotypes, prior and post-inoculation with *P. viticola.* Their results revealed a subtilisin-like protein, sharing sequence similarity with the tomato P69C subtilase, that was constitutively expressed in the resistant genotype and that increased its expression after inoculation with *P. viticola* (Figueiredo et al., 2012, 2008; Monteiro et al., 2013). In the present study, the expression profile of the fourteen selected subtilases was evaluated in a pathosystem involving two grapevine cultivars and *Plasmopara viticola*. The subtilase gene expression was analysed in leaves of two *Vitis vinifera* cultivars, Regent and Trincadeira (resistant and susceptible to this oomycete, respectively), at three time-points 6, 12 and 24 hours post inoculation (hpi). These inoculation time-points were chosen since they correspond to signalling events related to pathogen recognition in *V. vinifera*. Between 6 and 12 hpi stomatal penetration and development of stomatal vesicles with primary hyphae occur and at 24 hpi elongated hyphae invade the intercellular space of the mesophyll progressing to the branching stage in susceptible plants and stopping the development in resistant plants (Kortekamp and Zyprian, 2003; Unger et al., 2007).

The results showed that at 6 hpi with *P. viticola*, six subtilases (VtSBT4152, VtSBT4153, VtSBT3195, VtSBT7502, VtSBT8505 and VtSBT7672) have a high expression in *V. vinifera* cv. Regent comparatively to cv. Trincadeira (Table 10 and Supplementary Figure 1).

Table 10 - Expression profile for several subtilases in a *Vitis vinifera-Plasmopara viticola* pathosystem. Expression analysed in *V. vinifera* cv. Regent and *V. vinifera* cv. Trincadeira at three time-points after inoculation (6 hpi, 12 hpi and 24 hpi). Values between 0 and 1 represent down-regulation of expression, around 1 means basal expression and above 1 show up-regulation of expression. The corresponding graph is shown in appendix (Supplementary Figure 1).

	Fold change					
***Vitis* cultivars** / **Subtilase**	***V. vinifera* cv. Regent**			***V. vinifera* cv. Trincadeira**		
	6 hpi	**12 hpi**	**24 hpi**	**6 hpi**	**12 hpi**	**24 hpi**
VtSBT4101	0.25 ± 0.04	0.16 ± 0.11	2.07 ± 0.51	0.68 ± 0.08	1.81 ± 0.38	1.76 ± 0.49
VtSBT4152	1.77 ± 0.42	0.47 ± 0.30	2.55 ± 0.33	0.29 ± 0.02	0.65 ± 0.61	2.56 ± 1.02
VtSBT4153	4.27 ± 0.53	2.02 ± 0.71	0.67 ± 0.17	0.70 ± 0.35	1.21 ± 0.34	2.98 ± 1.00
VtSBT3195	5.30 ± 1.44	0.39 ± 0.49	4.76 ± 1.51	0.34 ± 0.27	0.76 ± 0.02	1.93 ± 0.85
VtSBT7502	10.06 ± 2.44	0.26 ± 0.13	2.18 ± 0.12	0.81 ± 0.10	1.93 ± 0.39	1.47 ± 0.27
VtSBT8505	324.63 ± 87.11	2.26 ± 0.38	2.00 ± 0.34	1.08 ± 0.20	2.56 ± 0.77	4.15 ± 2.08
VtSBT5381	0.09 ± 0.07	14.11 ± 8.62	0.06 ± 0.05	-	-	-
VtSBT7672	6.94 ± 0.10	0.34 ± 0.41	3.20 ± 0.17	2.31 ± 0.81	1.95 ± 0.69	1.54 ± 0.09
VtSBT5410	0.32 ± 0.04	1.04 ± 0.42	0.92 ± 0.08	-	-	-
VtSBT8450	0.09 ± 0.02	0.57 ± 0.25	0.66 ± 0.29	0.82 ± 0.11	1.09 ± 0.17	1.12 ± 0.29
VtSBT5471	0.39 ± 0.06	0.57 ± 0.47	1.36 ± 0.03	0.50 ± 0.21	0.94 ± 0.26	1.48 ± 0.31
VtSBT1922	0.07 ± 0.00	0.37 ± 0.29	1.46 ± 0.31	0.23 ± 0.09	1.04 ± 0.39	2.92 ± 2.53

The subtilase VtSBT7672 was one of the subtilases analysed by Cao and co-workers (2014) at different abiotic stimuli and in different tissues without stimulation. Their results showed that this subtilase has a constitutive high-level of expression in roots, leaves and stem. However, the expression was supressed in abiotic stress conditions (Cao et al., 2014), which may suggest that this subtilase could have a participation in response to biotic stimulus, like pathogen attack, but not in response to abiotic stimulus.

For the subtilases VtSBT4152, VtSBT3195, VtSBT7502 and VtSBT7672 was observed at 12 hpi in *V. vinifera* cv. Regent an abrupt down-regulation of its expression followed by an increase of expression at 24 hpi. For the subtilases VtSBT4153 and VtSBT8505, at 12 hpi in *V. vinifera* cv. Regent, besides the highly decrease of expression, they remain up-regulated. At 24 hpi, VtSBT4153 decreases its expression and VtSBT8505 maintains the expression level observed at 12 hpi. The subtilase VtSBT8505 showed to be the most interesting because of their extremely increased expression at 6 hpi followed by a drastic decrease at 12 hpi in the resistant cultivar (Table 10 and Supplementary Figure 1). This subtilase was previously identified by Figueiredo and co-workers (2008, 2012), showing a high expression level in *V. vinifera* cv. Regent comparatively to *V. vinifera* cv. Trincadeira, both at constitutive level and after inoculation of both cultivars with the *P. viticola* (Figueiredo et al., 2012, 2008). Thus, it was suggested an

involvement of this subtilase in a defence response of grapevine against *P. viticola* in the first few hours after infection.

These six subtilases belong to VvSBT1 (VtSBT8505), VsSBT3 (VtSBT3195, VtSBT4152, VtSBT4153 and VtSBT7672) and VvSBT4 (VtSBT7502) groups, which may suggest that subtilases showing similarity with cucumisin, SBT3.3/SBT3.5 and SBT5.3/SBT5.4 can have some role in defence response to pathogen attack.

A subtilase with an odd behaviour was VtSBT5381, exhibiting a down-regulation in *V. vinifera* cv. Regent at 6 hpi, with a high increase of expression at 12 hpi, becoming down regulated again at 24 hpi. This behaviour was exactly the opposite of that observed for the subtilases analysed, so far. In *V. vinifera* cv. Trincadeira this subtilase was not expressed in any time-point post-inoculation (Table 10 and Supplementary Figure 1). As mentioned, it was between 6 and 12 hours after inoculation with *P. viticola* that stomatal penetration and development of stomatal vesicles with primary hyphae occur. Curiously, this VtSBT5381 subtilase, a subtilisin-like protease SDD1 besides lacks a signal peptide (as predicted by SignalP, Table 7) was predicted located at apoplast/plant-type cell wall (Blast2GO prediction, Table 7), however, its function in grapevine and its role in mildew defence remains unknown. In *Arabidopsis thaliana*, the subtilases SDD1 have a function in stomatal density and distribution (Rautengarten et al., 2005). The subtilase VtSBT5410, also a subtilisin-like protease SDD1, was another protein without any detected expression in *V. vinifera* cv. Trincadeira. In *V. vinifera* cv. Regent it is down-regulated at 6 hpi, presented a basal expression level at 12 and 24 hpi (Table 10 and Supplementary Figure 1). Unlike the VtSBT5381, in this subtilase there was a predicted signal peptide (Table 7) and its predicted location was equally the apoplast or plant-type cell wall. The subtilases VtSBT5429 and VtSBT7899 (both subtilisin-like proteases) presented no expression neither in *V. vinifera* cv. Regent or *V. vinifera* cv. Trincadeira inoculated with *P. viticola*.

The subtilase VtSBT4101 presented a down-regulation of its expression in *V. vinifera* cv. Regent at 6 hpi and 12 hpi, and an increased expression at 24 hpi. In *V. vinifera* cv. Trincadeira this subtilase has apparently the same behaviour but a little early, increasing its expression at 12 hpi, (Table 10 and Supplementary Figure 1). Results from Cao and co-workers (2014), showed that this subtilase appears to be more expressed in berries and has a later behaviour in abiotic stress conditions, showing expression in all conditions (salt, cold, heat and drought) but at later time-points (Cao et al., 2014). Both results suggest an involvement of this subtilase in response to abiotic and biotic environmental stimulus, however at a later stage after stimulation.

The subtilases VtSBT5381, VtSBT5410 and VtSBT4101 belong at the VvSBT5 group which suggest that this subtilase group may have a function in grapevine immunity but with a later and less robust response.

The subtilases VtSBT8450, VtSBT5471 and VtSBT1922 were highly down-regulated in *V. vinifera* cv. Regent at 6 hpi. At 12 hpi, the three genes were less repressed and only at 24 hpi two of them (VtSBT5471 and VtSBT1922) were up-regulated in *V. vinifera* cv. Regent, while the other gene (VtSBT8450) remained its expression down-regulated at 24 hpi (Table 10 and Supplementary Figure 1). For all of these three subtilases, in *V. vinifera* cv. Trincadeira, the expression increased slowly over the three time-points, starting down-regulated at 6 hpi and finishing up-regulated at 24 hpi (Table 10 and Supplementary Figure 1). The subtilases VtSBT5471 and VtSBT1922 were also analysed by Cao and co-workers in response to abiotic stresses. VtSBT5471 showed low expression when submitted to abiotic stress conditions (heat and drought) but demonstrated high expression level in berries and stem (Cao et al., 2014). The subtilase VtSBT1922, despite having constitutive expression uniformly in all tissues, was suppressed in abiotic stress conditions (Cao et al., 2014), which together with the current results might suggest that this subtilase does not have a role in immunity.

Analysing the results focusing only on the compatible interaction (susceptible cultivar) it was observed that seven subtilases were down-regulated at 6 hpi (VtSBT4101, VtSBT4152, VtSBT4153, VtSBT3195, VtSBT7502, VtSBT8450, VtSBT5471 and VtSBT1922), with exception for VtSBT8505 and VtSBT7672, although for all of them the expression was lower than observed in the resistant cultivar Regent. At 12 hpi, some subtilases were down-regulated (VtSBT4152 and VtSBT3195), and others up-regulated (VtSBT4101, VtSBT4153, VtSBT7502, VtSBT8505 and VtSBT7672). At 24 hpi, all subtilases are up-regulated (VtSBT4101, VtSBT4152, VtSBT4153, VtSBT3195, VtSBT7502, VtSBT8505, VtSBT7672, VtSBT8450, VtSBT5471 and VtSBT1922), (Table 10 and Supplementary Figure 1). It is clear that the increase in subtilase expression in *V. vinifera* cv. Trincadeira presents a delay when compared to the resistant cultivar, in which several subtilases are highly expressed 6 hours after inoculation with the oomycete. This may be related to an attempt by the susceptible cultivar to initiate a defence strategy that is not fast or robust enough to prevent pathogen growth. Moreover, this results, may suggest a most early and robust response of grapevine subtilases that shared similarity with cucumisin, SBT3.3/SBT3.5 and SBT5.3/SBT5.4 in *Vitis vinifera* immunity, comparatively with other subtilases within the remaining groups.

The present results suggest that under pathogen attack, resistant grapevine cultivar have an early response increasing the expression of some subtilases. This early increase of subtilase expression may be related to the establishment of a defence strategy against the invading pathogen. Based on these promising results for *V. vinifera* cv. Regent (at 6 hpi), the expression of some subtilases (VtSBT4152, VtSBT4153, VtSBT3195, VtSBT7502, VtSBT8505 and VtSBT7672) was also analysed in other resistant grapevine species to understand if there is a constitutive expression of these subtilases that can distinguish them from susceptible species to *P. viticola*.

3.2.3 Subtilase expression profiles in *Vitis* species

Wild American *Vitis* species are naturally resistant (or tolerant) to *P. viticola*. Although not used for wine production, the resistance traits are an advantage in the development of hybrid species through cross-breeding programs with *Vitis vinifera* (Gessler et al., 2011). The cultivars Regent and Bianca, developed from a European *V. vinifera* and American vine species, is a successful example of this breeding strategy, combining quality traits and resistance: produces good quality wine and shows a broad resistance to the major fungal diseases, including downy mildew (Gessler et al., 2011). Based on the subtilase expression levels in the *V. vinifera* cv. Regent at 6 hpi in *V. vinifera-P. viticola* pathosystem, the expression profile of six subtilases (VtSBT4152, VtSBT4153, VtSBT3195, VtSBT7502, VtSBT8505 and VtSBT7672), that showed a high expression level after inoculation with *P. viticola*, was evaluated in several *Vitis* species showing different degrees of resistance towards *P. viticola*. These wild *Vitis* species were *V. labrusca, V. rupestris, V. rotundifolia, V. riparia, V. sylvestris and V. candicans*, described as resistant or tolerant (Table 1). This analysis was also performed in the resistant *V. vinifera* cultivar Regent and in the susceptible cultivar Trincadeira to evaluate the basal expression level of these subtilases, without infection. In this study, the cultivar Trincadeira was used as control, since it is a susceptible grapevine. So, all the results obtained for the resistant and tolerant species were normalized against the cultivar Trincadeira.

The results showed that the subtilases VtSBT4152, VtSBT3195 and VtSBT7502 were down-regulated in all species. The subtilase VtSBT4153 showed a high level of expression in *V. vinifera* cv. Regent. The subtilase VtSBT8505 was highly expressed in *V. sylvestris* and *V. vinifera* cv. Regent. As for the subtilase VtSBT7672, it has its expression more increased in *V. labrusca*, *V. rotundifolia* and *V. vinifera* cv. Regent. However, it was not possible see a pattern that distinguishes a specific subtilase and associate it with some kind of resistance marker of *Vitis*

species (Table 11 and Supplementary Figure 2). Comparing with the results obtained in the pathosystem analysis (Table 11 and Supplementary Figure 2), the subtilase VtSBT4153,), showed higher expression at basal level. The subtilases VtSBT8505 and VtSBT7672 in the same comparison, on the other hand, showed a high increase of its expression in *V. vinifera* cv. Regent when inoculated with the *P. viticola*, which may suggest a more important and highly response of these two subtilases in grapevine immunity. The subtilases VtSBT4152, VtSBT3195 and VtSBT7502, were down-regulated constitutively and up-regulated in V. vinifera cv. Regent at 6 hours post inoculation with P. viticola, though they seem to take only a function in immunity when the plants were subjected to a biotic environmental stimulus.

Table 11 - Subtilases expression profile in *Vitis* species and cultivars. Values between 0 and 1 represent down-regulation of expression, around 1 means basal expression and above 1 show up-regulation of expression. The corresponding graph is shown in appendix (Supplementary Figure 2).

	Fold change						
***Vitis* species** / **Subtilase**	***V. riparia***	***V. labrusca***	***V. rupestris***	***V. rotundifolia***	***V. candicans***	***V. sylvestris***	***V. vinifera* cv. Regent**
VtSBT4152	0.02 ± 0.00	0.10 ± 0.03	0.04 ± 0.03	-	0.18 ± 0.12	0.14 ± 0.11	1.01 ± 0.37
VtSBT4153	-	0.10 ± 0.08	-	-	0.01 ± 0.01	1.82 ± 0.94	17.92 ± 5.07
VtSBT3195	0.00 ± 0.00	0.06 ± 0.03	0.10 ± 0.07	0.16 ± 0.06	0.21 ± 0.14	0.73 ± 0.54	0.93 ± 0.25
VtSBT7502	0.39 ± 0.02	0.63 ± 0.32	0.58 ± 0.33	0.34 ± 0.08	0.52 ± 0.22	0.24 ± 0.15	1.17 ± 0.08
VtSBT8505	0.33 ± 0.03	1.58 ± 0.59	5.22 ± 2.02	0.32 ± 0.04	0.01 ± 0.00	12.08 ± 4.78	11.24 ± 3.39
VtSBT7672	1.26 ± 0.55	6.24 ± 1.62	0.81 ± 0.63	1.59 ± 0.41	0.38 ± 0.20	0.19 ± 0.07	2.52 ± 0.82

These results, together with results obtained from *V. vinifera-P. viticola* pathosystem analysis suggest that there is a differential expression of certain subtilases in resistant species but only after stimulation with the pathogen attack, which supports the hypothesis that some grapevine subtilases may have a role in defence response against *P. viticola*.

3.3 Cloning of subtilases putatively involved in *V. vinifera* immunity

The expression profile analysis of selected subtilases showed that, when submitted to a biotic environmental stimulus like *Plasmopara viticola* attack, the expression of some of these enzymes in *V. vinifera* cv. Regent leaves increased at the first hours after infection. This result suggests that certain subtilases may be involved in the defence response against pathogen

attack. Moreover, this response was more intense and early in the resistant *V. vinifera* cultivar comparatively to the susceptible one, which may be a characteristic of the resistance nature of this plant. With this in mind, it was necessary to characterize the most relevant subtilases that may play a crucial role in the defence response against *P. viticola*, for future functional and structural studies.

As a first approach, two subtilases were selected for cloning: VtSBT8505 and VtSBT7502. The subtilase VtSBT8505 was chosen due to its very high expression at 6 hpi in *V. vinifera* cv. Regent and because it showed a constitutively expression in the resistant genotype with an expression increase after inoculation with *P. viticola* (Figueiredo et al. 2008, Figueiredo et al. 2012). The subtilase VtSBT7502 was selected because it was the second subtilase with major expression in *V. vinifera* cv. Regent at 6 hpi. Both cDNAs were amplified from *V. vinifera* cv. Regent and the PCR products were analysed in agarose gel (Figure 9). The VtSBT7502 cDNA, with 1972 bp, and the VtSBT8505 cDNA, with 2307 bp, were isolated from the gel and digested with *EcoRI* and *XhoI* restriction enzymes.

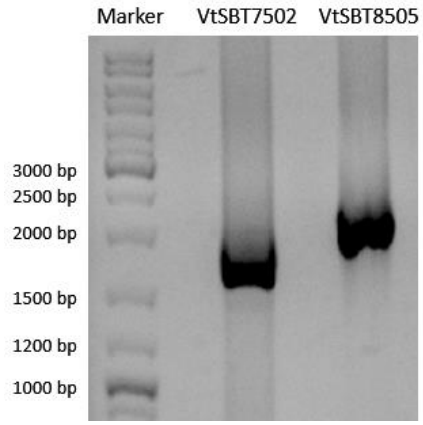


Figure 9 – PCR amplification of the VtSBT7502 and VtSBT8505 cDNAs (negative image). Marker: GeneRuler Ladder Mix, Thermo Scientific.

The expression vector pET28a (+) (Figure 3), with 5369 bp, was also digested with *EcoRI* and *XhoI* restriction enzymes and the digestion efficiency confirmed in agarose gel. This plasmid was chosen for bacterial protein expression because it expresses a His-tag (6xHis tag) at the C-terminal, facilitating the purification of the protein using immunoaffinity chromatography (IMAC).

Both cDNAs were cloned into the pET28a (+) vector (pET28a – VtSBT7502 and pET28a – VtSBT8505), (Figure 10), and the recombinant plasmids were transformed into *E. coli* One Shot TOP10 for plasmid propagation.

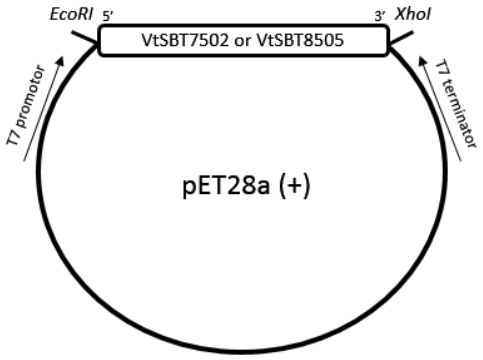


Figure 10 – Schematic representation of the vector-gene construction. The *EcoRI* and *XhoI* restriction sites and the direction of the T7 promotor and T7 terminator primers (used in sequencing) are shown.

The selection of the positive transformants was done by PCR screening of colonies (Colony-PCR) using the specific primers for each gene (Table 3) and the PCR products were analysed in agarose gel (Figure *11*).

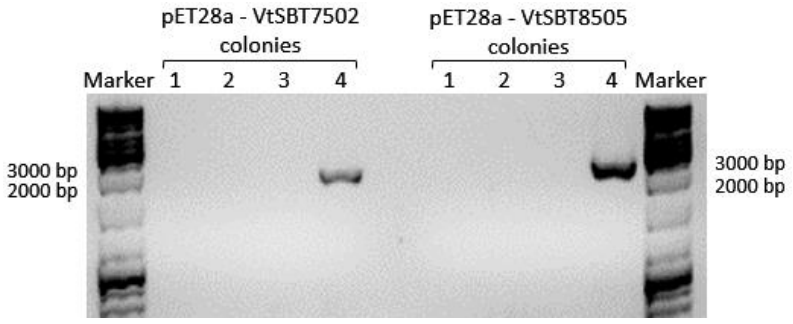


Figure 11 – PCR screening of colonies after *E. coli* transformation with the recombinant plasmids (pET28a – VtSBT7502 and pET28a – VtSBT8505), (negative image); Marker: GeneRuler Ladder Mix, Thermo Scientific.

The recombinant plasmids were extracted from the positive colonies (colony 4 in both cases) and the cDNA sequence was analysed using the T7 promotor and T7 terminator primers. The sequencing results confirmed the correct sequence for the cDNA VtSBT7502. However, the

VtSBT8505 cDNA cloned in the plasmid pET28a (+) was not the correct one, most probably due to unspecific amplification of the cDNA. This resulted in the amplification, and consequently isolation, of a different cDNA with the same apparent molecular weight.

With the correct cloning of VtSBT7502 into the plasmid pET28a (+), the recombinant plasmid can be used to transform bacterial expression cells for protein expression.

4 | Conclusion

Plant subtilases act as secretory enzymes, targeted to the endoplasmic reticulum and migrating to the cell plasma membrane. They are involved in many biological processes, being one of the most relevant their involvement in the plant response to biotic and abiotic environment stimulus. The main purpose of this project was to perform a thorough characterization of the grapevine subtilase gene family and associate some subtilase genes with Vitis vinifera defence responses towards *Plasmopara viticola*, the causative agent of downy mildew disease. Eighty-five subtilase genes were identified, unevenly distributed among 15 of the 19 grapevine chromosomes, coding for 97 subtilase proteins, as result of alternative *splicing*. These proteins were organized into 6 groups accordingly with their similarity. A subcellular location analysis showed that the majority of the grapevine subtilases were located in apoplast, cell wall or extracellular region. Comparison with *Arabidopsis thaliana* and *Solanum lycopersicum* subtilases showed that many of the grapevine subtilases shared high sequence similarity with SBT3.3 and P69C subtilases. These proteases have been already implicated in the defence response of *Arabidopsis* and tomato against biotic environment stimulus. Moreover, some of the grapevine subtilases genes have showed to be located near of locus associated to Vitis vinifera resistance against *Plasmopara viticola*.

The second goal of this project was to performed gene expression studies elucidating the role of selected grapevine subtilases in the grapevine resistance against *P. viticola*. First, subtilase expression was analysed in two *Vitis vinifera* cultivars (Regent and Trincadeira) after *P. viticola* inoculation. Then, the constitutive expression pattern of the selected subtilases was studied in several vine species and grapevine cultivars showing varying degrees of resistance towards *P. viticola*. It was observed, that under pathogen attack, resistant grapevine cultivar has an early increase of some subtilases' expression, which may be related to the quick establishment of a defence strategy against the invading pathogen. On the other hand, the susceptible grapevine cultivar showed a delay of the subtilase expression increase, which could be related to an attempt by the susceptible cultivar to initiate a defence strategy that was not fast or robust enough to prevent pathogen growth. At a constitutive level, in several vine species and grapevine cultivars, it was not observed a pattern of subtilases expression. Both results suggest that there was a differential expression of certain subtilases in resistant species but only after stimulation with the pathogen attack, which supports the hypothesis that some grapevine subtilases may have a role in defence response against *P. viticola*.

The last goal was the cloning of selected subtilases, based on their characteristics and on expression profiles, for recombinant protein expression. The sequencing results only confirmed the correct cloning for one of them. So this subtilase may proceed for further investigation.

4.1 Future perspectives

Once characterized the subtilase family in *Vitis vinifera*, many studies can be performed to increase the knowledge about these proteases. The reinforcement of the hypothesis that they may be involved in grapevine immunity against pathogen attack open all the doors for the study and characterization of these serine proteases in the grapevine-*P. viticola* interaction.

In the future it will be interesting to analyse the expression of other grapevine subtilases in *Vitis vinifera-Plasmopara viticola* interaction and at a constitutive level extending the search at other resistant and susceptible (grape)vine species, to understand if subtilases have only a role in plant defence after biotic stress or if some of them may be constitutively expressed, providing a profile that may allow to distinguish between resistant species and susceptible to downy mildew. For the most promising results it will be interesting to characterize the corresponding proteins at a structural and functional level, to know their substrates, to define their subcellular location and characterize their biological interactuants.

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6 | Appendix

6.1 Appendix 1

Supplementary Table 1 - Traits and allelles associated with *Vitis* resistance to *Plasmopara viticola*

Symbol	Associated marker	Chr	Position (MB)	Parent 1		Parent 2	Population size	Genotype of origin	Original species trait	Reference
Rpv1	VVIb32	12	10.3	Syrah	X	28-8-78		28-8-78	*V. rotundifolia*	Merdinoglu et al. 2003
Rpv2		18		Cabernet Sauvignon	X	8624	129	8624	*V. rotundifolia*	Wiedemann-Merdinoglu et al. 2006; Bellin et al. 2009
Rpv3	UDV-112	18		Regent	X	Lemberger	153	Regent		Welter et al. 2007; Bellin et al. 2009
	UDV-305		24.9	Chardonnay	X	Bianca	116	Bianca		
	VMC7f2		26.9							
Rpv4	VMC7h3	4	4.7	Regent	X	Lemberger	153	Regent		Welter et al. 2007
	VMCNg2e1		5.2		X					
Rpv5	VVIo52b	9	4.0	Cabernet Sauvignon	X	Gloire de Montpellier	138	Gloire de Montpellier	*V. riparia*	Marguerit et al. 2009
Rpv6	VMC8G9	12	20.4	Cabernet Sauvignon	X	Gloire de Montpellier	138		*V. riparia*	
Rpv7	UDV-097	7	11.4	Chardonnay	X	Bianca	116	Bianca		Bellin et al. 2009
Rpv8	Chr14V015	14	6.6	*V. amurensis* 'Ruprecht'	X	*V. amurensis* 'Ruprecht	232	*V.amurensis* 'Ruprecht	*V. amurensis*	Blasi et al. 2011
Rpv9	CCoAOMT	7	16.6	Moscato Bianco	X	*V. riparia*	174	Wr63	*V. riparia*	Moreira et al. 2011
Rpv10	GF09-46	9	3.7	Gf.Ga-52-42	X	Solaris	256	Solaris	*V. amurensis*	Schwander et al. 2012
Rpv11	VVMD27	4	4.5	Regent	X	Lemberger	153	Regent		Fischer et al. 2004; Bellin et al. 2009; Schwander et al. 2011
	CS1E104J11F			Chardonnay	X	Bianca	116	Chardonnay		
	VCHR05C			Gf.Ga-52-42	X	Solaris	256	Solaris		
Rpv12	UDV-014	14	8.0	99-1-48	X	Pinot Noir	180	99-1-48	*V. amurensis*	Venuti et al. 2013
	UDV-304		9.3	Cabernet Sauvignon	X	20/3		20/3	*V. amurensis*	
	rgvvin180									
	UDV-370		10.1							
Rpv13	VMC1G3.2	12	10.0	Moscato Bianco	X	*V. riparia*	174	Wr63	*V. riparia*	Moreira et al. 2011
Rpv14	GF05-13	5		*V.riparia*GM183	X	*V. cinerea* Arnold		*V. cinerea* Arnold		Ochssner et al. 2016
Rpv15		18		*V. piasezkii* (DVIT2027)	X	F2-35	94	*V. piasezkii* (DVIT2027)	*V. piasezkii*	Pap et al. (in preparation)

6.2 Appendix 2

Supplementary Table 2 – General features of grapevine subtilases. Adopted identifier, gene, nucleotide and protein accessions, protein name predicted, enzymes code, length, molecular weight and isoelectric point are represented.

			NCBI ID								
Adopted identifier	**Chr**	**Gene Locus**	**Start**	**End**	**Nucleotide**	**Protein**	**Sequence Description (Predicted)**	**Enzyme Codes**	**Length (aa)**	**Mol Wt. (kDa)**	**pI**
VtSBT5381	2	LOC100263349	4354053	4456526	XM_002275345.2	XP_002275381.2	subtilisin-like protease SDD1		1076	115	6.35
VtSBT5410	2	LOC100258212	4341005	4343452	XM_002275374.2	XP_002275410.2	subtilisin-like protease SDD1	EC:3.4.21	755	80	7.59
VtSBT5429	2	LOC100253079	4336989	4339321	XM_002275393.2	XP_002275429.1	subtilisin-like protease	EC:3.4.21	740	78	7.33
VtSBT5471	2	LOC100242816	4329190	4335224	XM_002275435.2	XP_002275471.2	uncharacterized protein	EC:3.4.21	1485	156	5.09
VtSBT5807	2	LOC100247874	4146149	4150511	XM_002275771.2	XP_002275807.1	cucumisin-like	EC:3.4.21	766	82	5.20
VtSBT8450	2	LOC100263381	2469920	2473555	XM_002278414.3	XP_002278450.2	subtilisin-like protease SBT3.3	EC:3.4.21	787	85	8.74
VtSBT1922	2	LOC100258241	2458478	2468930	XM_010663620.1	XP_010661922.1	subtilisin-like protease SBT3.5	EC:3.4.21	1488	157	6.27
VtSBT2304	3	LOC100255612	3842922	3845122	XM_002282268.3	XP_002282304.3	subtilisin-like protease	EC:3.4.21	732	79	5.75
VtSBT2333	3	LOC100267603	3833003	3835332	XM_002282297.2	XP_002282333.2	subtilisin-like protease	EC:3.4.21	746	81	5.62
VtSBT6965	3	LOC100854771	1535551	1538195	XM_010648663.1	XP_010646965.1	subtilisin-like protease	EC:3.4.21	774	82	8.76
VtSBT7672	3	LOC100260528	440921	445145	XM_010649370.1	XP_010647672.1	subtilisin-like protease SBT3.5	EC:3.4.21	776	83	8.08
VtSBT7741	3	LOC100265894	3852060	3854262	XM_010649439.1	XP_010647741.1	subtilisin-like protease	EC:3.4.21	732	79	5.17
VtSBT7877	3	LOC100260681	3848609	3850918	XM_010649575.1	XP_010647877.1	subtilisin-like protease	EC:3.4.21	693	75	5.65

VtSBT3237	4	LOC100246533	23760442	237565318	XM_002263201.3	XP_002263237.2	subtilisin-like protease SBT3.5 isoform X2	EC:3.4.21	763	82	8.52
VtSBT9319	4				XM_010651017.1	XP_010649319.1	subtilisin-like protease SBT3.5 isoform X1	EC:3.4.21	738	79	8.21
VtSBT9320	4				XM_010651018.1	XP_010649320.1	subtilisin-like protease SBT3.5 isoform X3	EC:3.4.21	726	78	8.22
VtSBT9321	4				XM_010651019.1	XP_010649321.1	subtilisin-like protease SBT3.5 isoform X3	EC:3.4.21	726	78	8.22
VtSBT1796	6	LOC100241012	12883750	12887018	XM_002271760.2	XP_002271796.2	cucumisin-like		727	77	6.21
VtSBT0348	6	LOC100243364	6420239	6423521	XM_002280312.3	XP_002280348.3	cucumisin-like	EC:3.4.21	735	79	8.54
VtSBT0971	6	LOC100241625	6456524	6461042	XM_010652669.1	XP_010650971.1	cucumisin-like		687	74	9.44
VtSBT0972	6	LOC100260464	6398346	6406337	XM_010652670.1	XP_010650972.1	cucumisin-like	EC:3.4.21	705	75	5.36
VtSBT1399	6	LOC100265607	344248	349212	XM_010653097.1	XP_010651399.1	subtilisin-like protease SBT3.5	EC:3.4.21	780	85	8.30
VtSBT1438	6	LOC100253594	6434842	6437933	XM_010653136.1	XP_010651438.1	cucumisin-like	EC:3.4.21	678	73	8.44
VtSBT1447	6	LOC100262117	5757473	5760376	XM_010653145.1	XP_010651447.1	cucumisin	EC:3.4.21	703	75	7.89
VtSBT1696	6	LOC100266702	12923126	12934637	XM_010653394.1	XP_010651696.1	cucumisin-like		746	79	5.63
VtSBT1909	6	LOC100251004	19891995	19896145	XM_010653607.1	XP_010651909.1	cucumisin-like	EC:3.4.21	756	81	7.04
VtSBT7899	7	LOC100266245	16714666	16718967	XM_002277863.3	XP_002277899.1	subtilisin-like protease		769	83	5.42
VtSBT4101	7	LOC100242388	15729038	15731985	XM_002284065.3	XP_002284101.1	subtilisin-like protease	EC:3.4.21	767	81	5.79
VtSBT2376	7	LOC100245280	3453021	3455498	XM_003632328.2	XP_003632376.1	subtilisin-like protease		784	83	8.36
VtSBT2423	7	LOC100265217	7494166	7497313	XM_010654121.1	XP_010652423.1	subtilisin-like protease SDD1	EC:3.4.21 EC:1.3.1.74	766	83	9.11
VtSBT7106	8	LOC100252726	17586256	17591115	XM_002277070.2	XP_002277106.1	xylem serine proteinase 1 isoform X2	EC:3.4.21 EC:1.3.1.74	744	80	9.57
VtSBT4056	8				XM_010655754.1	XP_010654056.1	xylem serine proteinase 1 isoform X1	EC:3.4.21	771	83	9.51

VtSBT7740	9	LOC100254813	21880756	21883400	XM_002267704.2	XP_002267740.1	subtilisin-like protease		762	82	5.99
VtSBT2769	9	LOC100251409	769298	771992	XM_002272733.2	XP_002272769.1	subtilisin-like protease	EC:3.4.21	771	82	6.03
VtSBT2775	9	LOC100852502	343508	346375	XM_003632727.2	XP_003632775.1	subtilisin-like protease	EC:3.4.21	787	83	8.74
VtSBT4422	9	LOC100251577	765220	767819	XM_010656120.1	XP_010654422.1	subtilisin-like protease	EC:3.4.21	788	84	7.34
VtSBT9375	10	LOC100250276	3915030	3919935	XM_002269339.2	XP_002269375.1	subtilisin-like protease SBT5.3	EC:3.4.21	778	84	9.41
VtSBT9555	10	LOC100252070	3990404	3995467	XM_002269519.3	XP_002269555.2	subtilisin-like protease SBT5.3	EC:3.4.21	777	84	9.42
VtSBT5857	10	LOC100242573	11406806	11423445	XM_010657555.1	XP_010655857.1	subtilisin-like protease SDD1	EC:3.4.21	842	90	7.35
VtSBT7221	10 (?)	LOC100243546	71072	76275	XM_002267185.3	XP_002267221.2	subtilisin-like protease SBT3.5	EC:3.4.21	822	87	5.70
VtSBT9786	11	LOC100245233	16593821	16604672	XM_002269750.2	XP_002269786.1	subtilisin-like protease isoform X4	EC:3.4.21	817	87	7.12
VtSBT8292	11	LOC100264662	814149	817194	XM_002278256.2	XP_002278292.1	subtilisin-like protease	EC:3.4.21	761	81	9.12
VtSBT6764	11	LOC100245233	16593821	16604672	XM_010658462.1	XP_010656764.1	subtilisin-like protease isoform X1	EC:3.4.21	841	90	7.37
VtSBT6765	11				XM_010658463.1	XP_010656765.1	subtilisin-like protease isoform X2	EC:3.4.21	834	89	8.02
VtSBT6766	11				XM_010658464.1	XP_010656766.1	subtilisin-like protease isoform X3	EC:3.4.21	833	89	7.70
VtSBT6767	11				XM_010658465.1	XP_010656767.1	subtilisin-like protease isoform X4	EC:3.4.21	817	87	7.12
VtSBT6768	11				XM_010658466.1	XP_010656768.1	subtilisin-like protease isoform X4	EC:3.4.21	817	87	7.12
VtSBT6728	12	LOC100252770	10151211	10162634	XM_002266692.2	XP_002266728.1	subtilisin-like protease SBT5.3 isoform X1	EC:3.4.21	769	82	8.81
VtSBT7502	12				XM_010659200.1	XP_010657502.1	subtilisin-like protease SBT5.3 isoform X2	EC:3.4.21	620	66	9.02
VtSBT3703	13	LOC100243842	15625502	15637951	XM_002273667.2	XP_002273703.1	CO(2)-response secreted protease	EC:3.4.21 EC:1.3.1.74	777	83	8.66
VtSBT7115	13	LOC100247847	3879716	3904781	XM_002277079.3	XP_002277115.3	cucumisin	EC:3.4.21	1359	145	5.07

VtSBT7563	13	LOC100244497	3718716	3722892	XM_002277527.1	XP_002277563.1	cucumisin-like	EC:3.4.21	742	79	5.32
VtSBT8504	13	LOC100256451	3818233	3821288	XM_010660202.1	XP_010658504.1	cucumisin-like	EC:3.4.21	702	74	5.54
VtSBT8505	13	LOC100259879	3740309	3743794	XM_010660203.1	XP_010658505.1	cucumisin isoform X1	EC:3.4.21	746	79	5.93
VtSBT8506	13				XM_010660204.1	XP_010658506.1	cucumisin isoform X2	EC:3.4.21	745	79	5.93
VtSBT8507	13	LOC104881130	3746539	3750191	XM_010660205.1	XP_010658507.1	cucumisin-like	EC:3.4.21	762	82	8.70
VtSBT8508	13	LOC100263317	3769095	3771614	XM_010660206.1	XP_010658508.1	cucumisin-like	EC:3.4.21	525	56	9.00
VtSBT8656	13	LOC100263269	3824838	3837200	XM_010660354.1	XP_010658656.1	cucumisin-like	EC:3.4.21	1364	144	5.34
VtSBT8658	13	LOC100258131	3768027	3808263	XM_010660356.1	XP_010658658.1	cucumisin	EC:3.4.21	1430	153	8.75
VtSBT8660	13	LOC104877291	3765728	3766409	XM_010660358.1	XP_010658660.1	cucumisin		178	19	7.75
VtSBT2598	15	LOC100259224	15340665	15344257	XM_002272562.3	XP_002272598.1	xylem serine proteinase 1	EC:3.4.21 EC:1.3.1.74	736	79	8.93
VtSBT2791	15	LOC100260739	15300073	15302466	XM_002272755.2	XP_002272791.1	subtilisin-like protease	EC:3.4.21	767	81	8.42
VtSBT2824	15	LOC100255668	15291930	15294968	XM_002272788.3	XP_002272824.2	subtilisin-like protease	EC:3.4.21	778	84	5.59
VtSBT2965	15	LOC100262514	15249300	15252310	XM_002272929.2	XP_002272965.1	subtilisin-like protease	EC:3.4.21	767	81	8.14
VtSBT2999	15	LOC100257393	15240668	15243478	XM_002272963.2	XP_002272999.1	subtilisin-like protease	EC:3.4.21	768	82	9.19
VtSBT1610	15	LOC100254106	15334997	15338703	XM_010663308.1	XP_010661610.1	xylem serine proteinase 1	EC:3.4.21 EC:1.3.1.74	737	79	9.13
VtSBT1611	15	LOC104881871	15327076	15330384	XM_010663309.1	XP_010661611.1	subtilisin-like protease	EC:3.4.21	768	81	5.54
VtSBT1612	15				XM_010663310.1	XP_010661612.1	subtilisin-like protease	EC:3.4.21	768	81	5.54
VtSBT1613	15	LOC100265949	15309082	15312107	XM_010663311.1	XP_010661613.1	subtilisin-like protease	EC:3.4.21	767	81	5.77
VtSBT1615	15	LOC100262514	15249300	15252310	XM_010663313.1	XP_010661615.1	subtilisin-like protease	EC:3.4.21	767	81	8.14
VtSBT1616	15	LOC100257393	15240668	15243478	XM_010663314.1	XP_010661616.1	subtilisin-like protease	EC:3.4.21	768	82	9.19
VtSBT2979	16	LOC100243906	15161710	15168245	XM_002262943.3	XP_002262979.2	cucumisin-like	EC:3.4.21	790	85	6.08
VtSBT4496	16	LOC100248908	15196653	15200153	XM_002264460.1	XP_002264496.1	cucumisin-like	EC:3.4.21	773	83	5.46

VtSBT0958	16	LOC100253196	14692526	14695285	XM_002270922.2	XP_002270958.1	subtilisin-like protease	EC:3.4.21	774	83	8.67
VtSBT3195	16	LOC100264034	21419829	21423364	XM_002273159.3	XP_002273195.2	subtilisin-like protease SBT3.5	EC:3.4.21	776	82	6.17
VtSBT4152	16	LOC100853857	21269828	21276081	XM_003634104.2	XP_003634152.2	subtilisin-like protease SBT3.5		776	82	6.74
VtSBT4153	16	LOC100853897	21257340	21261299	XM_003634105.2	XP_003634153.1	subtilisin-like protease SBT3.5	EC:3.4.21	776	83	6.31
VtSBT2319	16	LOC104882086	8177641	8178663	XM_010664017.1	XP_010662319.1	subtilisin-like protease	EC:3.4.21	312	34	5.36
VtSBT2545	16	LOC100267263	15356606	15359969	XM_010664243.1	XP_010662545.1	cucumisin-like	EC:3.4.21	736	79	5.46
VtSBT2559	16	LOC104882168	15378970	15380088	XM_010664257.1	XP_010662559.1	cucumisin-like	EC:3.4.21	241	26	4.69
VtSBT2562	16	LOC100853805	15307914	15311094	XM_010664260.1	XP_010662562.1	cucumisin-like	EC:3.4.21	750	81	5.87
VtSBT9753	18	LOC100257482	12908768	12912836	XM_002269717.2	XP_002269753.1	CO(2)-response secreted protease-like	EC:3.4.21	768	82	6.10
VtSBT2841	18	LOC100259061	8669672	8677405	XM_002282805.2	XP_002282841.2	uncharacterized protein	EC:3.4.21	1529	164	5.79
VtSBT2856	18	LOC100248833	8641061	8643507	XM_002282820.2	XP_002282856.2	subtilisin-like protease	EC:3.4.21	766	83	6.13
VtSBT3279	18	LOC100257444	7850312	7853095	XM_002283243.3	XP_002283279.2	subtilisin-like protease	EC:3.4.21	765	81	5.85
VtSBT4864	18	LOC100248944	8659550	8662566	XM_002284828.3	XP_002284864.1	subtilisin-like protease	EC:3.4.21	763	82	6.99
VtSBT4869	18	LOC100243797	8646067	8648420	XM_002284833.3	XP_002284869.3	subtilisin-like protease	EC:3.4.21	778	84	6.99
VtSBT4656	19	LOC100251507	9747950	9752833	XM_010646354.1	XP_010644656.1	subtilisin-like protease SBT5.4	EC:3.4.21	768	83	9.16
VtSBT9259	Un	LOC100254828	19571	23963	XM_002269223.3	XP_002269259.3	subtilisin-like protease SBT5.4	EC:3.4.21	749	81	6.55
VtSBT9456	Un	LOC100256591	106	2387	XM_002269420.3	XP_002269456.2	subtilisin-like protease SBT5.4, partial	EC:3.4.21	575	61	6.32
VtSBT5515	Un	LOC100853051	8	1592	XM_003635467.2	XP_003635515.1	subtilisin-like protease SBT5.4, partial	EC:3.4.21	347	38	6.45
VtSBT6374	Un	LOC104878187	158850	160083	XM_010648072.1	XP_010646374.1	subtilisin-like protease SBT3.5	EC:3.4.21 EC:1.3.1.74	251	27	8.79

VtSBT7020	Un	LOC100250428	11570	15932	XM_010648718.1	XP_010647020.1	subtilisin-like protease SBT5.3 isoform X1	EC:3.4.21	771	83	8.83
VtSBT7021	Un				XM_010648719.1	XP_010647021.1	subtilisin-like protease SBT5.3 isoform X2	EC:3.4.21	725	78	8.73
VtSBT7022	Un	LOC100255614	1255	5253	XM_010648720.1	XP_010647022.1	subtilisin-like protease SBT5.3	EC:3.4.21	758	82	6.79
VtSBT7023	Un	LOC104877405	16071	18643	XM_010648721.1	XP_010647023.1	subtilisin-like protease SBT5.4		354	38	9.11
VtSBT7520	Un	LOC100854364	20	2688	XM_010649218.1	XP_010647520.1	subtilisin-like protease SBT5.3		698	75	9.07

6.3 Appendix 3

Supplementary Table 3 - Domain prediction for grapevine subtilases.

	Domains			
Adopted identifier	**PA**	**S8 Peptidase**	**I9 Inhibitor**	**others**
VtSBT0348	0	1	1	0
VtSBT0958	1	1	1	0
VtSBT0971	1	1	1	0
VtSBT0972	0	1	1	0
VtSBT1399	0	1	1	0
VtSBT1438	0	1	1	0
VtSBT1447	0	1	1	0
VtSBT1610	0	1	1	0
VtSBT1611	1	1	1	0
VtSBT1612	1	1	1	0
VtSBT1613	1	1	1	0
VtSBT1615	1	1	1	0
VtSBT1616	1	1	1	0
VtSBT1696	0	1	1	0
VtSBT1796	0	1	1	0
VtSBT1909	0	1	1	0
VtSBT1922	2	2	2	0
VtSBT2304	1	1	1	0
VtSBT2319	0	1	1	0
VtSBT2333	1	1	1	0
VtSBT2376	1	1	1	0
VtSBT2423	1	1	1	0
VtSBT2545	0	1	1	0
VtSBT2559	0	1	0	0
VtSBT2562	0	1	1	0
VtSBT2598	0	1	1	0
VtSBT2769	1	1	1	0
VtSBT2775	1	1	1	0
VtSBT2791	1	1	1	0
VtSBT2824	1	1	1	0
VtSBT2841	0	2	2	0
VtSBT2856	1	1	1	0
VtSBT2965	1	1	1	0
VtSBT2979	0	1	1	0
VtSBT2999	1	1	1	0
VtSBT3195	0	1	1	0
VtSBT3237	1	1	1	0
VtSBT3279	1	1	1	0
VtSBT3703	0	1	1	0
VtSBT4056	0	1	1	0
VtSBT4101	1	1	1	0
VtSBT4152	0	1	1	0
VtSBT4153	0	1	1	0
VtSBT4422	1	1	1	0
VtSBT4496	0	1	1	0
VtSBT4656	1	1	1	0
VtSBT4864	0	1	1	0
VtSBT4869	0	1	1	0

VtSBT5381	2	2	1	0
VtSBT5410	1	1	1	0
VtSBT5429	1	1	1	0
VtSBT5471	2	2	2	0
VtSBT5515	0	1	1	0
VtSBT5807	0	1	1	0
VtSBT5857	0	1	1	0
VtSBT6374	0	1	0	0
VtSBT6728	1	1	1	0
VtSBT6764	0	1	1	fn3_5
VtSBT6765	0	1	1	fn3_5
VtSBT6766	0	1	1	fn3_5
VtSBT6767	0	1	1	fn3_5
VtSBT6768	0	1	1	fn3_5
VtSBT6965	1	1	1	0
VtSBT7020	1	1	1	0
VtSBT7021	1	1	1	0
VtSBT7022	1	1	1	0
VtSBT7023	1	1	0	0
VtSBT7106	0	1	1	0
VtSBT7115	0	2	2	0
VtSBT7221	0	1	1	0
VtSBT7502	1	1	0	0
VtSBT7520	1	1	1	0
VtSBT7563	0	1	1	0
VtSBT7672	0	1	1	0
VtSBT7740	0	1	1	0
VtSBT7741	0	1	1	0
VtSBT7877	0	1	1	0
VtSBT7899	0	1	1	0
VtSBT8292	1	1	1	0
VtSBT8450	0	1	1	0
VtSBT8504	1	1	1	0
VtSBT8505	1	1	1	0
VtSBT8506	1	1	1	0
VtSBT8507	1	1	1	0
VtSBT8508	1	1	0	0
VtSBT8656	0	2	2	0
VtSBT8658	0	2	2	0
VtSBT8660	0	1	0	0
VtSBT9259	1	1	1	0
VtSBT9319	1	1	1	0
VtSBT9320	1	1	1	0
VtSBT9321	1	1	1	0
VtSBT9375	1	1	1	0
VtSBT9456	1	1	0	0
VtSBT9555	1	1	1	0
VtSBT9753	0	1	1	0
VtSBT9786	0	1	1	fn3_5

6.4 Appendix 4

Supplementary Table 4 - Prediction of membrane localization, signal peptide and GO terms for grapevine subtilases.

Adopted identifier	Mempype			Blast2GO		
	Predicted membrane localization	Signal peptide				
		Start	End	Cellular Component	Molecular Function	Biological Process
VtSBT0348	Cell Membrane	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT0958	Internal Membranes	1	19	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis; asymmetric cell division
VtSBT0971	Cell Membrane	-	-	extracellular region	serine-type peptidase activity	
VtSBT0972	Internal Membranes	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT1399	Cell Membrane	-	-	extracellular region	serine-type endopeptidase activity	proteolysis; double fertilization forming a zygote and endosperm
VtSBT1438	Cell Membrane	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT1447	Cell Membrane	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT1610	Cell Membrane	1	26	extracellular region	serine-type endopeptidase activity; 2-alkenal reductase [NAD(P)] activity	proteolysis; oxidation-reduction process
VtSBT1611	Cell Membrane	1	20	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT1612	Cell Membrane	1	20	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT1613	Cell Membrane	1	20	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT1615	Cell Membrane	1	19	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT1616	Cell Membrane	1	20	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT1696	Cell Membrane	-	-	extracellular region	serine-type peptidase activity	

VtSBT1796	Cell Membrane	-	-	extracellular region	serine-type peptidase activity	
VtSBT1909	Cell Membrane	1	23	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT1922	Cell Membrane	1	36	extracellular region	serine-type endopeptidase activity	proteolysis; multicellular organism development
VtSBT2304	Cell Membrane	-	-	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT2319	Internal Membranes	-	-	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT2333	Cell Membrane	-	-	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT2376	Cell Membrane	1	29	apoplast; plant-type cell wall	serine-type peptidase activity	
VtSBT2423	Cell Membrane	1	21	apoplast; plant-type cell wall	serine-type endopeptidase activity; 2-alkenal reductase [NAD(P)] activity	proteolysis; stomatal complex morphogenesis; regulation of cell proliferation; oxidation-reduction process
VtSBT2545	Cell Membrane	1	25	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT2559	Internal Membranes	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT2562	Cell Membrane	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT2598	Cell Membrane	1	27	extracellular region	serine-type endopeptidase activity; 2-alkenal reductase [NAD(P)] activity	proteolysis; oxidation-reduction process
VtSBT2769	Cell Membrane	1	19	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis; secondary shoot formation
VtSBT2775	Cell Membrane	1	30	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT2791	Internal Membranes	1	20	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT2824	Cell Membrane	1	22	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT2841	Internal Membranes	1	27	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT2856	Organelle Membranes	1	32	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis

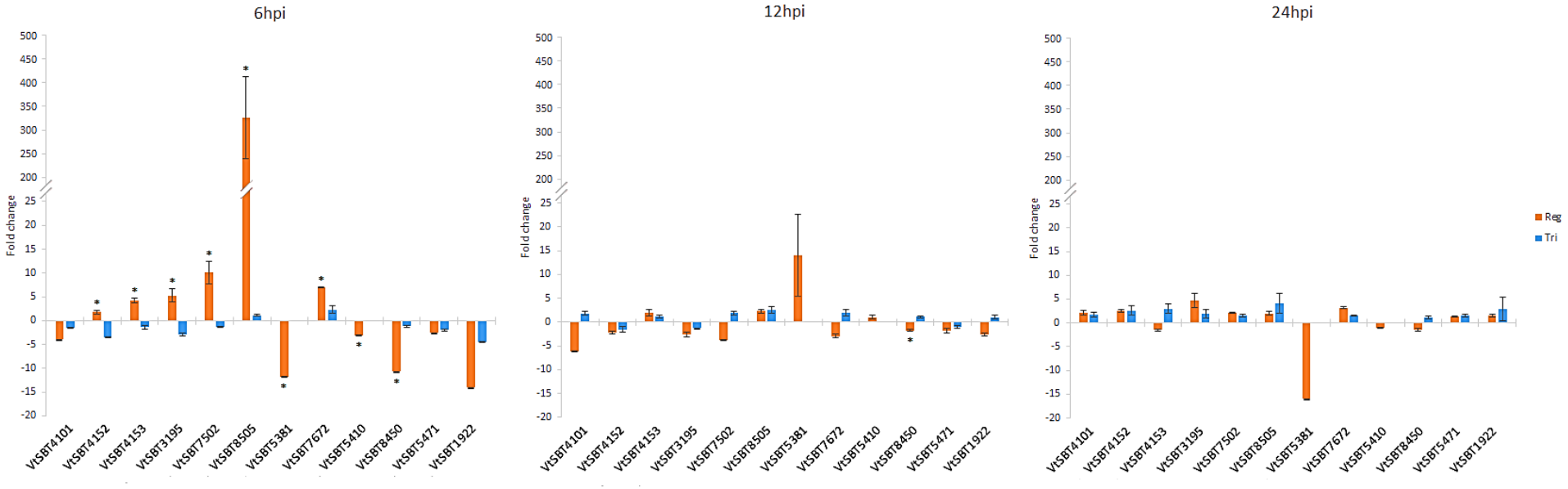
VtSBT2965	Cell Membrane	1	19	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT2979	Cell Membrane	1	24	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT2999	Cell Membrane	1	20	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT3195	Cell Membrane	1	33	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT3237	Internal Membranes	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT3279	Cell Membrane	1	21	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT3703	Cell Membrane	1	23	apoplast; plant-type cell wall	serine-type endopeptidase activity; 2-alkenal reductase [NAD(P)] activity	proteolysis; oxidation-reduction process
VtSBT4056	Cell Membrane	1	25	extracellular region	serine-type endopeptidase activity; oxidoreductase activity	proteolysis
VtSBT4101	Cell Membrane	1	24	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis; mucilage metabolic process involved in seed coat development; mucilage extrusion from seed coat
VtSBT4152	Cell Membrane	1	33	apoplast; plant-type cell wall	hydrolase activity	
VtSBT4153	Cell Membrane	1	30	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT4422	Cell Membrane	1	40	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis; secondary shoot formation
VtSBT4496	Cell Membrane	1	25	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT4656	Cell Membrane	1	25	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis; glucosinolate biosynthetic process
VtSBT4864	Internal Membranes	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT4869	Internal Membranes	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT5381	Cell Membrane	-	-	apoplast; plant-type cell wall	hydrolase activity	
VtSBT5410	Cell Membrane	1	31	apoplast; plant-type cell wall	serine-type endopeptidase activity; oxidoreductase activity	proteolysis
VtSBT5429	Cell Membrane	1	20	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis

VtSBT5471	Cell Membrane	1	31	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT5515	Internal Membranes	1	25	extracellular region	serine-type endopeptidase activity	proteolysis; maintenance of meristem identity
VtSBT5807	Cell Membrane	1	25	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT5857	Internal Membranes	1	23	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT6374	Cell Membrane	-	-	extracellular region	serine-type endopeptidase activity; 2-alkenal reductase [NAD(P)] activity	proteolysis; oxidation-reduction process
VtSBT6728	Cell Membrane	1	25	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis; glucosinolate biosynthetic process
VtSBT6764	Internal Membranes	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT6765	Cell Membrane	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT6766	Cell Membrane	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT6767	Cell Membrane	1	22	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT6768	Cell Membrane	1	22	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT6965	Cell Membrane	1	21	apoplast; plant-type cell wall	serine-type endopeptidase activity	sulfur amino acid metabolic process; polysaccharide catabolic process; starch metabolic process; proteolysis; microtubule nucleation; cellular amino acid biosynthetic process; serine family amino acid metabolic process; plant-type cell wall modification; plant-type cell wall biogenesis; regulation of meristem growth; glucosinolate biosynthetic process
VtSBT7020	Cell Membrane	1	25	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT7021	Cell Membrane	-	-	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT7022	Cell Membrane	1	25	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT7023	Cell Membrane	-	-	apoplast; plant-type cell wall		
VtSBT7106	Cell Membrane	1	25	extracellular region	serine-type endopeptidase activity; 2-alkenal reductase [NAD(P)] activity	proteolysis; oxidation-reduction process

VtSBT7115	Internal Membranes	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT7221	Cell Membrane	1	28	extracellular region	serine-type endopeptidase activity	proteolysis; cuticle development
VtSBT7502	Cell Membrane	-	-	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis; glucosinolate biosynthetic process
VtSBT7520	Cell Membrane	-	-	apoplast; plant-type cell wall	serine-type peptidase activity	
VtSBT7563	Cell Membrane	1	18	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT7672	Cell Membrane	1	27	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT7740	Cell Membrane	-	-	apoplast; plant-type cell wall	serine-type peptidase activity	
VtSBT7741	Cell Membrane	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT7877	Cell Membrane	1	25	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT7899	Cell Membrane	1	23	apoplast; plant-type cell wall	serine-type peptidase activity	
VtSBT8292	Internal Membranes	1	21	apoplast; plant-type cell wall	serine-type endopeptidase activity	RNA splicing, via endonucleolytic cleavage and ligation; proteolysis; methionine biosynthetic process; plant-type cell wall organization; plant-type cell wall biogenesis; regulation of meristem growth; anther development
VtSBT8450	Cell Membrane	1	39	extracellular region	nucleic acid binding; serine-type endopeptidase activity	proteolysis
VtSBT8504	Cell Membrane	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT8505	Cell Membrane	1	35	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT8506	Cell Membrane	1	35	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT8507	Cell Membrane	1	45	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT8508	Cell Membrane	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT8656	Internal Membranes	-	-	extracellular region	serine-type endopeptidase activity	proteolysis

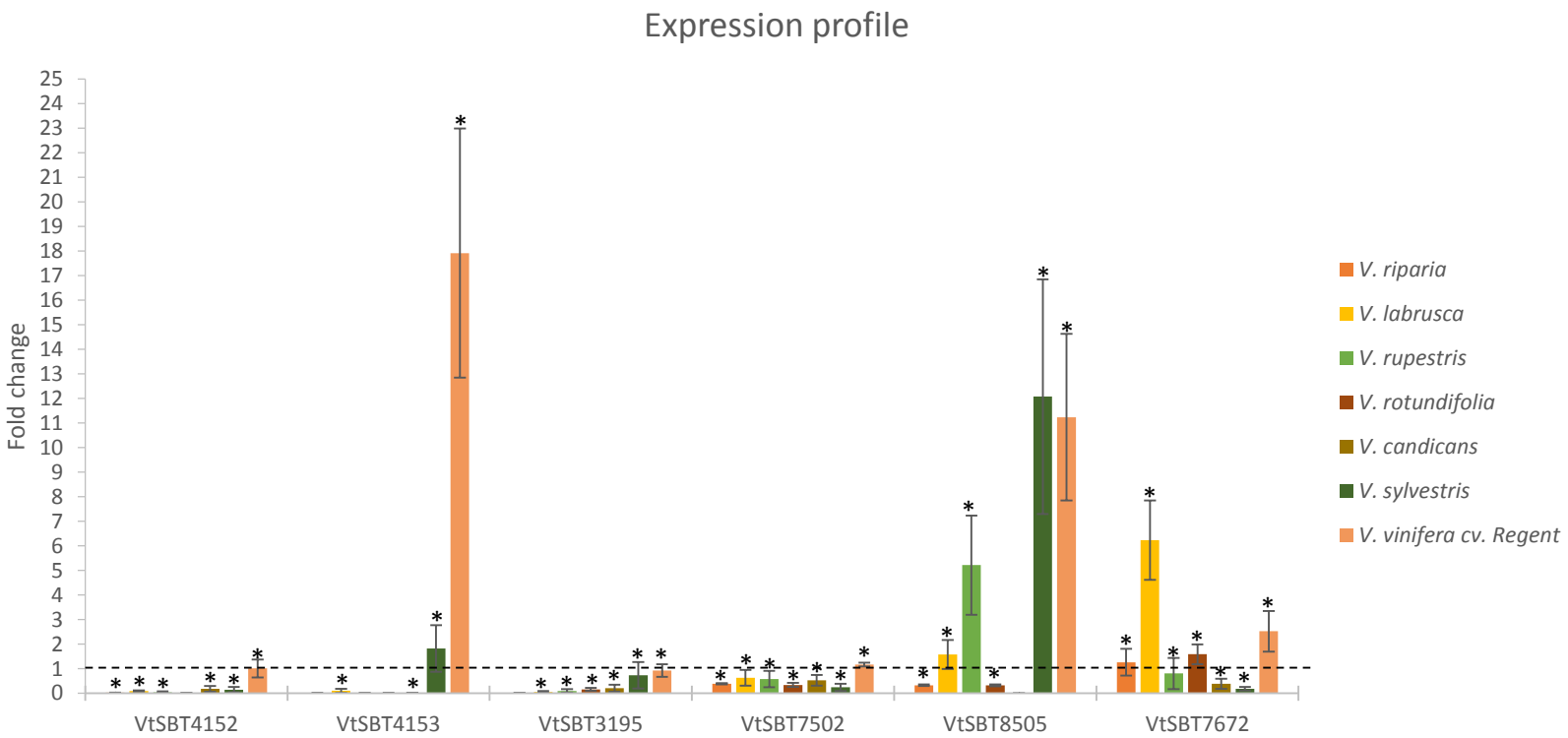
VtSBT8658	Cell Membrane	1	45	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT8660	Cell Membrane	-	-	extracellular region	serine-type peptidase activity	
VtSBT9259	Cell Membrane	-	-	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT9319	Internal Membranes	-	-	extracellular region	serine-type endopeptidase activity	proteolysis
VtSBT9320	Cell Membrane	-	-	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT9321	Cell Membrane	-	-	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT9375	Cell Membrane	1	18	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT9456	Organelle Membranes	-	-	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis; maintenance of meristem identity; glucosinolate biosynthetic process
VtSBT9555	Cell Membrane	1	18	apoplast; plant-type cell wall	serine-type endopeptidase activity	proteolysis
VtSBT9753	Cell Membrane	1	23	extracellular region	serine-type endopeptidase activity	proteolysis; response to carbon dioxide; negative regulation of stomatal complex development
VtSBT9786	Cell Membrane	1	22	extracellular region	serine-type endopeptidase activity	proteolysis

6.5 Appendix 5



Supplementary Figure 1 - Subtilases expression profile in *Vitis vinifera-Plasmopara viticola* pathosystem. Expression analysed in *V. vinifera* cv. Regent and *V. vinifera* cv. Trincadeira at three time-points after inoculation (6 hpi, 12 hpi and 24 hpi). Values lower than 0 correspond to gene down-regulation, values around 0 mean basal expression and values higher than 0 indicate up-regulation. Asterisks (*) represent significant difference ($p \leq 0.05$) between inoculated and control samples at the same time point (Mann–Whitney U test; SPSS Inc., USA, V20).

6.6 Appendix 6



Supplementary Figure 2 - Subtilases expression profile in *Vitis* species and cultivars. Values between 0 and 1 correspond to gene down-regulation, values around 1 mean basal expression and values higher than 1 indicate up-regulation. Asterisks (*) represent significant difference ($p \leq 0.05$) between target and control samples (Mann–Whitney U test; SPSS Inc., USA, V20).