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Statement of Integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Stresse biótico em videira – elucidação do papel dos transportadores SWEET na interação planta-patógeno

Resumo

Os açúcares desempenham funções vitais nos seres vivos, principalmente como fontes de carbono e de energia, mas também como reguladores osmóticos e como moléculas sinalizadoras. Em particular, na videira (*Vitis vinífera* L.), a qualidade do vinho depende dos níveis de açúcar nos bagos de uva porque determinam a concentração em etanol e influenciam a síntese de compostos secundários (incluindo pigmentos). Diferentes famílias de transportadores membranares presentes no genoma das plantas desempenham um papel essencial na translocação de açúcares entre os tecidos fotossintéticos e os tecidos de armazenamento. Entre eles, os transportadores denominados SWEET (*Sugars Will Eventually be Exported Transporter*), recentemente identificados, têm revelado diferentes papéis em mecanismos fisiológicos onde o efluxo de açúcar é fundamental, como nos nectários. Na videira, a família SWEET compreende 17 membros. No presente estudo pretendeu-se elucidar o papel dos VvSWEETs na resposta da videira à infeção por fungos (*Botrytis cinerea* e *Erysiphe necator*) e ao stresse abiótico, incluindo a secura. Além disso, para estudar os papéis fisiológicos do VvSWEET7 e VvSWEET15, foram aplicadas diferentes técnicas de engenharia genética de plantas, tal como CRISPR-Cas9. Em particular, na variedade Trincadeira, susceptível ao fungo *B. cinerea*, e na variedade Carignan, susceptível a *E. necator*, foram analisadas em detalhe as modificações no perfil de expressão dos *SWEETs* em bagos de uva infetados. Os resultados mostraram que a infeção por *E. necator* causa modificações mais pronunciadas na expressão dos *VvSWEETs* do que a infecção por *Botrytis*. Por outro lado, a maioria dos *SWEETs* da videira foram regulados negativamente em bagos de uva em resposta à secura, no entanto, o *VvSWEET10* e *VvSWEET11* foram regulados positivamente. Foi também observado que a expressão do *VvSWEET1*, *VvSWEET4* e *VvSWEET11* é regulada positivamente em folhas tratadas com caulino (filme inerte usado para proteger as videiras em situações de deficit hídrico, de radiação solar extrema e de ondas de calor), sugerindo que este mineral estimula a capacidade de transporte de sacarose entre os tecidos fotossintéticos e os tecidos de armazenamento. Estudos subsequentes mostraram que os genes *VvSWEET11* e *VvSWEET15* são positivamente regulados em bagos de uva submetidos a temperaturas de 50°C durante 7 dias, tratamento normalmente usado para a produção de uvas passas. Uma vez que os níveis de transcritos dos genes *VvSWEET7* e *VvSWEET15* foram elevados nos bagos de uva e aumentaram em resposta à infeção por *Botrytis*, as proteínas VvSWEET7 e VvSWEET15 foram alvo de estudos adicionais para se avaliar a sua localização sub-celular e função. As proteínas de fusão VvSWEET7-GFP e VvSWEET15-GFP foram transitoriamente expressas em células da epiderme de *Nicotiana benthamiana* e os resultados de microscopia confocal mostraram que ambas as proteínas se localizam claramente na membrana plasmática. Após expressão heteróloga numa estirpe mutante de *Saccharomyces cerevisiae* (*hxt-null*), a proteína VvSWEET7 foi caracterizada funcionalmente como um transportador de glucose e de sacarose (K_m =15,4 mM glucose e K_m = 40,1 mM sacarose). Ensaios de inibição competitiva mostraram que o manitol e o sorbitol inibem o transporte de D-[^{14}C(U)]-glucose, sugerindo que, além de mono- e de dissacarídeos, o VvSWEET7 medeia o transporte de polióis. No presente trabalho foram ainda identificados no genoma da videira 18 membros da família de transportadores de açúcares denominada ERD6like e a proteína VvERD6l13 foi alvo de um estudo mais aprofundado. A proteína de fusão VvERD6l13-GFP foi transitoriamente expressa em folhas de *N. benthamiana* após transformação mediada por *Agrobacterium* e os resultados de microscopia de fluorescência mostraram que se localiza na membrana plasmática. Estudos de transporte de açúcares marcados radioativamente, após expressão heteróloga em leveduras mutantes (*hxt-null*), mostraram que a proteína VvERD6l13 é um transportador de sacarose com protões (K_m = 33 mM). O gene *VvERD6l13* é fortemente regulado em bagos de uva infetados com *Botrytis* ou *E. necator*, sugerindo que a proteína VvERD6l13 tem um papel importante durante a interação planta-patógeno. Além disso, o *VvERD6l13* é expresso em diferentes tecidos da videira, em particular na raiz. Genericamente, os resultados mostraram que os transportadores VvSWEET e o VvERD6l desempenham um papel importante na mobilização de açúcares durante o desenvolvimento dos bagos de uva e que a sua expressão é regulada ao nível da transcrição em resposta ao stresse biótico e abiótico. No seu conjunto, estes resultados ajudam a compor o puzzle complexo dos mecanismos de resposta da videira aos stresses biótico e abiótico, abrindo ainda caminhos novos e desafiadores no tópico do transporte transmembranar em plantas.

Palavras-chave: Bago da uva; *Botrytis cinerea*; Oídio: Stresse biótico; Transportadores de açúcares.

Biotic stress in grapevine – elucidation of the role of the newly identified SWEET transporters on plant-pathogen interaction

Abstract

Sugars perform vital functions in the living world, primarily as sources of carbon and energy, but also as osmotic regulators and signaling molecules, among others. This is particularly relevant in the grapevine (*Vitis vinifera* L.) as the quality of the wine depends on the sugar concentration in the grape berry as it determines the final concentration in ethanol, but is also tightly related to the amount of secondary compounds (including pigments) synthesized during ripening. Different sugar transporter families are present in the genome of plants to fulfill the task of transmembrane sugar transport, which is pivotal for long distance transport between sources and sinks. Among these, the newly identified SWEETs transporters (from *Sugars Will Eventually be Exported Transporter*) have important roles in numerous physiological mechanisms where sugar efflux is critical. In grapevine, the SWEET family comprises 17 members. In this study, the main objective was to elucidate the role of VvSWEETs in grapevine response to fungal attack (*Botrytis cinerea* or *Erysiphe necator* infection) and abiotic stress, including drought. Also, to further study the physiological roles of VvSWEET7 and VvSWEET15, different plant genetic engineering techniques, such as CRISPR-Cas9, were used. In the *B. cinerea*-susceptible cv. Trincadeira and in the *E. necator*-susceptible cv. Carignan, modifications in the gene expression profile of *SWEETs* in infected grape berries were thoroughly analyzed. Overall, results showed that *E. necator* infection caused more pronounced modifications in *VvSWEET* gene expression than *Botrytis* infection. Moreover, the majority of grapevine *SWEET* genes were down-regulated in berries from drought-stressed vines of cv. Tempranillo, while *VvSWEET10* and *VvSWEET11* were up-regulated. In kaolin-treated leaves the expression of *VvSWEET1*, *VvSWEET4* and *VvSWEET11* was up-regulated, suggesting that this chemically inert mineral used to protect vines from radiation, drought and heat stimulates sucrose transport capacity improving source-to-sink transport of sucrose. Results also showed that *VvSWEET11* and *VvSWEET15* were strongly up-regulated in berries subjected to 50°C during 7 days, a protocol normally used to produce raisins. Following the observation that *VvSWEET7* and *VvSWEET15* were strongly expressed in berries and clearly up-regulated in response to *Botrytis* infection in cv. Trincadeira, they were subjected to additional studies to evaluate the subcellular localization and function of the encoded proteins. VvSWEET7-GFP and VvSWEET15-GFP fusion proteins were transiently expressed in *Nicotiana benthamiana* epidermal cells after *Agrobacterium*-mediated transformation and both proteins clearly localized to the plasma membrane, as assessed by confocal microscopy. VvSWEET7 was functionally characterized after overexpression in an *hxt*-null *Saccharomyces cerevisiae* strain as a low-affinity, high-capacity glucose and sucrose transporter, with a K_m of 15.4 mM for glucose and 40.1 mM for sucrose. Competitive inhibition experiments showed that mannitol and sorbitol also inhibited D-[^{14}C(U)]-glucose transport, suggesting that, besides mono- and disaccharides, VvSWEET7 mediates the transport of polyols. In the grapevine genome 18 members of the sugar transporter family ERD6l were identified and VvERD6l13 was selected for further characterization. The fusion protein VvERD6l13-GFP was transiently expressed in *N. benthamiana* leaves after *Agrobacterium*-mediated transformation. VvERD6l13 is localized in the plasma membrane. When VvERD6l13 was heterologously expressed in an *hxt*-null *S. cerevisiae* strain, it was observed that the protein mediates H^+-dependent sucrose transport with a K_m = 33 mM. *VvERD6l13* is strongly up-regulated in infected grape berries with *Botrytis* or *E. necator*, suggesting that it plays an important role during pathogen-host plant interaction. Moreover, *VvERD6l13* is expressed in different grapevine tissues, but its steady-state transcript levels were particularly high in roots. In sum, VvSWEET and VvERD6l transporters are important players in sugar mobilization during grape berry development and their expression is transcriptionally reprogrammed in response to biotic and abiotic stress. Together, these results constitute a new piece of the complex puzzle that is grapevine interaction with its surrounding environment and existing biological threats, while also opening new and exciting pathways in the plant sugar transporter research topic.

Keywords: Biotic stress; *Botrytis cinerea*; Grape berry; Grey mould; Sugar transporters.

Table of Contents

Abbreviations and acronyms

3-OMG - 3-O-Methyl-D-Glucose;

aa – Amino acids;

ABA - Abscisic acid;

ADP – Adenosine diphosphate;

Avr – Avirulence;

bp – base pair;

Ca^{2+} - Calcium ion;

CCCP - Carbonyl cyanide m-chlorophenylhydrazone;

CSB - Cabernet sauvignon berry;

cv. – Cultivar;

CWDE - Plant cell wall-degrading enzymes;

DAF – Days after flowering;

DAMPs - Damage associated molecular patterns;

DEFL - Defensin-like genes;

DNS - 3,5-dinitrosalicylic acid;

Dpm – Disintegrations per minute;

Egfp/GFP - Green fluorescent protein;

ERD6-like – Early-responsive to dehydration 6-like transporters;

ET – Ethylene;

ET_c – Evapotranspiration;

ETI - Effector-triggered immunity;

FRET - High-sensitivity fluorescence resonance energy transfer;

GA – Gibberellin;

GOI - Gene of interest;

H_2O_2 – Hydrogen peroxide;

HR - Hypersensitive response;

Hsp - Heat shock proteins;

HSTs - Host-specific toxins;

HXT/HT - Hexose transporters;

INT - Myo-inositol transporters;

INV/CwINV – Invertase/Cell-wall Invertase;

JA - Jasmonic acid;

Kb – Kilobase pairs;

K_m - Michaelis constant;

LysM - Lysin domain-containing;

MAMPs - Microbial associated molecular patterns;

MAPK - Mitogen-activated protein kinase;

MeJA - Methyl jasmonate;

MFS - Major facilitator superfamily;

MLO - Mildew Locus O;

ORFs – Open reading frame;

OSR - Oxidative stress responsive;

PAMP - Pathogen-associated molecular pattern;

PCD - Programmed cell death;

pGlcT - Plastidial glucose transporters;

PIP – Plasma membrane intrinsic protein;

PM - Powdery mildew;

PMT/PLT - Polyol/monosaccharide transporters;

PRRs - Pattern recognition receptors;

PRs - Pathogenesis-related proteins;

PTI - PAMP-triggered immunity;

RbcS - Ribulose bisphosphate carboxylase small chain;

RFP – Red fluorescente protein;

RH – Relative humidity;

RNAi - RNA interference;

ROS - Reactive oxygen species;

SA - Salicylic acid;

SAR - Systemic acquired resistance;

sRNAs - Small RNAs;

STP - Sugar Transport Protein;

SUT/SUC – Sucrose transporters;

SWEET - Sugars will eventually be exported transporters;

TAL - transcription-activator like effector;

THB - Triple-helix bundles;

TM - Transmembrane domain;

TMT - Tonoplast monosaccharide transporters;

VGT - Vacuolar glucose transporters;

V_{max} - Maximum rate of transport;

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Chapter 1
Introduction

1.1 - Fungal-induced biotic stress in plants

Plant pathogenic fungi cause great economic damages in the agriculture sector with estimated losses of more than 200 billion euros (Birren et al., 2002). These pathogens can cause massive reductions in crop yield and quality, problems that are expected to worsen in the context of the ongoing climate change (Gonzalez-Lamothe et al., 2006). Additionally, farmers massively use fungicide treatments to combat fungal infections, a practice with adverse effects on the environment. Furthermore, present agricultural practices rely on monoculture which promote the quick selection of fungal strains that overcome plant genetic resistance (Strange and Scott, 2005). In this regard, the study of fungal pathogenicity mechanisms is of utmost importance not only for the food/agriculture sector but also to contribute towards sustainability in a context of climate change.

Parasitic interactions between fungi and plants were established in the Lower Devonian, approx. 400 million years ago (Taylor et al., 1992). The authors of this study observed several infectious stages of a parasitic plasmodiophoromycete fungus in cells of the prehistoric algae *Paleonitella* and, remarkably, the host symptoms are strikingly similar to the actual interactions between a modern plasmodiophoromycete *Sorodiscus* and its hosts *Chara contraria* and *Chara delicatula*. As the establishment of parasitic fungal-plant interactions occurred a long time ago, a large number of plant pathogens have evolved and an even large number of diseases have developed (Horbach et al., 2011). This is evident as approximately 10% of the known fungal species (ca. 10.000 species) are plant pathogens while only 50 fungal species cause diseases in animals (Agrios et al., 2005). Moreover, the highly diverse types of defense responses exhibited by plants may have further increased the great complexity of the mechanisms of fungal pathogenicity, as reflected by the morphology of highly specialized infection structures (Mendgen and Deising, 1993; Mendgen et al., 1996) or by the vast array of secondary metabolites that fungal pathogens produce during infection (Friesen et al., 2008; Bräse et al., 2009; Daub and Chung, 2009).

1.1.1 - Fungal host attachment and penetration

Fungal infection often begins with the germination of spores dispersed by wind, water or insect vectors. When fungal spores come into contact with the host surface, they attach through the secretion of an adhesive extracellular matrix, preventing them from being washed away before penetration. That mucilage is stored in a periplasmic compartment at the conidial apex that is released upon the hydration of conidia, breaking the spore wall (Hamer et al., 1988). When favorable conditions are present, the spore germinates and a filamentous germ tube is formed, a step that requires a total reprogramming of the spore cell molecular and biochemical mechanisms. This runner hyphae grows along the host plant surface in a polarized manner dependent on the recognition of distinct physical (surface hardness, hydrophobicity) and

chemical (cutin monomers, leaf waxes) stimuli (Ebbole, 2007). These initial phases of infection - spore adhesion to the plant surface, growth of the germ tube and differentiation of infectious structures (appressoria or hyphopodia) - are similar in all plant-colonizing fungi, however, they differ in the surface signals they perceive, the chemical composition of epicuticular waxes or the hydrophobicity characteristics of the spore surface (Tucker and Talbot, 2001; O'Connell and Panstruga, 2006; Rich et al., 2014). Different fungi develop different types of appressoria. Fungal pathogens like *Magnaporthe oryzae*, *Colletotrichum spp.* and *Alternaria spp.* form a dome-shaped appressoria that accumulate turgor pressure and allow a mechanical entry of the infection hyphae into the host (Tucker and Talbot, 2001). The appressorium has a differentiated cell wall, which is enriched in chitin and contains a distinct melanin rich layer. The chemical composition of the cell wall is essential for the turgor generation that is produced by the accumulation of glycerol in the cell (Howard et al., 1991; de Jong et al., 1997; Wilson and Talbot, 2009). The turgor-driven mechanical force (which can reach 8.0 MPa) is applied by a thin penetration peg that, together with some degree of degradation of the host cuticle, caused by secreted enzymes, breaks the plant cuticle and allows the appressorium to grow into the underlying epidermal cell (Howard et al., 1991). During the formation and growth of *M. oryzae* appressoria different signaling cascades are sequentially activated and a high degree of cellular reorganization occurs (Saunders et al., 2010). Other pathogens, mainly necrotrophs, developed slightly different mechanisms to break the host cuticle. Besides a different appressoria, whose structure is not separated by a cell wall nor fortified by melanization, penetration of the plant cuticle is mainly caused by secretion of large amounts of plant cell wall-degrading enzymes (CWDEs) (Gourgues et al., 2004; Schirawski et al., 2005; Mendoza-Mendoza et al., 2009). Another group of plant pathogenic fungi, which includes species like *Cladosporium fulvum* and most rust fungi, penetrate the host plants by the stomata (Maheshwari and Hildebrandt, 1967; Thomma et al., 2005). They have evolved directional growth patterns on the host leaf surface to locate and recognize stomatal guard cells. The orientation and formation of the appressorium are controlled through topographical signals perceived by the fungus. Subsequently, the pegs differentiate into invasive hyphae that rapidly colonize to epidermal and mesophyll tissues (Hoch et al., 1987).

1.1.2 – Plant pathogenic fungi lifestyles

In addition to differences in the strategy of penetration into the host plant surface, plant pathogenic fungi are classified by their nutritional lifestyles and the way they feed on the host (Figure 1.1). Biotrophic pathogens feed on nutrients provided by a living host, forming intimate and fascinating interactions with plants. To promote its survival and reproduction, these pathogens massively modulate the metabolism, physiology and even morphology of plants as they can develop specialized organs, such as pseudo-flowers (rust) or plant tumors (smuts). Other pathogens, the necrotrophs, kill the host cells by secreting toxic

secondary metabolites in order to obtain host cells nutrients. This pathogenesis strategy was thought to have a rather indiscriminate action, however, recent findings showed that these fungi elegantly manipulate and exploit crucial biological processes in host plants for their own success. In addition to these groups, hemibiotrophic pathogens show an initial biotrophic phase followed by a final necrotrophic stage.

Necrotrophic pathogens cause necrosis and eventually the death of infected plants whereas biotrophs diseases symptoms can, in many cases, appear mild. Nevertheless, some of the economically most devastating pathogens belong to this group (Doehlemann et al., 2017).

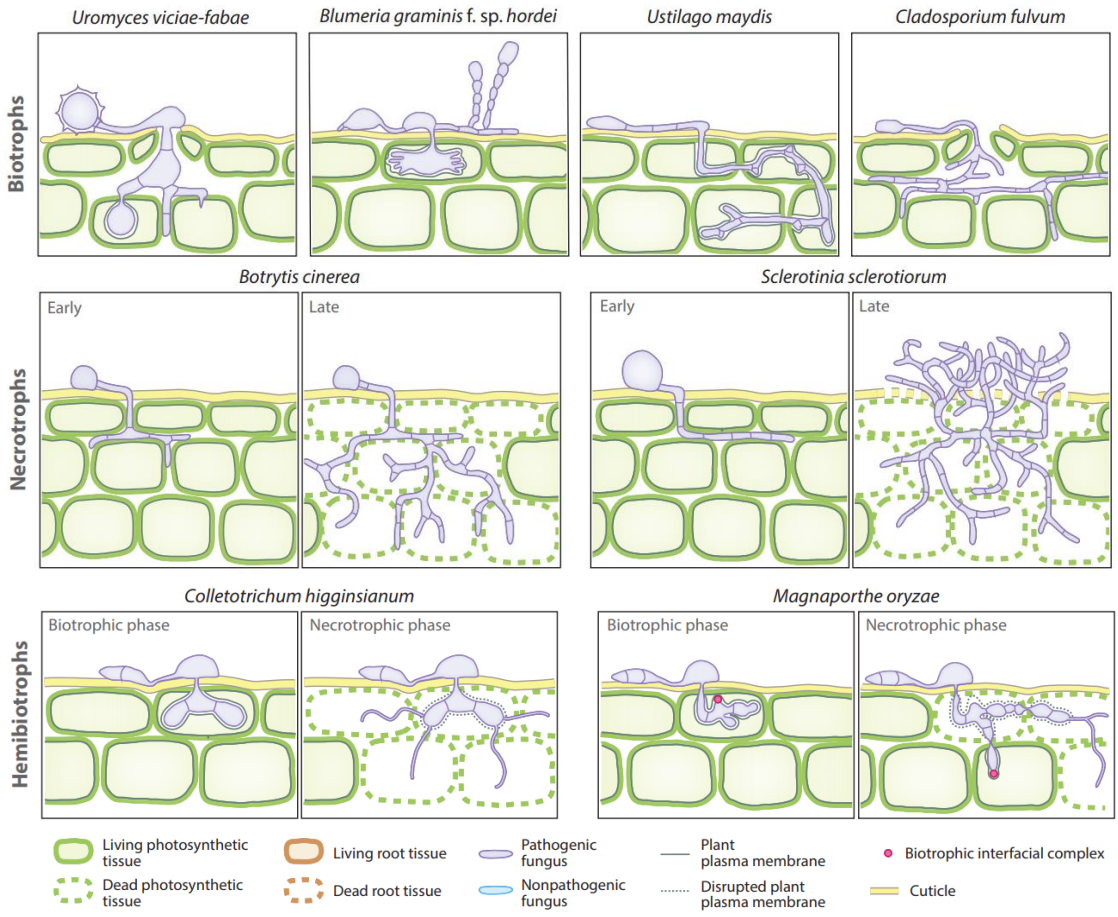

Figure 1.1 – Plant pathogenic fungi lifestyles. Biotrophic pathogens such as rust fungi (*Uromyces viciae-fabae*) and powdery mildew (*Blumeria graminis* f. sp. *hordei*) initially form a haustorium. *Ustilago maydis* at late stages growth predominantly in the intercellular space, forming large tumors. *C. fulvum* colonizes the extracellular compartment of leaves and later growth block the stomata which can cause chlorosis or cell death. Necrotrophic fungi such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* generally grow under the cuticle and kill epidermal cells by secreting toxic metabolites and proteins. Hemibiotrophic fungi such as *Colletotrichum* spp. and *M. oryzae* initially develop a biotrophic hyphae switching later to a necrotrophic hypha. Both biotrophic and necrotrophic phases are shown. Image adapted from Lo Presti et al. (2015).

Interaction between plants and pathogens occurs through a variety of molecules, normally between microbial effectors and host immune receptors. As microbial molecules can be recognized by the host and

trigger an immune response, the success of a pathogen colonization is dependent on the pathogen ability to suppress that immune response. Pathogens ability to suppress the host immune response as well as manipulate the host biological processes is attained by the secretion of effectors (Hogenhout et al., 2009). Therefore, effectors play an integral role in host-pathogen interactions and can impact the outcome of an infection both positively and negatively depending on the host genotype. Each of these molecules have a specific role during infection, thus its expression is tightly regulated (Skibbe et al., 2010; Rouxel et al., 2011; Kleemann et al., 2012; Hacquard et al., 2013; Palma-Guerrero et al., 2016; Gervais et al., 2017). Also, these molecules need to be translocated to either the host apoplast or cytoplasm (Lo Presti and Kahmann, 2017). As genes encoding for an effector suffered great evolutionary pressures, they generally lack conserved domains or homologs in other species (Figure 1.2) (Holub, 2001; Woolhouse et al., 2002; Brown and Tellier, 2011). However, unrelated effectors in different pathogen species often have common targets (Mukhtar et al., 2011) and share structural similarities and other characteristics features, such as small size and a signal peptide for secretion (de Guillen et al., 2015; Franceschetti et al., 2017). Effectors have highly diverse functions, including protecting the fungal cell wall from hydrolytic enzymes secreted by the plant (van den Burg et al., 2006; de Jonge et al., 2010). Also, other molecules and enzymes, as cell wall–degrading enzymes (Pryce-Jones et al., 1999), protease inhibitors (Dong et al., 2014), interactors with the ubiquitin-proteasome system (Park et al., 2012), and disruptors of the hormone signaling pathway (Djamei et al., 2011) can act as effectors. Moreover, many effectors can be functionally redundant. This characteristic can help in pathogen resistance against host evolution as new host genotypes, which can recognize a specific effector, can be highly detrimental to the pathogen ability to infect that plant. In that way, if a new plant genotype gains the ability to recognize a pathogen effector, as several effectors target the same host pathway, the pathogen population can adapt by losing that effector gene. Thus, the loss of the recognized effector gene can help the pathogen to evade recognition without compromising the pathogen infecting ability (Win et al., 2012; Lo Presti et al., 2015). As an example, two LysM effectors of *Zymoseptoria tritici* prevent chitin degradation by the host defense hydrolytic enzymes (Marshall et al., 2011).

During the infectious process, pathogenic fungi undergoes a tightly controlled transcriptional reprogramming as different effectors are required at different infection stages. As example, in the hemibiotrophic pathogen *Colletotrichum higginsianum* the expressed effectors during the first phase of infection are related with cell viability and are expressed in the penetration appressoria. However, as the infection develops, effectors related with host cell death are expressed (Kleemann et al., 2012; O'Connell et al., 2012). In *L. maculans*, *U. maydis*, *Puccinia striiformis*, *Melampsora larici-populina*, *Z. tritici*, and *M. oryzae*, expression of effectors also follows a similar transcriptional control of effectors (Skibbe et al., 2010; Hacquard et al., 2012; Cantu et al., 2013; Dong et al., 2015; Mirzadi Gohari et al., 2015; Rudd et al., 2015). Effector gene expressions are also regulated by the host genotype and the nature of the interaction. In *Z. tritici* and *B. graminis f.* sp. *hordei*, a large set of effectors are highly expressed during compatible

interaction whereas in incompatible interactions most of the infection gene repertoire is down-regulated (Hacquard et al., 2013; Kellner et al., 2014).

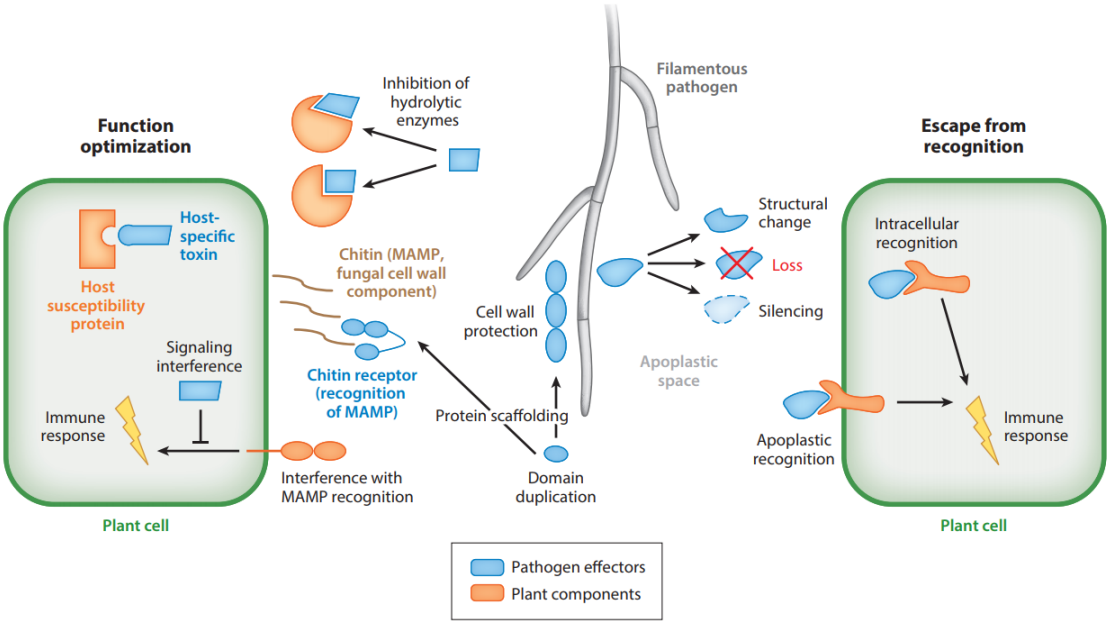

Figure 1.2 – Evolution of effectors is directed towards the optimization of their function (left panel) or escaping recognition (right panel). Effectors can acquire new conformations, gaining new targets, such as the effectors of *Phytophora infestans* that evolved their structural conformation, inhibiting hydrolytic enzymes of new hosts. Also, effectors can evolve to escape host recognition, slightly changing its conformation to evade recognition but maintaining its essential functions. Adapted from Sanchez-Vallet et al. (2018).

Biotrophic fungi

As referred above, biotrophic pathogens are characterized by living with nutrients that are provided by a living host, and they can be either obligated or non-obligated. The obligated biotrophic group is composed by the casual agents of powdery mildew (Ascomycota) and rust (Basidiomycota) diseases. These organisms are dependent on their host for completion of their life cycle, having evolved to match the life cycle of their host plants (Doehlemann et al., 2017). Rust disease causing agents are filamentous fungi that belong to the order Pucciniales of the *Basidiomycete phyla*. They developed different types of spores and developmental patterns and can infect alternative hosts upon senescence of the primary host (Petersen, 1974; Duplessis et al., 2011). The spores (eurediniospores) attach to the host leaf surface and germinate, developing an invading hypha that grows through the stoma into the mesophyll. Then, the hyphae differentiate into substomatal vesicles and produce the primary hyphae.

Haploid filamentous ascomycetes of the order Erysiphales are the cause of powdery mildew (PM). Their conidia germinate on the leaf surface and develops an appressoria to penetrate into the leaf surface (Spanu et al., 2010; Hückelhoven and Panstruga, 2011). After the hyphae of these pathogens penetrate the host organisms they generate a unique and specialized structure, the haustoria. This structure, which

serve as an interface for nutrient acquisition, breach the plant cell wall and form an invagination inside the plant cell that do not disrupt the plasma membrane. A fungus-plant hybrid is then formed, which encompasses the haustoria, the plant cell and an extra-haustorial matrix (Heath and Skalamera 1997). This specialized fungal structure is rich in a range of transporters that mediate the uptake of nutrients from the host (Voegele and Mendgen 2011; Struck, 2015) and also secrete fungal effectors that can suppress plant biotic defense mechanisms and keep the invaded cell alive (Kemen et al., 2005; Kemen et al., 2013; Petre and Kamoun, 2014; Petre et al., 2016). These pathogens, as obligatory parasites, lack the ability to use common substrates as energy sources, depending on their host to survive (Schulze-Lefert and Panstruga, 2003; Wernegreen, 2005). In fact, members of this group lack genes involved in key metabolic pathways, as in nitrogen and sulfur assimilation, and in carbohydrate metabolism (Spanu et al., 2010; Duplessis et al., 2011; Links et al., 2011; Kemen and Jones, 2012; Fernandez et al., 2014). However, it is yet not clear if these organisms are obligatory biotrophs because of the loss of some biosynthetic and metabolic capacity (Both et al., 2005; Spanu, 2006). An alternative hypothesis is that these organisms developed as obligatory biotrophs because of their dependence on signal gradients found in host plants for the regulation of the expression of key metabolic genes. Evidence that support this hypothesis were observed in barley PM development and pathogenic attack (Both et al., 2005) and in rusts haustoria metabolic and uptake functions (Sohn et al., 2000; Voegele et al., 2001; Jakupović et al., 2006).

The non-obligated biotrophic fungi group is phylogenetically more disperse being formed by the casual agents of smut (Basidiomycota, Ustilaginales) and certain species of *Claviceps* (Ascomycota, Claviceptacea). These organisms can survive without a host, easily growing in laboratory conditions (Tudzynski and Scheffer, 2004). In *Claviceps* species the infection process begins when the wind-dispersed ascospores reach the pistil surface of grass florets during anthesis. The invading hyphae penetrates through stigmatic hairs reaching the ovarian tissue. Then, it grows towards the base of the ovary and develops a specific and persisting host-pathogen interface. This intracellular hypha is not a classical haustoria, nonetheless it can have haustorial functions (Tudzynski and Tenberge, 2003). Then, a mycelial stroma develops and produce conidiospores that are exuded into a sugar-rich fluid derived from the phloem sap. For overwinter these fungi form sclerotia. Among the smut fungi, the pathogen *U. maydis* infects all aerial maize plant structures where it induces the formation of tumors, in which fungal spores develop (Brefort et al., 2009).

Biotrophic pathogens have a “survive or die” stage at the first encounter with the plant defense system. As they thrive to keep the tissue alive, the plant defense strategy at this critical stage is a suicidal one in the form of a hypersensitive response (HR), while the pathogen strategy is prevention of this response. In fact, for biotrophic pathogens the suppression of the host programmed cell death (PCD) mechanisms is crucial.

Biotrophic pathogens have a big array of effector molecules to inhibit plant defense responses and/or to keep the host alive during infection. During epidermal penetration the maize smut *U. maydis* secretes the effector Pep1 (Doehlemann et al., 2009), which can suppress the activity of the maize peroxidase POX12 that is involved in reactive oxygen species (ROS) generation (Hemetsberger et al., 2012; Hemetsberger et al., 2015). Another secreted effector, Pit2 can inhibit the activity of maize cysteine proteases which can induce plant defenses by the salicylic acid (SA) signal pathway (Mueller et al., 2013), and *pit2* mutants severely attenuated tumors and virulence (Doehlemann et al., 2011). SA signal pathways can also be disrupted by the chorismate mutase Cmu1, as this effector is secreted into the plan cell cytoplasm and reduces SA precursor chorismate, thus, disrupting the synthesis of SA in the host cell (Djamei et al., 2011). Another *U. maydis* effector, which is crucial for tumor progression, is See1 that interfere with the MAPK-triggered phosphorylation of maize SGT1, thus inhibiting its activity and resulting in modulation of reactivation of DNA synthesis in leaf cells (Redkar et al., 2015). Maize secondary metabolism is also altered by *U. maydis*. In fact, Tin2 effector interacts and stabilizes maize kinase ZmTTK1. In its active state, ZmTTK1 controls the activation of anthocyanin biosynthesis genes, lowering the biosynthesis of lignin, thus facilitating the infection (Brefort et al., 2014; Tanaka et al., 2014). In the model pathosystem *C. fulvum-Solanum lycopersicum*, almost all the identified pathogen effectors have a corresponding resistant gene (Stergiopoulos and de Wit, 2009). Avr4 protects the fungal cell wall against plants chitinases (Stergiopoulos et al., 2010), Ecp6, also a chitin binding protein, sequesters chitin fragments that are released from the fungal cell wall to prevent the elicitation of chitin-induced plant defenses (de Jonge et al., 2010), and Avr2 binds and inhibits the tomato Rcr3 and Rcr3pim cysteine protease activity (Krüger et al., 2002; Rooney et al., 2005). *B. graminis* f. sp. *hordei* effector arsenal is composed with the effector CSEP0055, which can interact with PR1 and PR17, two plant pathogenesis-related proteins. Silencing this effector resulted in a decreased frequency of fungal penetration sites (Zhang et al., 2012). Likewise, silencing the RNAase-like effectors BEC1011 and BEC1054 indicated that these effectors are involved in pathogen virulence, and specifically BEC1011 may be involved in interfering with pathogen-induced host cell death (Pliego et al., 2013). Also, the putative effector BEC4 was shown to interact with the ADP ribosylation factor-GTPase-activating protein and it may interfere in defense-associated host vesicle trafficking (Schmitz and Harrison 2014). Silencing of CSEP0105 and CSEP0162, two effector proteins which can interact with small heat shock proteins Hsp16.9 and Hsp17.5 of barley, resulted in a decrease rate of haustoria formation, compromising the infectious process. Specifically, CSEP00105 compromise the chaperone activity of Hsp16.9, which is involved in defense and stress responses against pathogen infection (Ahmed et al., 2015).

Necrotrophic fungi

Necrotrophic fungi are characterized by their capacity to kill the host cell to feed on dead or dying tissue, contrarily to saprophytic fungi which feed on already dead tissue or plant organs that have been previously weakened, e.g. by other pathogens, injury, or abiotic effects. During the early stages of infection, necrotrophic pathogens need to subvert the plant biotic defense mechanisms using a "sneaking in" strategy to subsequently generate a necrotic zone. This strategy allows the pathogen to feed on the dead tissue while creating a buffer zone protected from the host defense mechanisms. After the initial infection stage the fungus expands the necrotic zone as the disease progress. Necrotrophic fungal infection was long thought to rely mainly on the fungus capacity to kill the host plant and degrade plant tissue, however, there is evidence that asymptomatic colonization occurs (van Kan et al., 2014; Shaw et al., 2016) and that these organisms use an array of specific molecules (effectors) and mechanisms to cope with and manipulate the host defense during infection, in a manner that is not less sophisticated than biotrophic pathogens. True necrotrophic pathogens are much more taxonomically diverse than biotrophic fungi and have varied host-exploiting strategies being divided between narrow-host-range and broad-host-range classes (Mengiste, 2012). Narrow-host-range pathogens are characterized by having a narrow set of hosts which they infect. These pathogens produce host-specific toxins (HSTs) which are essential for their pathogenicity. *Cochliobolus* species secrete HST toxins like the HC-toxin from *Cochliobolus carbonum*, the T-toxin from *Cochliobolus heterostrophus*, and victorin from *Cochliobolus victoriae* (Wolpert et al., 2002). The pathogens *Pyrenophora tritici-repentis* and *Parastagonospora nodorum*, that cause wheat tan spot and blotch diseases, also produce HSTs like the ToxA toxin (Faris et al., 2010). These toxins share many characteristics with avirulence gene (Avr) products: they have a primary virulence function, are specifically recognized by host resistance counterparts, and can be recognized by the plant immune system as virulence factors.

The other group of necrotrophs, the broad-host-range class, being able to infect a broad range of plant species, lack the production of HSTs. *Botrytis* and *S. sclerotiorum* are the most known pathogens of this group causing great economic losses worldwide (Bolton et al., 2006; Williamson et al., 2007; Amselem et al., 2011). These pathogens are harder to control than the narrow-host-range species. As resistance to HST producing pathogens is provided by HST-blocking genes, the genetic basis of resistance against broad-host-range necrotrophic pathogens is much more complex (Oliver and Solomon, 2010; Mengiste, 2012). These pathogens develop melanized sclerotia which are overwinter structures central in their life cycle. The sclerotia normally initiate infection by germinating vegetatively or carpogenically, initiating the sexual cycle of the pathogen and producing ascospores. During the initial infection stage necrotrophic pathogens try to induce the host PCD pathways by the secretion of necrosis inducing effectors as Neps, cerato-platanin and certain glycosyl (Govrin and Levine, 2000; Oliver and Solomon, 2010; Shlezinger et al., 2011; Mengiste, 2012; González et al., 2016). At later stages, other types of effectors are also produced and secreted. *S. sclerotiorum* produces oxalic acid which induces PCD in the host while suppressing autophagy (Kim et al.,

2008; Kabbage et al., 2013). Activation of autophagy or apoptosis pathways result in cell death and the appearance of necrotic plant tissue. However, the difference between PCD through apoptosis or through autophagy has enormous consequences for infection with necrotrophic fungi. The capacity of *S. sclerotiorum* to suppress autophagy was shown to be crucial for disease progression (Williams et al., 2011). Mutants of *S. sclerotiorum* lacking the ability to produce oxalic acid are unable to suppress autophagy and consequently trigger a massive necrotic response that results in immunity of the plant to the fungus. Oxalic acid exogenous supplementation restored the virulence of the mutant. However, is important to note that the role of autophagy in pathogenicity is still puzzling: in some cases, is probably a salvation mechanism that alleviates damage and rescues host cells following PCD, but in other situations it serves as a pro-death mechanism (Minina et al., 2014). Other crucial element to necrotrophic fungi is the production of ROS (Govrin and Levine, 2000; Choquer et al., 2007; Kim et al., 2008; Heller and Tudzynski, 2011; Williams et al., 2011). *B. cinerea* and *Leptosphaeria maculans* are known to exploit the plant's oxidative burst and contribute to ROS production (Li et al., 2008). *Botrytis* also achieve suppression of plant defense mechanisms by producing small RNAs (sRNAs) that hijack the host RNAi machinery to silence plant genes involved in immune responses (Weiberg et al., 2013). Necrotrophs also use hydrolytic enzymes and toxins to macerate the host tissue (Cole, 1956). In fact, a relatively large number of genes coding for CWDE and other hydrolytic enzymes are present in necrotrophic fungi genome (Soanes et al., 2008; Amselem et al., 2011). Successful necrotrophs rely on massive secretion of such hydrolytic enzymes, however, current studies show that these enzymes do not work alone and they might have additional roles other than sheer hydrolysis of plant polymers. Moreover, different reports show that a large number of these enzymes are produced and secreted at various stages of the infection process (González et al., 2016; Kim et al., 2016; McCotter et al., 2016).

Necrotrophic fungi effectors can alter host metabolism, being their virulence factors mainly aimed to induce necrosis. *B. cinerea* induces a HR in several host plants by secreting the effector cerato-platanin *BcSpl1* and deletion of this gene causes severe reduction in virulence (Frías et al., 2011; 2014). *B. cinerea* can also manipulate different host plant immune pathways by using the effector exopolysaccharide β-(1,3)(1,6)-D-glucan which activate the SA signal pathway in tomato. The activation of the SA pathway inhibits the jasmonic acid (JA) signaling pathway through NPR-1, which increase plant susceptibility to *Botrytis* (Frías et al., 2013). *S. sclerotiorum* can also manipulate plant defense signaling pathways and trigger host cell death. The fungus effector SSITL, an integrin-like protein, can directly or indirectly suppress the JA/ethylene (ET) signal pathway which mediates plant immune response. Knock-out *ssitl* mutants have its virulence capacity severely affected (Zhu et al., 2013). Ss-Caf1, which has a putative Ca^{2+}-binding motif, functions as a pathogenicity factor by triggering host cell death during the early stages of infection (Xiao et al., 2014). The effector *SsSSVP1* functions as another inducer of plant cell death and the silencing of this gene reduces the infection capacity of the mutant. This effector can also interact with and hijack the activity of QCR8

(cytochrome b-c1 complex subunit 8) by changing its subcellular localization from mitochondria to the cytoplasm, hence disabling its biological functions and causing cell death (Lyu et al., 2016). In *Arabidopsis*, *S. sclerotiorum* effector SCFE1 (sclerotinia culture filtrate elicitor 1), induces a typical pathogen-associated molecular pattern PAMP-triggered immune response thought the RLP30/SOBIR1/BAK1-dependent signaling pathway (Zhang et al., 2013). This fungus also secretes a cysteine-rich secretory protein, SsCVNH, which is essential for the virulence and sclerotial development (Lyu et al., 2015). Also, *S. sclerotiorum* effectors SsNep1 and SsNep2 are related to necrosis induction and pathogen virulence (Dallal Bashi et al., 2010).

Hemibiotrophic fungi

Classically, hemibiotrophs are defined as fungal species that have a first variable biotrophic phase, followed by a necrotrophic one (Perfect et al., 1998; O'Connell et al., 2012; Yi and Valent, 2013). Different species belong to this class of pathogen fungi, among them *Fusarium* spp. (Ma et al., 2013; Ploetz, 2015), *Verticillium* spp. (Fradin and Thomma, 2006), *Mycosphaerella* spp. (Churchill, 2011; Goodwin et al., 2011), and many others, however the rice blast fungus *M. oryzae* and species within the genus *Colletotrichum* spp. are the model hemibiotrophic organisms (Yi and Valent, 2013). Following penetration of the plant cuticle, specialized hyphae develops inside plant cells, forming a close contact with the plasma membrane. After a certain period of time (from one to several days), the fungus shifts to a classical necrotrophic mode. A new type of hyphae is developed and secretes different enzymes and toxins. These organisms have evolved successful infection strategies, being among the most aggressive plant pathogens. *M. oryzae* can cause disease in a limited number of grasses; however, it is most well-known for the rice blast disease, the most destructive disease in rice (Talbot, 2003; Wilson and Talbot, 2009; Yi and Valent, 2013). *Colletotrichum* spp. cause anthracnose diseases in more than 600 dicot and monocot plant species and include severe pathogens of important crops (Kleemann et al., 2012). Infection by these pathogens typically begins by the germination of conidia. This process is complex and involves a well-orchestrated cell cycle, cell architecture and a strict sensing of the leaf surface by the spore (Barhoom and Sharon, 2004; Barhoom et al., 2008; Nesher et al., 2011). A dome-shaped appressorium is then formed and it pokes an extremely thin hole in the cuticle by applying a physical force. The fungus then moves to a transient biotrophic phase. *M. oryzae* appressorium immediately differentiates into a thin filamentous primary hypha that grows in the cell lumen and invaginates the host plasma membrane (Heath et al., 1990; Kankanala et al., 2007; Khang et al., 2010). An invasive hyphae occupies the adjacent cells by growing trough the plasmodesmata while switching to a necrotrophic behavior in the first invaded cell. These sequences of events are closely coordinated with the host defense, therefore *M. oryzae* utilizes a range of stage-specific effectors to affect the host throughout the infection, starting at the stage of appressorium formation until the entire course of interaction (Mosquera et al., 2009; Giraldo et al., 2013; Yi and Valent, 2013; Zhang and Xu, 2014). The

genome of hemibiotrophic pathogens has a large number of putative effectors genes and their expression is highly orchestrated (Kleemann et al., 2012). Also, the genomes of these species are enriched in genes encoding for plant CWDE, which are expected to be necessary at the necrotrophic phase (O'Connell et al., 2012). High steady-state transcript levels of several genes involved in secondary metabolism were also observed, which may play an important role during the infection process (O'Connell et al., 2012; Gan et al., 2013).

Hemibiotrophic fungi display a different set of effectors depending on the infectious stage. During the biotrophic phase, effectors that counter pathogen cell wall fragments recognition are mainly displayed. In rice, the chitin elicitor binding protein (CEBiP) recognizes chitin oligosaccharides released from the cell walls of fungal pathogens and promotes chitin triggered immune responses. The rice blast fungus (*M. oryzae*) secretes the effector protein LysM Protein1 (Slp1), which binds to chitin and suppress chitin-induced plant immune responses (Mentlak et al., 2012). *M. oryzae* also secretes the cytoplasmic effector AvrTpiz-t which interacts with the rice RING E3 ubiquitin ligase APIP6 and inhibits its activity, thus suppressing oxidative burst and PAMP-triggered immunity (PTI), promoting virulence of *M. oryzae* (Park et al., 2012). After appressorium formation and during the development of the invasive hyphae, this pathogenic fungus expresses the virulence-associated effector MC69. The deletion of the orthologous gene in *C. orbiculare* reduced its pathogenicity during infection of cucumber and tobacco leaves (Saitoh et al., 2012). During the necrotrophic phase of hemibiotrophic pathogens other set of effectors are expressed and secreted. MSP1, a member of the cerato-platanin family, is secreted during the necrotrophic phase of *M. oryzae* and contributes to the pathogenicity of these fungi in barley leaves. Over-expression of this gene in *Escherichia coli* induces autophagy cell death, H_2O_2 production, and defense responses in rice leaves (Wang et al., 2016). Recently, by genomic and transcriptomic analysis, a number of novel effectors were uncovered in the most aggressive *M. oryzae* strain, 98-06. Five of the tested effectors inhibited BAX-mediated apoptosis-like PCD in *Nicotiana benthamiana* leaves. The overexpression of the newly discovered effectors *Iug6* and *Iug9* suppressed the expression of defense-related genes in rice, suggesting their roles in the inhibition of host SA and ET signal pathways. This proved the crucial role of these effectors in fungal propagation and pathogenicity (Dong et al., 2015). The xylem-infecting tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* secretes 14 SIX (secreted-in-xylem proteins) effectors (Six1-14) during infection (Houterman et al., 2007; Schmidt et al., 2013). Tree members of the SIX family were already reported as required for pathogenicity of susceptible tomato lines (Rep et al., 2004; Houterman et al., 2009; Takken and Rep, 2010). In resistant lines these effectors also function as Avr proteins, which trigger the activation of HR (Takken and Rep, 2010). Interestingly, some studies reported interactions between members of this family (Ma et al., 2015). In the *Colletotrichum* species, several effectors candidates have been studied (Bhadauria et al., 2011; Kleemann et al., 2012; O'Connell et al., 2012; Gan et al., 2013; Bhadauria et al., 2015). *C. gloeosporioides* secretes the effector CgDN3 during its early phase of infection which prevent the HR of

Stylosanthes guianensis and *cgdn3* mutant lines failed to infect and reproduce in intact host leaves (Stephenson et al., 2000). Interestingly, in *C. orbiculare* the necrosis-inducing effector NIS1 was suppressed by CgDN3, which revealed a remarkably regulation between the infectious phases of hemibiotrophic pathogens (Yoshino et al., 2012). Other effectors are expressed during the transition from the biotrophic to the necrotrophic phase. In *C. truncatum*, the gene *CtNUDIX*, that has a nudix hydrolase domain, can elicit HR-like cell death in tobacco leaves being expressed during the late biotrophic phase of the pathogen. In overexpressing *CtNUDIX* mutants, a HR response was triggered already at the beginning of infection, thus causing incompatibility with the host plant. This indicates that the pathogen uses CtNUDIX as a signal for the transition from biotrophy to necrotrophy (Bhadauria et al., 2013). Other *Colletotrichum* species, *C. graminicola*, secretes the effector CgEP1, which targets the nucleus of the host cells. This effector binds to the promoter of several genes and regulates their expression (Vargas et al., 2016).

1.1.3 – Pathogen-driven plant metabolic alterations

In any pathogenic interaction nutrients move from the plant to the microbe that normally the plant cells try to restrict by repressing the carbon export and metabolism (Figure 1.3)(Chen et al., 2010; Kretschmer et al., 2017). During this clash a severe alteration in the secondary metabolism of the plant cell is also observed (Piasecka et al., 2015; Pusztahelyi et al., 2015). Interestingly, some of the plant secondary metabolites are precursors of phytohormones (such as SA and jasmonates) and defense-related compounds (including phytoalexins) (VanEtten et al., 1994; Dixon and Paiva, 1995; Bolton, 2009; Wojakowska et al., 2013; Piasecka et al., 2015; Pusztahelyi et al., 2015). Likewise, during pathogen infection, the photosynthetic machinery is affected (Berger et al., 2006). A decrease of photosynthetic activity was observed in infected *Arabidopsis* plants by biotrophic fungi, such as *Albugo candida*, *Golovinomyces orontii* or *Erysiphe cichoracearum* (Chou et al., 2000; Zimmerli et al., 2004; Chandran et al., 2010). Photosynthetic activity is also impaired upon infection by necrotrophic pathogens, such as during *B. cinerea* infection in plants like *Arabidopsis*, tomato or lettuce (Berger et al., 2004; Windram et al., 2012; De Cremer et al., 2013; Smith et al., 2014). Moreover, hemibiotrophic fungal pathogens like *Colletotrichum lindemuthianum* and *Mycosphaerella graminicola* also inhibit photosynthesis during its necrotrophic phase on beans and wheat, respectively (Lopes and Berger, 2001; Meyer et al., 2001; Scholes and Rolfe, 2009). It is somewhat apparent that photosynthesis repression during necrotrophic fungal infection occurs faster than during biotrophic interactions (Rolfe and Scholes, 2010). Likewise, genes of the respiratory process, i.e., glycolysis, tricarboxylic acid cycle (TCA cycle) and mitochondrial electron transport chain are up-regulated in the infected tissues (Doehlemann et al., 2008; Parker et al., 2009; Chandran et al., 2010; Voll et al., 2011; Teixeira et al., 2014; Xu et al., 2015). Thus, during infection, there is a clear reprogramming of the host carbohydrate metabolism. *U. maydis*, a biotrophic pathogen, causes significant alterations in the soluble

sugar contents of infected maize leaves (Doehlemann et al., 2008; Horst et al., 2008). Curiously, mutants with defects in sugar accumulation (*id1*: *indeterminate1*; increased accumulation of sucrose) or starch metabolism (*su1*: *sugary1*; altered starch metabolism) are more tolerant against *U. maydis* infection (Kretschmer et al., 2017). In tomato, the concentration ratios of different sugars seem to play a determinant role in plant defense against *Botrytis* and *Sclerotinia* fungi (Lecompte et al., 2013; Lecompte et al., 2017).

Modifications of the sugar metabolism, as the down-regulation of photosynthetic genes and photosynthetic activity and the up-regulation of respiratory machinery genes, favors the establishment of a sink-type environment in the infected tissue (Teixeira et al., 2014; Dhandapani et al., 2016). Furthermore, sugar hydrolysis and uptake mechanisms are modulated in the infected tissues, which tends to increases the sink-type environment (Fatima and Senthil-Kumar, 2015; Oliva and Quibod, 2017). The up-regulation of the plant cell wall invertases during pathogen attack influences the hexose to sucrose ratio, also increasing sink strength in the infected tissue (Chou et al., 2000; Fotopoulos et al., 2003; Hayes et al., 2010). Additionally, pathogenic fungi can up-regulate their own invertases during pathogenesis (Voegele et al., 2006; Chang et al., 2017). Other enzymes that are also involved in the breakdown of sucrose, such as sucrose synthase, are equally up-regulated in some pathosystems (Hren et al., 2009; Brzin et al., 2011; Cabello et al., 2014). Likewise, both pathogen and plant-host sugar transporters are key players during infection.

As stated above, pathogen sugar transporters gene expression is highly modulated during infection. The hexose transporter and sensor HXT1 from the pathogen *U. maydis* is required for infection as the mutant *Δhxt1* strains shows decreased virulence (Schuler et al., 2015). Hexose transporters (HXT) from *C. graminicola* are differentially regulated during the different phases of infection in maize (Lingner et al., 2011). *CgHXT1* and *CgHXT3* are expressed in the first phases of infection, during the biotrophic stage and others as the *CgHXT2* and *CgHXT5* are induced during the necrotrophic phase. Sugar transporters from other families, as the *MFS1*, are also up-regulated during this pathogen necrotrophic phase (Pereira et al., 2013). Interestingly, these transporters are co-regulated with cell wall invertases, which suggest that the expression of these proteins is coordinated to increase sugar uptake by the pathogen (Sutton et al., 2007; Essmann et al., 2008). In *Uromyces fabae* a similar mechanism is also active as both invertase (*UflNV1*) and hexose transporter (*UfHXT1*) are induced to promote hexose uptake (Voegele et al., 2001; 2006). Sucrose transporters, as the *U. maydis Srt1* are also expressed during infection, being essential to this fungus virulence (Wahl et al., 2010).

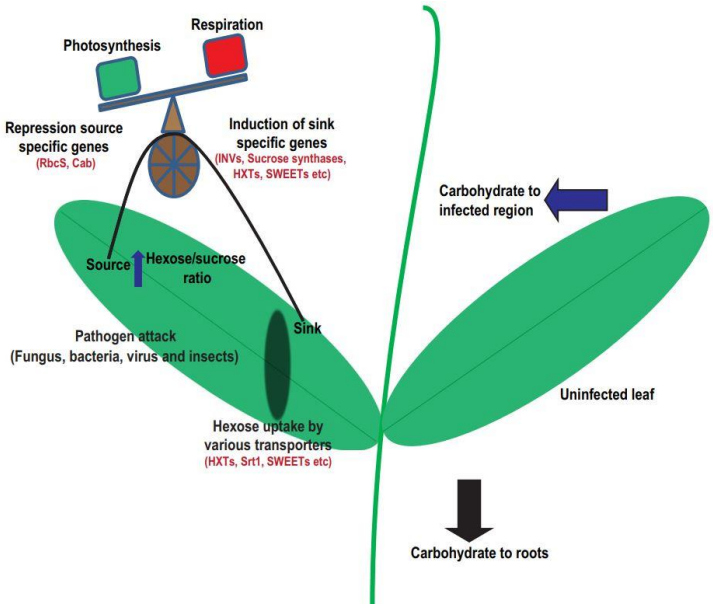

Figure 1.3 – Plant source-tissue transition during pathogen attack. Extensive metabolic changes occur, leading to the increase of hexose/sucrose ratio, causing the source tissue to evolve toward a sink-type tissue. Hexose transporters and respiratory metabolism genes are up-regulated while photosynthesis genes are down-regulated. Adapted from Kanwar and Jha, (2018).

In wheat, *Lr67* (*STP13*) gene encodes for a hexose transporter that confers partial resistance to all three wheat rust pathogen species and PM. A different variant of the same protein with two different amino acids does not confer resistance to these pathogens. The dominant resistant variant, *Lr67res* encodes for a protein that is not capable to transport sugars whereas the susceptible variant, *LR67sus* encodes a fully functional hexose transporter and both variants are up-regulated when plants are challenged by pathogens. Intriguingly, even though the Lr67res protein lacks glucose transport activity, grain yield is not affected in plants carrying this allele. Alterations in the hexose transport capacity, depending of the existing allele, may explain the ability of *Lr67res* to resist to multiple pathogenic species (Moore et al., 2015). In infected leaves of *A. thaliana* challenged with *B. cinerea*, *STP13* gene expression increased. *stp13* mutant plants exhibited enhanced susceptibility and a reduced rate of glucose uptake. Conversely, plants constitutively expressing this transporter showed a resistant phenotype and a higher capacity to transport glucose. It was postulated that STP13 participate in the active resorption of hexoses from the apoplast, depriving the fungus from its sugar source (Lemonnier et al., 2014). Remarkably, this transporter physically interacts with the bacterial flagellin receptor FLS2 and the LRR-RK co-receptor protein BAK1, which are crucial components of plant defense mechanism (Chinchilla et al., 2007). When the FLS2 binds to the bacterial flagellin protein Flg22 the kinase BAK1 phosphorylate STP13 increasing its sugar uptake capacity from the apoplast region, reducing the available sugar to the pathogen and increasing plant resistance (Yamada et al., 2016). Furthermore, the tomato *hexose transporter 1* (*HT1*) is only expressed in resistant lines to yellow leaf curl

virus (TYLCV) and *ht1* mutant lines became susceptible to the infection (Eybishtz et al., 2010), however the role of *LeHT1* in tomato plant defense mechanisms is still unknown. Also, the grapevine *hexose transporter 5* (*VvHT5*) is highly induced in coordination with the cell wall invertase (*VvcwINV*) during powdery (*Erysiphe necator*) and downy mildew (*Plasmopara viticola*) infection. This coordinated up-regulation appears to enhance sink strength during infection (Hayes et al., 2010). In maize, expression of *ZmSUT1* (sucrose transporter) is enhanced when challenged with the pathogen *Colletotrichum graminicola* (Vargas et al., 2012).

In the section 1.3.2. and 1.3.3 the role of mono and disaccharide transporters specifically in grapevine response to pathogen attack will be further addressed.

More recently a new type of sugar transporters, coined as SWEET (from Sugars Will Eventually be Exported Transporters), were identified (Chen et al., 2010). They are strongly induced upon pathogen invasion (both bacteria and fungi) in order to promote sugar leakage to the apoplast for pathogen nutrition. This topic will be addressed below (section 1.2.3) after a brief biochemical and molecular characterization of these new class of transporters.

1.1.4 – Grapevine fungal pathogens

Grapevine (*Vitis vinifera* L.) is socially and economically one of the most important plant species in the world and, as other economically important crops, grapevine faces great challenges in a rapid changing environment. This crop is greatly affected by a large number of pathogenic microorganisms that cause great damage during pre or post-harvest periods, affecting production and quality. Viruses, bacteria and oomycetes attack the grapevine, however the most important diseases are caused by pathogenic fungi including powdery mildew (*E. necator*) and gray mould (*B. cinerea*). To combat these diseases, winemakers apply in the field high amounts of chemical fungicides. Indeed, in the European Union over 2001–2003 period a staggering 81,000 tonnes of active substances were applied annually to grapevines which represented 67% of all fungicides applied to crops in the EU (EUROSTAT, 2007). This translates into increased production costs for growers, in negative impacts over beneficial organisms in the vineyard (Gadino et al., 2011), as well as, in environmental pollution which compromises human health and global biodiversity (Le Moal et al., 2014).

Erysiphe necator (powdery mildew)

Powdery mildew is one of the most widespread and destructive grapevine diseases worldwide (Figure 1.4). The fungus *E. necator* is the causal agent of this disease, appearing as a white-grayish powder on the surface of the infected tissue, generally affecting leaves and stem tissues (Gadoury et al., 2001; Calonnec et al., 2004). This infection causes great reduction of grape berry yield, weight, and total anthocyanin

content and a small increase in sugar concentration and in total acidity (Calonnec et al., 2004). *E. necator* is an obligatory biotrophic fungi, thus depending on its host to survive. After the germination of conidium on the plant tissue a primary germ tube forms and an appressorium develops to enter the plant tissue. Subsequently, a hypha grows into the host cells and a haustorium is formed to facilitate the exchange of molecules. It is through this interface that the fungus secretes effector proteins to suppress the plant host defenses and retrieves sugars, amino acids, vitamins, and other nutrients from the host cells (Qiu et al., 2015). Pathogenesis continues with the development of secondary hyphae along the infected tissue and with the production of asexual reproductive bodies (conidiophores and conidia). In the presence of adverse conditions, this pathogen develops cleistothecia, fruiting bodies in which ascospores are developed (Gadoury et al., 2012).

As in other obligate biotrophic pathogens, nutrient acquisition from the host is essential for *E. necator* development and reproduction due to gene loss related to secondary metabolism (i.e., polyketide synthase, non-ribosomal peptide synthase, dimethylallyl tryptophan synthase and terpene synthase), nitrate and sulfate metabolism, amino acid metabolism, fermentation, channels/transporters, stress response, among others (Jones et al., 2014). Accordingly, in the susceptible grapevine cultivar Cabernet Sauvignon, but not in the resistant cultivar Norton, the nitrate transporter VvNPF3.2 is up-regulated after PM infection, which may account for an increased nitrate or nitrite uptake (Pike et al., 2014). Also, an increase in the abundance of proteins related to amino acid metabolism (i.e., alanine aminotransferase and alanine glyoxylate aminotransferases), vitamin biosynthesis and lipid/sterol biosynthesis was described in PM-infected *V. vinifera* cv. Cabernet Sauvignon (Marsh et al., 2010). In fact, during *E. necator* infection, a transcriptionally reprogramming of genes involved in photosynthesis, primary metabolism, protein destination and synthesis occurs, suggesting that this pathogen manipulates plant energy processes. In addition, PM can exploit plant components for its successful penetration and establishment in the host cell (Marsh et al., 2010). The gene family *Mildew Locus O* (*MLO*), which encodes for plant-specific proteins that are related to metazoan G-protein couple receptors (GPCRs) and are likely to be implicated in the perception of calcium-dependent stimuli, is required for successful host-cell invasion (Kim et al., 2002; Devoto et al., 2003). Accordingly, loss-of-function *mlo* alleles in barley and *A. thaliana* lead to enhanced resistance to PM (Freialdenhoven et al., 1996; Consonni et al., 2006). Interestingly, during the establishment of fungal penetration in grape leaves, three members of the grapevine MLO family (*VvMLO3*, *VvMLO4*, and *VvMLO17*) are strongly induced (Feechan et al., 2009).

V. vinifera is classified as a susceptible species, however other species of the *Vitis* genus, as *V. riparia*, are more resistant to PM. In the *Vitaceae* family, different *loci* are identified as related with disease resistance. The grapevine *PENETRATION* (*PEN*) genes (*PEN1*, *PEN2*, *and PEN3*) are important components of PAMP-triggered immunity being related to resistance against PM (Qiu et al., 2015). Also, other *loci* were identified in this family as conferring resistance to PM, as *REN1* (Hoffmann et al., 2008), *REN2* (Dalbó et

al., 2001), *REN3* (Welter et al., 2007), *REN4* (Ramming et al., 2011), *REN5* (Blanc et al., 2012), *REN6* and *REN7* (Qiu et al., 2015), *RUN1* (Barker et al., 2005), *RUN2.1* and *RUN2.2* (Riaz et al., 2011) (reviewed by Armijo et al., 2016). These genes are related to pathogen effector recognition and signal amplification (McHale et al., 2006). Interestingly, grape species from central Asia that have partial resistance against *E. necator* were previously shown to carry a REN1-like local haplotype. In these species, pathogen resistance is related with recognition of the pathogen and activation of HR and PCD pathways, slowing fungal spread (Amrine et al., 2015). JA along systemic acquired resistance (SAR) mechanisms and accumulation of phytoalexins also play an important role in grapevine resistance against *E. necator*. In fact, during pathogen response of the resistant Chinese wild grape (*V. pseudoreticulata*), alterations in the expression profile of numerous genes related to SA, JA, SAR, HR, flavonoid biosynthesis and plant hormone signal transduction were observed (Weng et al., 2014). Defense-related genes encoding for glycosyl hydrolases, lipases, PR-5 thaumatin-like proteins and proteinases, among others, have been reported to be up-regulated in response to *E. necator*. As example, *V. pseudoreticulata* expresses the nuclease *VpPR-10.1* that has been correlated with resistance to pathogens. This nuclease has the ability to degrade both DNA and RNA and can have a dual function by inducing the host PCD or by degrading pathogen RNA (Xu et al., 2014a). Also, the transcription factor *VpWRKY1* is highly induced during pathogen attack in *V. pseudoreticulata* (Li et al., 2010). Other genes differentially expressed in PM-resistant wild *Vitis* species and susceptible *V. vinifera* cultivars have been associated to resistance to PM, thus the overexpression of these genes in grapevine leaves or in mutant lines of *A. thaliana* increased resistance levels to this disease (Qiu et al., 2015). The *EDS1* (*Enhanced Disease Susceptibility 1*) gene, which is a positive regulator of SA pathway, is constitutively expressed in *V. aestivalis* and induced after *E. necator* attack in *V. vinifera*. Thus, *V. aestivalis* SA levels are constitutively higher than in *V. vinifera*, which correlates with a higher resistance to the pathogen (Fung et al., 2008). This gene, along with *EDL2*, were considered as necessary components of the regulatory node EDS-PAD4 in a SA-mediated pathway (Gao et al., 2014). Also, the production of lignin, flavonoids, phytoalexins and phenolic compounds is related with a defense response against PM. Among them, the production of viniferins and resveratrol appears to enhance PM resistance. In the resistant cultivar *V. aestivalis*, after inoculation with PM, the enzyme stilbene synthase (SDS) is highly induced, accumulating more transcripts than the susceptible *V. vinifera* (Dai et al., 2012). Altogether, resistance or susceptibility to *E. necator* is mainly orchestrated by an effector-triggered immunity (ETI) response (Qiu et al., 2015), followed by a strong plant defense response observed in a strong HR reaction, along with a strong oxidative burst, the accumulation of callose, lignin and increase antimicrobial compounds, accumulation of SA and JA, induction of PCD and expression of pathogenesis-related proteins (PRs) (Thatcher et al., 2005; Hammond-Kosack and Jones, 2015; Qiu et al., 2015). However, the molecular mechanisms underlying plant immune response against grapevine PM are far from being fully understood.

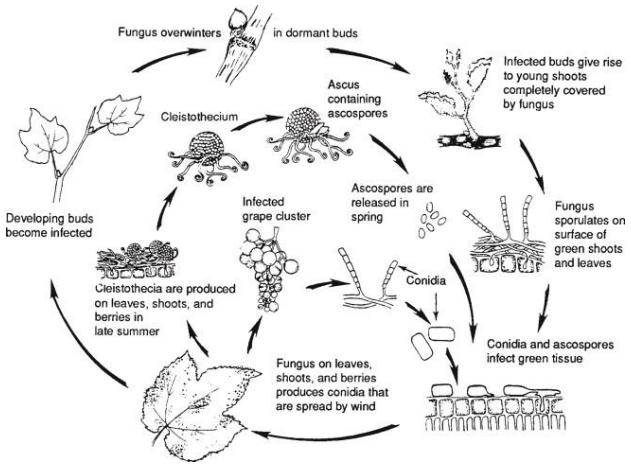

Figure 1.4 – Life and disease cycle of the biotrophic grapevine pathogen *Erysiphe necator* (powdery mildew). Image adapted from Pearson and Goheen, (1988) (drawing by R. Sticht Kohlage).

Botrytis cinerea (gray mould)

Botrytis is a necrotrophic fungus that can live as a parasite in green tissues and as saprophyte in decaying ones. This fungus is the causal agent of gray mould and infects more than 1000 plant species (Veloso and van Kan, 2018). It is one of the most important grapevine fungal pathogens and causes great productivity and quality losses (Dean et al., 2012).

Botrytis infection can be initiated in two major ways (Figure 1.5). Spores of the fungi can germinate and penetrate through skin pores and injuries or can invade the flower receptacle, and to a lesser extent the stigma and styles, remaining latent until the grape berry develops (Viret et al., 2004). In the grape berry, conidia germination can occur at different developmental stages. In fact, appressoria development and expression of *Botrytis* genes related with virulence, such as *endopolygalacturonase* (*BcPG2*) or *pectin methyl esterase* (*BcPME2*), among others, are induced during the first 16–24 h (Kelloniemi et al., 2015). However, infection generally occurs during the mature stage. Regardless of the infection route, germination and penetration of the conidium is influenced by the presence of free surface water or high relative humidity (above 93 %) (Kars and van Kan, 2007). The appressorium is then formed, breaching the host cuticle by the action of secreted lytic enzymes and by a penetration peg (Rolke et al., 2004). The secretion of a large set of proteins, toxins, phytotoxic secondary metabolites and small molecules, which are necessary for progression of the infection from early to late stages, are tightly regulated by complex signaling networks. Several molecular factors are important during infection, as genes associated with different processes, such as signaling, metabolism, catabolism, adherence and cell cycle and architecture, have been proposed to encode virulence factors (Choquer et al., 2007; Nakajima and Akutsu, 2014). As initial defense mechanisms, plants unleash high amounts of ROS, which *Botrytis* exploits and even contribute to it by forming its own ROS. Therefore, the fungus has a robust oxidative stress responsive (OSR) system to cope with ROS, including several antioxidant enzymes. Also, transcription factors such as the *Botrytis* activator

protein (Bap1) and the response regulator Skn7 were shown to be involved in the OSR (Temme and Tudzynski, 2009; Schumacher, 2016). Secretion of CWDE (Kars et al., 2005a; 2005b), is crucial during the infection process. The most secreted CWDE are pectin-degrading enzymes, such as xyloglucan transglucosylase/hydrolases and glucanases (Blanco-Ulate et al., 2014). Breakdown of pectin increases the porosity of the host cell wall, facilitating further degradation. Oxalic acid is also secreted in large amounts by the fungus (Verhoeff et al., 1988), acidifying the infected region, which increases the activity of pectinases and laccases. Besides, it favors hyphal growth and induces signaling cascades for the infectious structure, which promotes the host PCD machinery (van Kan, 2006; Kim et al., 2008). Moreover, *Botrytis* secretes a variety of secondary metabolites, being botrydial and botcinic acids, which have nonspecific phytotoxic activities, the most predominant (Cutler et al., 1993; 1996; Rebordinos et al., 1996, Collado and Viaud, 2016). Beyond those acids, *B. cinerea* produces structurally unique terpenes and polyketides (Collado et al., 2000; 2007; Shiina and Fukui, 2009, Collado and Viaud, 2016).

Furthermore, manipulation of the plant HR has been proposed to play a central role in the pathogenic strategy of *B. cinerea.* The molecular mechanisms on how *Botrytis* manipulates the plant PCD machinery are largely uncharacterized, although, it is possible that some of the secreted proteins and metabolites can function as effectors that target PDC (González et al., 2016; Veloso and van Kan, 2018). Also, *B. cinerea* infection causes substantial metabolic and transcriptional changes in the grape berry. Infected berries have higher levels of gluconic acid, arginine, alanine, proline, glutamate, glycerol, succinate, degraded phenylpropanoids, flavonoid compounds and sucrose (Hong et al., 2012). More recently it was observed in infected berries at the veraison stage a substantial reprogramming of its carbohydrate metabolism (Agudelo-Romero et al., 2015).

The skin composition, such as the number of cell layers, cuticle, and wax content of the fruit, along with the quantity and density of stomata and leaf trichomes are the first line of grapevine defense against *Botrytis* primary infection processes, such as appressoria formation and plant tissue penetration (Gabler et al., 2003). If the fungus penetrates the plant cuticle, inducible responses can be triggered after the recognition of the pathogen by pathogen derived microbial associated molecular patterns (MAMPs) and host damage associated molecular patterns (DAMPs), via recognition by host receptor-like kinases dubbed pattern recognition receptors (PRRs) (Boller and Felix, 2009, Windram et al., 2016). In the Chinese wild *Vitis*, minimal production of ROS and a timely elevation of antioxidative capacity were correlated with a high level of resistance, contrarily to the susceptible "Red Globe" cultivar that did not activate any antioxidative mechanisms, which resulted in massive infection (Wan et al., 2015). Activation of the JA/ET pathway and the induction of genes related with phytoalexin biosynthesis are also observed in the infected grapevine, together with an increased expression of secondary metabolism key enzymes (Chong et al., 2008; Girault et al., 2008; Thaler et al., 2012; Wang et al., 2015). Treatment with methyl jasmonate (MeJA) has been linked to increased resistance by an increment of H_2O_2 concentration, enhanced expression of defense

related enzymes, such as VvNPR1.1 and chitinases (fungal cell-wall degrading enzyme) and accumulation of secondary defense-related metabolites such as trans-resveratrol (Wang et al., 2015; Jia et al., 2016). Also, to reduce the extensive pectin degradation caused by fungal attack, *V. vinifera* synthesize and secrete proteins such as polygalacturonase-inhibiting proteins (De Lorenzo et al., 2001). In fact, in *B. cinerea*-infected grapevine cell suspensions, a transcriptomic study showed that genes involved with response to oxidative stress, cell wall modification and protein folding were up-regulated (Dadakova et al., 2015). Moreover, in infected grape berries defensin-like genes (*DEFL*) were up-regulated as defensins inhibited conidia germination (Giacomelli et al., 2012). Transcription factors also have been identified in grapevine pathogen defense responses against *Botrytis* (Le Henanff et al., 2013; Merz et al., 2015). During *Botrytis* infection the activation of the SA-dependent defense pathway, the reinforcement of the cell wall and a formation of papillae underneath the appressoria were also observed (Armijo et al., 2016), however, towards ripening, grape berry loses resistance to pathogen attacks. In mature infected grape berries, the activation of the JA-dependent pathway was observed, however it was not able to stop the infection (Kelloniemi et al., 2015). In the *Vitis* genus, higher levels of resistance are found in the species *Muscadinia rotundifolia* (*V. rotundifolia*), *V. labrusca*, and other grape hybrids, whereas *V. vinifera* genotypes varied between low and no resistance to *Botrytis*. The number and thickness of the epidermal and hypodermal cell layers and cuticle and the wax contents appear to be the principal resistance-related characteristics (Gabler et al., 2003), however, no genetic studies have been conducted to date in these resistant plants. These primarily defense characteristics seem to be important against *Botrytis*, along with the early activation of defense-mechanisms mediated by SA or JA/ET pathways, together with an appropriate response between ROS production/antioxidant defense systems.

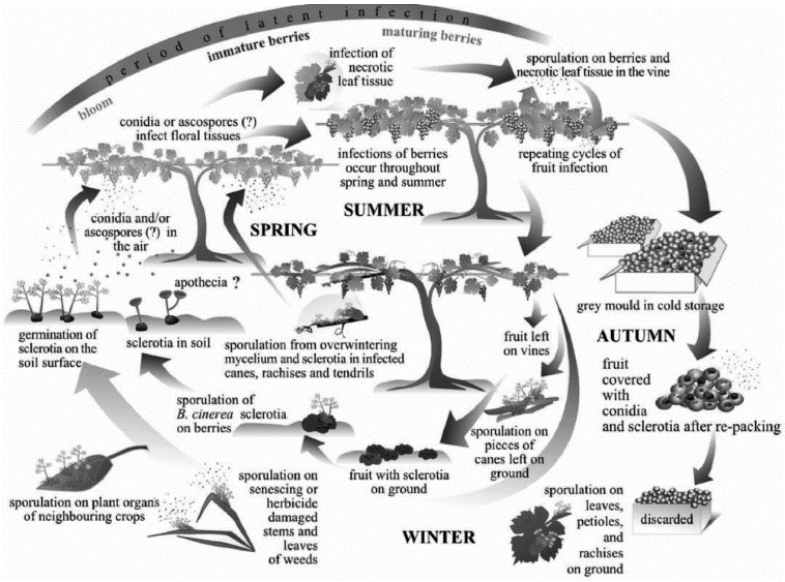

Figure 1.5 - Life and disease cycle of *Botrytis* in the grapevine. Adapted from Elmer and Michailides, (2007).

1.2 – The newly identified SWEET transporter family

1.2.1. Structure and transport dynamics

This new class of transporters were firstly identified in *Arabidopsis* by Chen and co-workers (Chen et al., 2010) who tried to find the molecular basis that could explain sugar efflux mechanisms, which remained puzzling until then (Thorens et al., 2000; Stümpel et al., 2001; Hosokawa and Thorens, 2002; Lalonde et al., 2004). They screened genes encoding uncharacterized polytopic membrane proteins from the *Arabidopsis* membrane protein database Aramemnon (2010) using a new mammalian expression system (Takanaga and Frommer, 2010). Candidate genes were co-expressed with the high-sensitivity fluorescence resonance energy transfer (FRET) glucose sensor FLIPglu600mD13V in human HEK293T cells, with low endogenous glucose uptake activity (Takanaga et al., 2008; Takanaga and Frommer, 2010). AtSWEET1 (*AT1G21460*) was the first characterized SWEET transporter as a glucose bidirectional uniporter/facilitator. To determine the bidirectional capacity of the transporter the FRET glucose sensor FLIPglu600mD13VER was expressed in the lumen of the endoplasmic reticulum.

SWEET transporters belong to a novel transporter family (PFAM PF03083) whose members are highly conserved from the super kingdoms Archea and Bacteria (SemiSWEET) to Fungi, Protista and Metazoa. They are also present in Streptophyta (green plants), Chlorophyta (green algae), and other algae and, even in the Oomycota class (Figure 1.6) (Jia et al., 2017). This family is ubiquitously present in plants. In *A. thaliana* is constituted by 17 members (Chen et al., 2010), 21 in *O. sativa* (Yuan and Wang, 2013), 23 in *Sorghum bicolor* (Mizuno et al., 2016), 52 in *Glycine max* (Patil et al., 2015), 35 in *Solanum tuberosum* (Manck-Gotzenberger and Requena, 2016), 29 in *Solanum lycopersicum* (Feng and Frommer, 2015), 33 in *Malus domestica* (Wei et al., 2014), among others.

These transporters are structurally different from the classic 12 transmembrane-domains sugar transporters previously characterized of the MFS (Major Facilitator Superfamily). They are composed by two internal triple-helix bundles (THB) linked by a linker-inversion transmembrane domain (TM), comprising 7 TM in total (Figure 1.7) (Chen et al., 2010). Bacterial SemiSWEET are formed by only three TM and structural resolution studies showed that two individual semiSWEET transporters form oligomers in parallel orientation to create a functional pore for translocation. Therefore, SWEETs possibly arose by gene duplication of SemiSWEET unit in concert with the insertion of an inversion linker-helix (Xuan et al., 2013; Xu et al., 2014b; Wang et al., 2014). More recently, following an extensive phylogenetic analysis, Hu and co-workers (2016) proposed that a fusion of archeal and bacterial SemiSWEETs formed eukaryotic SWEETs, which potentially explains the asymmetry of eukaryotic SWEETs. Still, how the least conserved TM4 was inserted in the structure remains elusive.

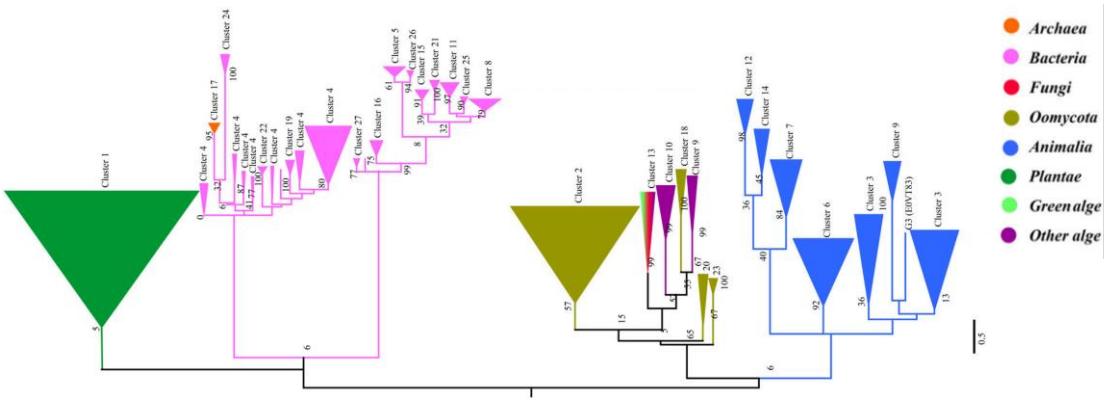

Figure 1.6 – Phylogenetic tree of SWEET and SemiSWEETs proteins present is different organisms. Bootstraps values are showed nest to the branches (1.000 replicates). The color of each branch corresponds to its cluster. Adapted from Jia et al. (2017).

SWEET proteins have been characterized as uniporters capable to mediate both uptake and efflux of sugars and other molecules, such as gibberellins (Kanno et al., 2016), in a low affinity and high capacity manner and relative pH independence (Chen et al., 2010; Eom et al., 2015). After crystal structures and molecular dynamic simulations were published, the detailed mechanism of sugar transport was unveiled. Different important conserved amino acids have been recognized in both SemiSWEET and SWEET transporters. SemiSWEETs form symmetrical parallel dimers. A conserved PQ motif is present at the TM1 which stabilize the L1-2 loop conformation and bring the L1-2 loop to the dimer interface.

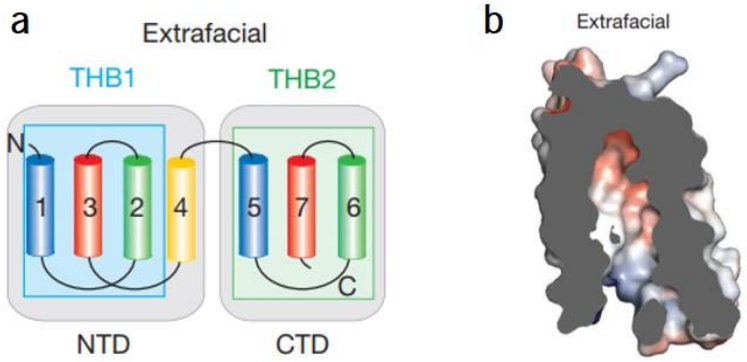

Figure 1.7 – a) Typical membrane topology of SWEET sugar transporter proteins. TM4 and THB1 form the N-terminal domain while the THB2 forms the C-terminal domain. Representation of a slab view of VvSWEET2b in an inward (cytoplasmic) open conformation. Adapted from Tao et al. (2015).

Also, it serves as a molecular hinge that enables a "binder clip-like" motion during sugar transport (Lee et al., 2015). The binding pocket is formed by tryptophan and asparagine residues and facilitate transport (Xu et al., 2014b). In *Arabidopsis* SWEET proteins, four conserved prolines, which are located in

equivalent position as the PQ motif of SemiSWEETs, have a significant role in transport mechanisms. In AtSWEET1, replacing any of the four prolines caused loss of AtSWEET1 activity. These proline rings might facilitate a precise structural rearrangement related to the shift between the conformation states acting as hinges for gating the sugar transportation pathway (Tao et al., 2015). SWEET transporters can also form oligomers, as structural and biochemical analyses showed that OsSWEET2b forms homomeric trimers. When compared with SemiSWEET sequences, SWEETs do not have the tryptophan pairs that form the binding pocket site, rather, OsSWEET2b cavity is formed by a cysteine and a phenylalanine and in AtSWEET1 by a serine and a tryptophan (Tao et al., 2015). The crystal structure of AtSWEET13, a multi-substrate transporter, was also reported in the inward facing conformation with a sucrose analog bound in the central cavity (Han et al., 2017). These authors observed that different parts (e.g., loops 2–3 and 5–6 vs. TM7) of the cytosolic side of AtSWEET13 move independently, instead of forming rigid bodies, in response to substrate binding. Thus, Han and co-workers (2017) postulated a revolving-door like mechanism for transport by an AtSWEET13 dimer, in which a substrate-carrying conformational transition in one protomer is coupled to the substrate-free opposite transition in the other protomer. Additionally, SWEETs contain multiple phosphorylation sites at the cytosolic C terminal end with an average length of approx. 45 amino acids. The cytosolic C terminus may act as a hub for binding of other proteins (e.g., regulatory components), or it could function in transmission of signals to the cell if SWEETs also work as sugar receptors (or transceptors) (Chen et al., 2015b).

1.2.2 - SWEET transporters physiological roles

Plant SWEET sugar transporters have different physiological roles during plant growth and development. On average, in angiosperms, this family is constituted by 20 paralogs, differentially expressed in several tissues. SWEET members are phylogenetically divided in four clades, however membership in a clade does not predict the physiological process the protein is involved in, but it slightly defines its preferent transported substrate. In *Arabidopsis*, clade I (SWEET1-2) members preferentially transport 2-deoxyglucose, clade II mostly monosaccharides (SWEET3-8), clade III (SWEET9-15) mainly uses sucrose and finally, clade IV (SWEET16-17) predominantly transports monosaccharides (Chen et al., 2015b). Likewise, SWEET transporters can localize in different cellular compartments, mainly in the plasma membrane (SWEET1, 8, 9, 11, 12, and 15) (Seo et al., 2011; Kryvoruchko et al., 2016), in the tonoplast (SWEET2, 16 and 17) (Chardon et al., 2013; Klemens et al., 2013; Guo et al., 2014; Chen et al., 2015a) and even in the Golgi membrane (SWEET9) (Lin et al., 2014; Chen et al., 2015b).

SWEET transporters have important roles in plant reproductive organs where the efflux of sugar from the autotrophic tissues is of utmost importance. RNA sequencing experiments showed that SWEET genes are variably expressed in pineapple (*Ananas comosus*) having an important role during fruit development,

being *AnmSWEET5* and *AnmSWEET11* the most expressed members (Guo et al., 2018). During apple development, 9 *MdSWEET* genes were highly expressed, among them *MdSWEE9b* and *MdSWEET15a* were the most expressed members and they are likely to be involved in the regulation of sugar accumulation (Zhen et al., 2018). Also, during pea germination and seed development SWEET genes are strongly up-regulated (Jameson et al., 2016). In expression studies and analysis of the promoter region of *Gossypium hirsutum* SWEET genes, these transporters were shown to be likely involved during development and abiotic stress resistance (Li et al., 2018). *Arabidopsis SWEET15*, along with *SWEET11* and *12*, is highly expressed in the seed coat. Triple knockout mutants showed a severe delay in embryo development and a wrinkled seed phenotype at maturity due to lower starch and lipid content and a smaller embryo. Thus, these proteins are involved in the transport of sucrose from the seed coat to the embryo in a coordinated manner (Chen et al., 2015c). Likewise, in pollen grains, *AtSWEET15* (also known as *Vegetative Cell Expressed 1*, *VEX1*) is highly expressed and is involved in the transport of sugars, especially in the vegetative cells. This transporter is also continuously expressed along pollen maturation and even in germinating pollen grains (Engel et al., 2005), indicating an important physiological role of this transporter during pollen development. Likewise, AtSWEET8 has an important role in the transport of glucose for pollen nutrition. This transporter is highly expressed in the tapetum membrane and *atsweet8* mutant lines showed male sterility, which resulted in non-viable pollen grains (Guan et al., 2008). In tomato maturing pollen grains, *SlSWEET5b/Lestd1* is highly expressed, which can also have a crucial role in pollen viability (Salts et al., 2005). In rice, the sucrose transporter OsSWEET11, which is highly expressed in pollen grains, has a prominent role in pollen viability as pollen grains of *ossweet11* knock-out mutants showed reduced starch contents which may lead to male sterility (Yang et al., 2006). In *Arabidopsis* flower, 8 SWEET genes are highly expressed (*AtSWEET15*, *AtSWEET14*, *AtSWEET13*, *AtSWEET8*, *AtSWEET7*, *AtSWEET5*, *AtSWEET4* and *AtSWEET1*). *AtSWEET14* and *AtSWEET13* predominate in the stamen; *AtSWEET8* are abundant in the microspores; *AtSWEET15* and *AtSWEET1* are abundant in the petals and *AtSWEET4* in the sepals (Moriyama et al., 2006). *AtSWEET1* is also highly expressed in the stamen primordia and during early stages of floral development (Wellmer et al., 2006).

SWEET transporters have also a great importance in nectar secretion in nectaries. SWEET9 is an essential nectar specific transporter in *A. thaliana*, *Brassica rapa* and *Nicotiana attenuate* (all eudicots) acting in sugar efflux. Loss-of-function mutants lead to loss of nectar secretion in all the studied plants. This transporter is expressed in the nectary parenchyma and participates in the secretion of sucrose into the extracellular space which then is hydrolyzed into glucose and fructose to maintain the concentration gradient (Lin et al., 2014). AtSWEET9 homolog in *Petunia*, NEC1, is also nectary-specific and its expression pattern correlates inversely with nectarial starch content. Likewise, silencing this gene triggered male sterility (Ge et al., 2000; 2001).

Sucrose is the principal carbohydrate translocated from the leaves at long distances in the veins of higher plants to support the growth and development of roots, flowers, fruits and seeds. Proper control of carbohydrate partitioning is of utmost importance for plant yield, fitness and development. Sucrose is synthesized in the leaf mesophyll cells and diffuses by plasmodesmata towards the phloem veins. In the majority of crop plants, phloem loading cannot be completed by a symplastic way as phloem's companion cells and sieve elements are not connected by plasmodesmata. Therefore, sucrose should be transported to the apoplastic space and incorporated by plasma membrane active sucrose transporters (SUT/SUC) of the companion cells or sieve element cells (Lalonde et al., 2004; Sauer, 2007; Kühn and Grof, 2010; Slewinski et al., 2010; Ainsworth and Bush, 2011; Ayre, 2011). This last unresolved step in sucrose translocation was elucidated by Chen et al. (2012) who observed that the sucrose transporters *AtSWEET11* and *12* are highly expressed in a subset of leaf phloem parenchyma cells, proximal to the companion cells and sieve elements. Double mutant *atsweet11;12* lines showed moderate defects in sucrose phloem transport and an excessive accumulation of sugars in the leaves and delayed root development. Likewise, *OsSWEET11* was found to be expressed in the phloem of rice leaves (Chu et al., 2006), indicating that *OsSWEET11* may play a similar role in phloem loading. In sorghum, *SbSWEET13a*, *13b* and *13c* are mostly expressed in leaves and stems and their expression pattern correlates with sucrose accumulation in the stem (Makita et al., 2015; Bihmidine et al., 2016). In *Setaria viridis* homologue, *SvSWEET13b* is strongly expressed in mature stem tissues (Martin et al., 2016; McGaughey et al., 2016) and may play an important role in sustaining cell turgidity in a concerted manner with aquaporins (Moore and Cosgrove, 1991; McGaughey et al., 2016). In maize, similarly to *ZmSUT1*, *ZmSWEET13a*, *13b* and *13c* are preferentially expressed in the bundle sheath/vein of leaves. Triple knockout mutants of *zmsweet13a,b,c* presented a severely stunted phenotype, showing impaired phloem loading, reduced photosynthetic activity and accumulation of high levels of soluble sugars and starch in leaves. Furthermore, RNA-seq analysis revealed a deep transcriptional deregulation of genes associated with photosynthesis and carbohydrate metabolism (Bezrutczyk et al., 2018a).

Vacuolar sugar transport and storage are tightly related with resistance to different environmental constraints (Martinoia et al., 2007). In tea plant (*Camellia sinensis*), the tonoplast sugar transporter CsSWEET16 accumulates sugar in the vacuole and is repressed during cold-acclimation. *Arabidopsis* plants transformed with *CsSWEET16* showed an increase tolerance to cold by different vacuolar sugars contents, compared to wild type lines (Wang et al., 2018). Likewise, *AtSWEET16* expression, a multi-substrate vacuolar transporter, is repressed during cold and osmotic stresses and also under low nitrogen. Overexpressing lines (*35S$_{Pro}$:SWEET16*) showed a number of peculiarities related to differences in sugar accumulation. Under cold stress, mutant lines were able to accumulate fructose, while during nitrogen starvation they accumulated glucose and fructose, but no sucrose. Remarkably, *35S$_{Pro}$:SWEET16* lines showed improved germination, increased freezing tolerance and improved nitrogen use efficiency (Klemens

et al., 2013). The vacuolar sugar transporter AtSWEET2 limits carbon efflux from roots into the rhizosphere (Chen et al., 2015a). The sucrose transporter *AtSWEET15* is also highly expressed during senescence and may be implicated in sugar remobilization (Quirino et al., 1999). Also, lines overexpressing *AtSWEET15* exhibited enhanced leaf senescence and higher cell viability under high salinity and other osmotic stresses (Seo et al., 2011). In banana, *MaSWEET* genes also play an important role in response to cold, salt and osmotic stress (Miao et al., 2017), and in *Brassica oleracea* var. *capitata* L. some *SWEET* genes are likely involved in chilling tolerance (Zhang et al., 2019a).

SWEET proteins can also be involved in hormone regulation. In *Arabidopsis*, AtSWEET13 and 14 can transport different gibberellins (GAs) and *atsweet13;14* double-mutant lines were incapable to transport exogenous GA and showed altered responses during seed germination (Kanno et al., 2016). OsSWEET5, a galactose transporter, is mainly expressed in the floral organs at the heading stage and is also expressed in stem, root and senescing leaves. *OsSWEET5*-overexpressing plants showed growth retardation, precocious senescing leaves and changed sugar contents in leaves. Remarkably, auxin concentration, signaling and translocation were inhibited. *OsSWEET5* is possibly an important player in the sugar and auxin crosstalk (Zhou et al., 2014).

1.2.3 – SWEET roles in plant-pathogen interaction

Different SWEET transporters are up-regulated by various species of the genus *Xanthomonas* that cause bacterial blight disease (Yang et al., 2006; Antony et al., 2010; Chen et al., 2010; Liu et al., 2011; Yu et al., 2011). These bacteria secrete several transcription-activator like (TAL) effectors (Bogdanove, 2014) to directly enhance the expression of specific *SWEET* genes. Thus, *Xanthomonas oryzae* pv. *oryzae* secretes the TAL effector PthXO1 that targets rice *OsSWEET11* (Yang et al., 2006; Chen et al., 2010), and an African *Xanthomonas* strain secretes the effector TALC that increases the transcript abundance of *OsSWEET14* transcripts (Yu et al., 2011). *OsSWEET13* is also up-regulated during *X. oryzae* pv. *oryzae* infection, however no effector was yet identified (Liu et al., 2011). SWEET transporter activity hijacked by *Xanthomonas* species appears to be crucial for the growth and proliferation of these pathogens because the lack of induction in most cases results in disease resistance. Bacterial mutant strains carrying truncated versions of TAL effectors or even plant mutations in the promoter region where TAL effectors bind result in reduced infection (Chen et al., 2010; Liu et al., 2011; Yu et al., 2011). For instance, the strain PXO99A mutated in its *pthxo1* gene cannot induce *OsSWEET11* and fails to infect rice plants (Chen et al., 2010). Interestingly, the same SWEET member can be targeted by different effectors of different pathogen species, as these effectors can bind to different regions of the gene promoter. Thus, recessive mutations in the promoter region of *SWEET* genes can increase pathogen resistance without losing the sugar transport function (Antony et al., 2010). It seems that these pathogens mainly target clade III SWEET transporters, as their physiological function is

normally related with sucrose efflux to the apoplastic space surrounding the phloem, as AtSWEET11 and AtSWEET12 (Chen et al., 2012). These bacterial pathogens also target SWEET transporters of other plant species. In Cassava, *MeSWEET10a* is induced by *X. axonopodis*, promoting its virulence (Cohn et al., 2014), and in citrus, the pathogen *X. citri* ssp. *citri*, which causes bacterial canker disease, induces *CsSWEET1* by a TAL effector-dependent method (Hu et al., 2014). However, *SWEET* gene expression can be altered not only by bacteria from the *Xanthomonas* species but also by other bacterial and fungal pathogens. *Pseudomonas syringae* induces different *SWEET* genes (*AtSWEET4*, *AtSWEET5*, *AtSWEET7*, *AtSWEET8*, *AtSWEET10*, *AtSWEET12*, and *AtSWEET15*) in infected *Arabidopsis* leaves, especially *AtSWEET15* during the early stages of infection (Chen et al., 2010). Also in *Arabidopsis*, the obligate biotrophic PM pathogen *Golovinomyces cichoracearum* enhanced the expression of *AtSWEET12* in infected leaves during the formation of the primary haustorium and during hyphal growth and development of the reproductive structures (Chen et al., 2010). *B. cinerea* infection also induces expression of different *AtSWEETs*, principally *AtSWEET15* (Chen et al., 2010). *Botrytis* also induces *VvSWEET4* in grapevine and *SlSWEET15* in tomato (Chong et al., 2014; Asai et al., 2016). Interestingly, *VvSWEET4* is only responsive to infection by *Botrytis*, as its expression is not altered during infection with pathogens with other lifestyles (*P. viticola and E. necator*) (Chong et al., 2014). This sugar transporter is also induced by ROS production, cell death and virulence factors from necrotizing pathogens, all hallmarks of necrotrophic interactions. SWEET family members are also induced upon plant-interaction with mycorrhizal fungi (Perotto et al., 2014). Overall it seems that most of the pathogens induce host SWEET transporters to gain access to the plant sugar resources for nourishment (Chen et al., 2010; Cohn et al., 2014) as there is no doubt that host-derived sugars are absorbed by the pathogen (Aked and Hall, 1993; Sutton et al., 1999). However, in some cases, up-regulation of these transporters does not result in higher plant susceptibility towards infection. In roots of *A. thaliana*, infection by the soil-borne oomycete *Pythium irregulare* caused an increase of more than 10-fold in *AtSWEET2* gene expression, nonetheless loss-of-function *sweet2* mutants were more susceptible to the infection, showing impaired growth when challenged with the oomycete (Chen et al., 2015a). In sweet potato (*Ipomoea batatas*), infection with *Fusarium oxysporum* Schlecht. f. sp. *batatas* significantly up-regulated the gene expression of the sucrose transporter *IbSWEET10*. Unexpectedly, *IbSWEET10*-overexpressing sweet potato lines were more resistant against this fungal pathogen that control ones and also RNAi lines showed higher susceptibility (Li et al., 2017b). The mechanisms of how higher levels of SWEET activity can increase plant resistance to pathogenic attacks are unknown. One hypothesis is that, as sugars can act as signaling molecules, SWEET up-regulation can act as a defense mechanism, altering sugar levels at the infection site and triggering signaling cascades that result in SA pathway activation which leads to up-regulation of defense genes, ultimately generating physiological changes that repel pathogens (Herbers et al., 1996; Herbers and Sonnewald, 1998; Morkunas and Ratajczak 2014; Gebauer et al., 2017; Bezrutczyk et al., 2018b; Kanwar and Jha, 2018). Also, they can possibly function as sugar sensors much like other sugar transporters such

as SUC2 and SUT1 in *Arabidopsis* (Lalonde et al., 1999; Barker et al., 2000; Ho et al., 2009; Thevelein and Voordeckers, 2009), however this hypothesis is highly speculative (Bezrutczyk et al., 2018b).

During pathogen attacks, the most common modification in *SWEET* gene expression is one of induction, however a recent study (Asai et al., 2016) demonstrated the down-regulation of several *SWEET* genes in tomato cotyledons when challenged with *B. cinerea*. In fact, over twenty-one of the thirty *SlSWEET* genes were significantly down-regulated after 16 h post inoculation. The physiological importance of down-regulation of *SWEET* genes during infection is still puzzling. It was reported that upon pathogen attack various sugar signaling cascades are disrupted (Berger et al., 2006, Sade et al., 2013; Morkunas and Ratajczak, 2014) eventually due to the down-regulation of *SWEET* genes. Therefore, pathogens can repress these transporters to diminish plant defense responses resulting in a more beneficial environment for pathogen growth.

1.3 - Grapevine sugar transporters

In the grapevine, efficient assimilation and use of nutrients is of prime importance for plant growth and development. Thus, the process of assimilation, transporting and distributing sugars from source tissues to sink tissues (sugar partitioning) is of utmost importance (Braun and Slewinski, 2009; Bihmidine et al., 2013; Yadav et al., 2015). This complex mechanism is tightly controlled by a vast network of sugar transporters. In grapevine, 79 ORFs encoding putative sugar transporters are identified. Among these, 4 encode previously described sucrose transporters from the SUT/SUC family (Davies et al., 1999; Ageorges et al., 2000; Manning et al., 2001) and the other 58 ORFs seem to encode putative monosaccharide transporters (MST), which is composed by 7 distinct sub-families (Afoufa-Bastien et al., 2010). The other 17 genes correspond to the grapevine SWEET family (Chong et al., 2014; Lecourieux et al., 2014).

1.3.1- Grapevine SWEET transporters

The grapevine SWEET family was firstly identified by Lecourieux et al. (2014), however the first characterization of a VvSWEET transporter was performed by Chong and co-workers (2014). This family is composed by 17 members, the same number as in *A. thaliana,* and were named on the basis of their identity percentage with *A. thaliana* SWEET proteins. VvSWEETs clearly separate in the classic four clades, however, clade III appears to be underrepresented. *VvSWEET* genes are differentially expressed in each organ of the grapevine and only *VvSWEET9* and *VvSWEET17b* expression was not detected so far. Several members are highly expressed in reproductive organs and fewer in vegetative ones. None of the *VvSWEET* genes is significantly induced after infection of leaves by biotrophic pathogens (*E. necator and P. viticola*), however, the infection by *B. cinerea* strongly induces *VvSWEET4* while *VvSWEET2a* and *VvSWEET7* are

moderately induced. VvSWEET4 was further characterized as a plasma membrane glucose transporter which is also induced by ROS production and PCD, indicating a possible involvement in sugar redistribution during pathogen-induced cell-death. Recently, VvSWEET10 was also characterized as a plasma membrane hexose transporter. Its over-expression in grapevine *calli* and tomato plants increased their glucose, fructose and total sugar levels (Zhang et al., 2019b).

1.3.2 – Grapevine SUT/SUC (SUcrose Transporter) family

V. vinifera sucrose transport family is constituted only by four members (Afoufa-Bastien et al., 2010). Three of them were functionally characterized as proton-dependent sucrose symporters by heterologous expression in *S. cerevisiae* (Davies et al., 1999; Ageorges et al., 2000; Manning et al., 2001; Zhang et al., 2008). VvSUC11/VvSUT1 and VvSUC12 are intermediate affinity sucrose transporters (K_m of 0.9 mM and 1.4 mM, respectively) (Ageorges et al., 2000; Manning et al., 2001), and VvSUC27 is a low affinity sucrose transporter (K_m of 8–10 mM) (Zhang et al., 2008). *VvSUT2/VvSUC3* sequence is close to *VvSUC27*, both being around 2380 bp long and containing 4 exons and 3 introns, however it was not yet functionally characterized and is weakly expressed in the grape berries (Afoufa-Bastien et al., 2010). The physiological roles of these transporters are yet to be fully understood. In grape berries, sucrose transporters are likely to be involved in the maintenance of sucrose in the conducting bundles until it reaches the site of unloading. As they function as proton-sucrose symporters they are expected to mediate only uptake functions, however in certain conditions these types of transporters are likely to mediate sugar efflux (Carpaneto et al., 2005). The expression of both *VvSUC11* and *12* genes increases along grape berry development in contrast to *VvSUC27* which significantly decreases at veraison (Davies et al., 1999). This differential expression along with their different kinetic parameters suggests that they play different roles during fruit development and maturation. Their sub-cellular localization is not yet known, which hampers further conclusions about their roles.

1.3.3 – Grapevine HT (Hexose Transporter) family

This family is constituted by 22 members, showing high similarity with the STP (Sugar Transport Protein) family from *Arabidopsis*. Only 5 members were studied so far (VvHT1, 2, 3, also named VvHT7, 4 and 5) (Fillion et al., 1999; Vignault et al., 2005; Hayes et al., 2007) and more recently 17 new ORFs in the grapevine genome were identified (Afoufa-Bastien et al., 2010) and named VvHT8 to VvHT24. Nevertheless, as some identified sequences share a high similarity with others - VvHT8 share 99.4% similarity with VvHT1 and their chromosomal location is unknown – or are only partially annotated - *VvHT22*, *VvHT23* and *VvHT24*, their sequences do not seem to be fully sequenced, missing either the N-terminal or

the C-terminal region, or both – this family can contain only 20 members instead of 24 (Afoufa-Bastien et al., 2010). Interestingly, some groups of genes, such as *VvHT9*, *10*, *11* and *VvHT14* to *24*, form a cluster of genes, being located in a tandem repeat region in chromosome 14 and 13. Their chromosomal location and sequence similarity suggest that this family evolved by duplication events. The exon-intron organization in the *VvHT* family seems to be conserved and it is sub-divided in two clades. The most expressed hexose transporters in all grapevine tissues are *VvHT1*, *3* and *11*. *VvHT2* is highly expressed in roots and grape berries and *VvHT5* in mature leaves. *VvHT4*, *12* and *13* are weakly detected in all the tissues. Throughout grape berry development, *VvHT1*, *2* and particularly *3* are the most expressed hexose transporters (Afoufa-Bastien et al., 2010).

All the so far characterized transporters (VvHT1, 4 and 5) localize at the plasma membrane and are high affinity, proton-dependent hexose transporters (Vignault et al., 2005; Hayes et al., 2007). VvHT1 shows a high affinity for glucose (K_m = 70 μM) and VvHT4 and 5 a slight lower affinity (K_m approx. 150 and 100 μM, respectively). VvHT1 can also transport other hexoses, such as fructose, galactose, xylose and glucose analogs as 3-*O*-methyl-d-glucose, however with a lower affinity (Conde et al., 2006). This transporter is more abundant during the early stages of grape berry development thus indicating that it is not responsible for the post-veraison sugar accumulation (Vignault et al., 2005; Conde et al., 2006). However, it can be involved in the retrieval of minor amounts of hexoses that leak from the conduction complex of the phloem because VvHT1 transcripts are abundant in this region of the phloem at leaves, petioles and berries (Vignault et al., 2005). *VvHT3* is highly expressed in the grape berry during the green and ripening stages (Hayes et al., 2007; Afoufa-Bastien et al., 2010) but upon heterologous expression in yeast it was not able to transport any of the tested radiolabeled substrates (Vignault et al., 2005; Hayes et al., 2007).

1.3.4 - Other sugar transporters in grapevine

The grapevine tonoplast monosaccharide family (TMT) is composed by three members with high similarity with the *Arabidopsis* TMT family (Wormit et al., 2006). All the members show an extended middle loop between TM 6 and 7. VvTMT1 was previously named VvHT6, however it shows higher similarity with AtTMT (58.8 to 70.9%) than with the VvHT family proteins (15 to 26.3%) (Afoufa-Bastien et al., 2010). The exon-intron gene structure is conserved in this family, having each member 5 exons and 4 introns. This family is closely related with the myo-inositol transporters (VvINT) and vacuolar glucose transporters (VvVGT) families. All the members are weakly expressed in the vegetative organs of the grapevine. VvTMT1 was functionally characterized as a hexose-proton antiporter and is highly expressed in the mesocarp cells of the grape berry at the early stages of development, decreasing along maturation (Zeng et al., 2011). Contrarily, *VvTMT2* expression increases with during maturation (Terrier et al., 2005; Deluc et al., 2007), suggesting

that it can mediate sugar accumulation and *VvTMT3* is weakly expressed in the grape berry (Afoufa-Bastien et al., 2010).

The polyol/monosaccharide transporter (PMT) in *Vitis* is composed by 5 members with a high similarity (41.4 to 72.1%) with *Arabidopsis* PMT family (Afoufa-Bastien et al., 2010). Between themselves, these proteins share 40 to 76.8% homology and present all the same genetic structure with 2 exons. *VvPMT* genes are weakly expressed in all the vegetative organs with the exception of *VvPMT5,* which is highly expressed in the mature leaf, the petiole and the tendril (Afoufa-Batien et al., 2010). In the grape berry, only *VvPMT5* is expressed specifically at the early stages of development. VvPMT5/VvPLT1 is a plasma membrane, proton-dependent polyol symporter that can transport both mannitol (K_m = 5.4 mM), sorbitol (K_m = 9.5mM) and a broad range of other polyols and monosaccharides. This transporter is highly expressed during water-deficit conditions (Conde et al., 2015).

The ERD6-like sugar transporter family (Early Responsive to Dehydration 6-like) is one of the least investigated sub-clades of sugar transporters within the MST family. Curiously, this family forms one of the largest sugar transporter subfamilies in *Vitis* as in *Arabidopsis* (Büttner, 2007) and in *Arabidopsis* three members were characterized as monosaccharide vacuolar transporters (Yamada et al., 2010; Poschet et al., 2011; Klemens et al., 2014). The grapevine ERD6-like transporter family is constituted by 22 ORFs, sharing between 36.2 and 93.2% similarity (Afoufa-Bastien et al., 2010). Several identified ORFs correspond to partial sequences and are not clearly recognized which can suggest inaccurate identifications. Fourteen members are located on chromosome 14, in a region of tandem gene duplications, three other members are located at chromosome 5 and other two at chromosome 12. Two other members are located at chromosome 7 and 4. Phylogenetic analysis showed that ERD6-like genes are distributed in 4 clades.

The vacuolar glucose transporter family (VvVGT) is constituted by two ORFs, named VvGT1 and 2 that share high similarity with *Arabidopsis* VGT family. In *Arabidopsis*, AtVGT1 is an energy-dependent vacuolar glucose transporter (Aluri et al., 2007). The inositol transporter family (VvINT) is composed by 3 ORFs. These transporters are putative plasma membrane H^+/symporters specific for myo-inositol. However, in *Arabidopsis* these transporters can also localize in the tonoplast (Schneider et al., 2008). The plastidial glucose transporter (pGlcT) family is composed by 4 members in the grapevine. This family is composed by putative monosaccharide transporters that localized in the membranes of plastids and also in the Golgi apparatus (Weber et al., 2000; Wang et al., 2006). *VvGlcT1* coding sequence is 1629 long and codes for a glucose transporter localized at the plastid (Zeng et al., 2013).

1.4 – Research objectives

Current areas of the scientific activity in our group include plant-environment interactions and plant stress biology. More recently we have focused on grapevine source to sink interactions in response

to environment and on key biochemical and molecular events occurring during fruit development and ripening. In the present study we took advantage from the work in progress in collaboration with different national and international research groups and from funding provided by different ongoing I&D projects ("MitiVineDrought"- PTDC/BIA-FBT/30341/2017 and POCI-01-0145-FEDER-030341; "BerryPlastid" - POCI-01-0145-FEDER-028165 and PTDC/BIA-FBT/28165/2017; "GrapInfectomics" - PTDC/ASP-HOR/28485/2017; "CherryCrackLess" - PTDC/AGR-PRO/7028/2014). Of particular interest for the present PhD project was the work performed by Prof. Ana Fortes at FCUL on the transcriptome and metabolome reprogramming in *V. vinifera* cv. Trincadeira berries upon infection with *B. cinerea* (Agudelo-Romero et al., 2015). In an attempt to identify the molecular and metabolic mechanisms associated with the infection, peppercorn-sized fruits were infected in the field. Green and veraison berries were collected following infection for microarray analysis complemented with metabolic profiling of primary and other soluble metabolites and of volatile emissions. The results provided evidence of a reprogramming of carbohydrate and lipid metabolisms towards increased synthesis of secondary metabolites involved in plant defense, such as trans-resveratrol and gallic acid. Taking advantage from these data (in particular after an analysis in their Micro-array database we observed great differences in gene expression of several sugar transporters) and from biological samples from infected and control plans kindly provided by Ana Fortes' group, we performed an extensive study aimed at testing the hypothesis that the grapevine infection by *B. cinerea* or *E. necator* extensively affected the transcriptional profile of key genes of VvSWEET family in berry tissues. These studies were complemented with data from *in vitro* cultures, when Cabernet Sauvignon Berry (CSB) cells were cultivated in mineral medium supplemented with sucrose. We aimed at testing the hypothesis that SWEET transporters were transcriptionally regulated by the amount of external sugars, much like classical monosaccharide and disaccharide transporters (William et al., 2000; Conde et al., 2006; Conde et al., 2007).

Our previous collaboration with Manuela Chaves' group at ITQB was particularly fruitful on the study of grapevine response to water-deficit stress. Field studies conducted in vineyards from Alentejo (where drought stress is particularly severe) subjected to different irrigation treatments allowed us to perform a metabolomic profiling of the berry pulp by GC-TOF-MS which revealed many changes in berry composition induced by water deficit, particularly in the content of sugar-alcohols like mannitol (Conde et al., 2015). An extensive reprogramming of genes coding for polyol transporters and polyol dehydrogenases was observed and correlated with the observed changes in the fruit content of polyols. We took advantage from these results - and from the corresponding biological samples made available - to test the hypothesis that grapevine SWEET transporters were also regulated in response to drought stress, which could bring new hints to a topic that has been relatively less explored.

With the group of José Moutinho Pereira from UTAD we have set an extensive, long and fruitful collaboration aimed at studying - and modulating - grapevine response to the particularly harsh

environmental conditions of Douro region. It is widely accepted that, particularly in the context of the ongoing climate changes, drought, elevated air temperature, and high evaporative demand are increasingly frequent during summer in grape growing areas like the Mediterranean basin, limiting grapevine productivity and berry quality. In this context, the foliar exogenous application of kaolin, a radiation-reflecting inert mineral, has proven effective in mitigating the negative impacts of these abiotic stresses in grapevine and other fruit crops. In the context of this collaboration, we have studied the influence of kaolin on key molecular mechanisms and metabolic pathways notably important for grape berry quality parameters (Conde et al., 2016; 2018a). In particular, in the present study we wanted to test the hypothesis that Kaolin particle film application stimulated carbohydrate metabolism and modified the primary metabolome of grape leaves through modifications in high-capacity monosaccharide transporters like VvSWEET1, VvSWEET4 and VvSWEET11, for which an ability to also transport sucrose should not be ruled out.

Postharvest dehydration causes changes in texture, color, taste and nutritional value of food due to the high temperatures and long drying times required. In grape berries, a gradual dehydration process is normally utilized for raisin production and for making special wines. We aimed at analyzing particular changes in the primary metabolism during the dehydration process using molecular and biochemical analyses to understand the role of sugar transporters, which are known to be (or putatively, in some cases) involved in sugar allocation from berry apoplast into the cells as well as post-phloem transport in the berry, polyol transporters, aquaporins, and the role of the metabolism of organic acids and polyols, due to their involvement in berry/wine flavor and in water deficit stress tolerance (Conde et al., 2018b). Particularly, in the context of the present thesis we wanted to address if VvSWEET transporters were also involved in sugar transport during dehydration.

From the above referred studies on the transcriptional analysis of VvSWEETs in response to *Botrytis* and *E. necator* infection in the field results showed that *VvSWEET7* and *VvSWEET15* are highly expressed in the grape berry and transcriptionally regulated in response to infection, suggesting that they have important physiological roles in sugar import during maturation and in plant-pathogen interactions. Thus, subsequent studies were performed to functionally characterize these two genes and to evaluate their sub-cellular localization. For the sub-cellular localization studies, a transient expression in tobacco leaves of a chimeric protein, composed by our gene of interest (GOI) and a fluorescent protein, was performed. For functional characterization, VvSWEET7 and VvSWEET15 were heterologously expressed in an *hxt-null S. cerevisiae* mutant strain and uptake experiments were performed with radiolabeled sugars.

To further elucidate the physiological roles of VvSWEET7 and VvSWEET15, different plant genetic engineering techniques were also applied taking advantage from the expertise of the group of Prof. Antonio Granell from the Polytechnic University of Valencia (UPV). For this purpose, overexpressing Micro-TOM tomato mutant lines of our GOI (*VvSWEET7* and *VvSWEET15*) were firstly established. Also, using the newly discovered genetic engineering system CRISPR-Cas9, knockout mutant tomato lines were generated to

silence these genes. The third approach aimed at performing tissue localization of our GOI. To fulfill this objective, tomato mutant lines expressing a fluorescent reporter protein regulated by our GOI promoter were created. All these relevant studies were performed during a 3-month staying period of the proponent in the laboratory of Polytechnic University of Valencia and the work is still in progress.

In the work by Agudelo-Romero et al. (2015) referred above, members of Early-Response to Dehydration 6-like (ERD6l) were overexpressed in response to *Botrytis* infection, thus we decided to study in more detail this sugar transporter family. In this regard, a deep *in silico* analysis was performed, which allowed to identify the members of this family in the grapevine genome, and to study the VvERD6l phylogenetic relations with other plant species. Moreover cis-acting elements present in the promoter regions of all of the members were also identified. The subsequent work aimed at addressing the hypothesis that VvERD6l13 was a true sugar transporter of the plasma membrane of grape cells. VvERD6l13 function was evaluated after heterologous expression in yeast and its sub-cellular localization was evaluated in tobacco. Lastly, we wanted to evaluate if the expression of this protein was transcriptionally regulated in response to *Botrytis* and *E. necator* infection and the tissue-specific localization of this protein.

In summary, in the present study, we wanted to address the main following scientific questions in the context of grapevine-environment interactions and plant stress biology:

i) Does *B. cinerea* or *E. necator* infection induce a transcriptional reprogramming of VvSWEETs in the grape berry?

ii) What are the most expressed *VvSWEETs* in the grape berry and how their steady-state transcript levels are changed in response to infection?

iii) Does extracellular sugar levels regulate the expression of *VvSWEETs*?

iv) Does drought stress regulate the expression of *VvSWEETs*?

v) How *VvSWEETs* are transcriptionally regulated upon application of kaolin in the vineyard to mitigate abiotic stress such as heat and high light?

vi) How dehydration of the grape berry affects the sugar metabolism in particular regarding the expression of *VvSWEETs*?

vii) Are VvSWEET7 and VvSWEET15 true sugar transporters?

viii) What is the specificity for monosaccharides and disaccharides of these transporter proteins?

ix) Are VvSWEET7 and VvSWEET15 plasma membrane transporters?

x) What are the phenotypes of transformed Micro-TOM tomato overexpressing *VvSWEET7* or *VvSWEET15* or knocked-out-lines for these tomato homologous transporters?

xi) What are the biochemical properties of Micro-TOM tomatoes from transformed tomato plants overexpressing *VvSWEET7* or *VvSWEET15* or from knocked-out lines for these tomato homologous transporters?

xii) What members of *Early-Response to Dehydration 6-like* (ERD6l) are present in the grapevine genome and how are they phylogenetically related to the members of other plant species?

xiii) Is VvERD6l13 a true sugar transporter of the plasma membrane in grapevine?

xiv) How *Botrytis* and *E. necator* infection affect *VvERD6l13* expression at the transcriptional level?

xv) Is *VvERD6l13* differentially expressed in different grapevine tissues?

Chapter 2
Materials and Methods

2.1 - Biological material

2.1.1 – Grapevine cell suspension culture growth conditions and *Botrytis cinerea* elicitation experiment

Cell suspensions of *V. vinifera* L. (Cabernet Sauvignon Berry - CSB) were freshly established from somatic callus that had been previously initiated from Cabernet Sauvignon berry pulp according to Calderón et al. (1994). They were maintained in 250 mL flasks at 25 °C in the dark on a rotator shaker at 100 rpm, on modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962; Decendit et al., 1996), supplemented with 2% (w/v) sucrose as carbon and energy source. The suspension-cultured cells were sub-cultured weekly by transferring 10 ml aliquots into 40 ml of fresh medium.

Cell culture samples were obtained at 2, 7 and 10 days after sub-culture. Suspension cell aliquots (1–5 mL) were filtered through pre-weighed GF/C filters (Whatman) and washed with deionized water and weighed. Liquid media was collected for sugar quantification. For RNA isolation, cell samples were immediately frozen in liquid nitrogen and grounded to a fine powder with a mortar and a pestle. For all the sampled times, three biological replicates were used.

For elicitation experiments, grapevine suspension cells were harvested at the mid-exponential growth phase, centrifuged at 5.000 xg during 5 minutes and resuspended in MS medium at a final density of 0.1 g F.W. mL^{-1}. *Botrytis* mycelia extract was added to the suspension cell culture at a final concentration of 2 mg mL^{-1}. Then, the elicited suspension cultures were incubated in the dark, at room temperature on a rotatory shaker at 100 rpm. After 48 h of incubation, cells were filtered through GF/C filters (Whatman) and samples were washed with deionized water and immediately frozen in liquid nitrogen and grounded to a fine powder with a mortar and a pestle. For both *Botrytis*-elicited and control suspension cell cultures, three biological replicates were used. *B. cinerea* growth and mycelia harvest were performed according to Azevedo et al. (2006). The fungus was cultivated in potato dextrose (PD) liquid medium, at 25 °C with agitation (150 rpm). Mycelia were harvested from 12 d cultures, by centrifugation at 5,000 xg for 5 min, followed by resuspension in sterile water. The mycelia were autoclaved and then lyophilized during 48 h and ground with a mortar and pestle to a fine powder.

2.1.2 - Grape berry infection with *Botrytis cinerea* or *Erysiphe necator*

Field experiments were performed in a vineyard located at the Instituto Superior de Agronomia, Universidade de Lisboa, Portugal. The vineyard is arranged to the Lys training systems (3.0×1.0) and pruned to two buds.

For the *Botrytis* experiment, *B. cinerea* was isolated from contaminated vines and maintained in potato dextrose agar (Difco, Detroit, MI, USA) at 5 °C. For the production of conidia, Petri dishes were exposed to fluorescent light at 24 °C and after 14-18 days of treatment conidia were collected from Petri dishes by rubbing the culture with phosphate buffer (0.03 M KH_2PO_4). The suspension was then filtered through a cheesecloth and the concentration was adjusted to 10^5 conidia mL^{-1}. Clusters of Trincadeira cv. (a *Botrytis*-susceptible cultivar) grapes were infected in very well-established and standardized conditions according to Agudelo-Romero et al. (2015) and Coelho et al. (2019). Grape berries at the peppercorn size stage (EL29) were sprayed with the conidial suspension while control clusters were sprayed with phosphate buffer. After the inoculation, each cluster was enclosed by a plastic bag during one week to maintain 100% RH. Inoculation was performed, at the same time, in multiple clusters in very similar conditions, particularly in size, appearance, exposure to light, canopy densities, and plant orientation between them and also identical to control clusters. Samples were harvested at three developmental stages, at green (EL33), veraison (EL35) and mature (EL38) (Coombe, 1995). *Botrytis* infection was evaluated by visual inspection and additionally by PCR. Results are depicted in figure 1 and figure 2 of Coelho et al., 2019, as the plant material is common to that of the present work.

Also, grape berries of Carignan cultivar (*Erysiphe necator* susceptible cultivar), naturally infected with powdery mildew, and non-infected ones, were sampled at two developmental stages, at green (EL33) and veraison (EL35). *E. necator* infection was evaluated by visual inspection.

For each treatment (infected and control) three biological replicates were collected, each one constituted by a composite pool of at least 12 berries collected from different clusters from three different plants, at around 10 a.m.. Every collected infected berry had the same infection appearance and visual symptoms that were in fact similar between all infected clusters, as the inoculation was performed at the same time in all berry clusters. Thus, both control and infected collected grape berries were well representative of their physiological condition. The collected samples were frozen in liquid nitrogen and stored at -80 °C. Prior to RNA extraction, the seeds of each the three sampled biological replicates were removed and the remaining tissues ground in liquid nitrogen to a fine powder. For all experiments performed on grape berry tissues in this work, each of these 3 representative pools was used as a different biological replicate, and each biological replicate was used for a different experimental repetition (meaning three different RNA extractions, one from each biological replicate and three different qPCR analyses), with each qPCR analysis having its own internal triplicates.

2.1.3 – Drought-stressed grapevine field experiments

Field-grown grapevines of the Tempranillo cultivar, located in Estremoz (38°48'N, 7°29'W), Alentejo (South Portugal) that were used in Conde et al. (2015) were also used in the present study. In the region,

climate is Mediterranean with hot and dry summers and precipitation concentrated in the autumn and winter (Kottek et al., 2006). Two different irrigation treatments were applied to the grapevines: full irrigation (FI; 100% evapotranspiration (ET_c)) and non-irrigation (NI; rain fed only). The irrigation was applied accordingly to the grapevine ET_c and soil water content. Treatments started after flowering and ended at harvest (early September). Grape berries clusters from 4 to 6 plants, located in different rows and subjected to different irrigation regimes were collected at the green (44 days after flowering), veraison (62 DAF) and mature (77 DAF) stages. After harvest, grape berries were frozen in liquid nitrogen and stored at -80 °C. Prior to RNA extraction, seeds were removed and the tissue was ground to a fine powder with liquid nitrogen.

2.1.4 – Kaolin particle application experiments

Grapevines, from the cultivar Touriga Nacional grafted onto 110-R from the commercial vineyard "Quinta do Vallado", in the Douro Demarcated Region (Denomination of Origin Douro/Porto), located at Peso da Régua, Portugal (41°09′44.5″N 07°45′58.2″W), were used in this study. In this region the climate is typically Mediterranean with hot and dry summers and warn-temperate and relatively rainy during winter (Kottek et al., 2006). Standard cultural practices, as applied by commercial farmers and no irrigation was performed in the vineyard. The monthly maximum temperature (T_{max}) and precipitation values from April to October are reported in Dinis et al. (2016a).

Kaolin (Surround WP; Engelhard Corp., Iselin, NJ) at a concentration of 5% (w/v) was applied at three vineyard rows, with twenty plants each, in July 17th 2014, during the late green-phase. In the same day, another application was performed to ensure kaolin adhesion uniformity and to impede removal by precipitation. Similar structured control vines were maintained without kaolin application. At late afternoon (7 p.m.), 20 to 25 mature leaves were collected, two weeks after kaolin application (2 WAA) from different positions within a plant (and from different plants), with apparent different sun exposure (at the time of sampling), of several kaolin-treated and control plants, located in three different rows. The collected leaves were sampled accordingly on two criteria: (i) similar leaf size and surface area and, in the case of the kaolin-treated (ii) totally covered by the kaolin particle film in a uniform, homogenous way, without excess kaolin deposition. Kaolin residue was totally removed from the treated leaves by rapid washing on site at the sampling time. Collected leaves were rapidly frozen in liquid nitrogen and grounded to a fine powder in liquid nitrogen and stored at -80 °C until further use.

2.1.5 – Grape berry post-harvest dehydration experiments

The cultivar Sémillon, which is used to make dry and sweet wines but also raisins, was used in this study. Clusters were harvested in a particular vineyard in Fafe, Portugal, with a typical Mediterranean

climate. The vineyard was managed without any irrigation and with standard cultural practices applied in commercial farms. Mature grape clusters were randomly, carefully and representatively harvested. A set of grape clusters were placed in small perforated boxes and, in laboratory conditions, were subjected to raisin production, mimicking the industrial process (50 °C), while other set was immediately frozen in liquid nitrogen (control). After 5 and 11 days of dehydration, grapes were randomly sampled and immediately frozen in liquid nitrogen. None of the sampled berries present signs of biotic contamination. Prior to RNA extraction, seeds were removed and the berry was ground to a fine powder under liquid nitrogen refrigeration. Dry weight was always used for normalization purposes to avoid negative effects introduced by water content and berry weight variations.

2.2 - RNA extraction method

An initial amount of 200 mg of ground biological material was used for total RNA extraction, following the method described by Reid et al. (2006) combined with in-column purification using the RNeasy Plant Mini Kit (Qiagen). After isolation and verification of RNA purity, treatment with DNase I (Qiagen) was performed and cDNA was synthesized from 1 µg of total RNA using the Xpert cDNA Synthesis Master-mix Kit (GRISP).

2.3 - Gene expression analysis by qPCR

The genes studied in this work (*SWEET*/ *SUC*/ *HT*/ *ERD6-like*) were analyzed in all plant materials by real-time qPCR. For that, real-time qPCR was performed with Xpert Fast SYBR Blue (GRISP) using 1 µL of diluted cDNA (1:10) in a total of 10 µL of reaction mixture per well. For reference genes, *VvACT1* (actin) and *VvGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) were used as they are considered extremely adequate reference genes for gene expression normalization purposes in qPCR analyses in grapevine (Reid et al., 2006). Specific primers, used for each studied gene are listed in the Annex Table 4. Melting curve analysis was performed for specific gene amplification confirmation. Stability of the reference genes was confirmed by the automatic M-value analysis performed by the Bio-Rad® CFX Manager 2.0 Software. For each gene, the relative gene expression values were obtained following calculation by the Bio-Rad® CFX Manager 2.0 Software. For each of the three biological replicates, after RNA extractions and cDNA synthesis, an independent qPCR analysis was performed with internal triplicates.

2.4 - Determination of reducing sugar concentration in the medium by DNS methods

Concentration of reducing sugars present in the liquid media samples was determined with the 3,5-dinitrosalicylic acid (DNS) reagent method (Miller, 1959). In each reaction, 100 µL of diluted sample was mixed with 100 µL DNS solution, vortexed and boiled during 5 min in a water bath. After boiling, the samples were immediately placed on ice and 1 mL of cold water was added to each sample to stop the reaction. The absorbance of each sample was measured at 540 nm in a spectrophotometer (Shimadzu Pharmaspec UV-1700). To determine the concentration of reducing sugars the absorbance value was interpolated from a previously obtained calibration curve prepared with different known concentrations of D-glucose.

2.5 - Extraction and quantification of major sugars by HPLC

Sugar extraction from grape berries samples infected by *Botrytis* was performed following an adaptation of the method described by Eyéghé-Bickong and co-workers (2012) as reported in Conde et al. (2018b). To 80 mg of frozen grape berry lyophilized powder, 800 µL of deionized H_2O and 5% (w/v) insoluble PVPP were added and the mixture was vigorously vortexed. Then, an equal volume of chloroform (800 µL) was added and the mixture vortexed during 5 minutes followed by an incubation at 50 °C during 30 minutes with continuous shaking. This step was followed by a centrifugation at 17500 ×g for 10 minutes at room temperature and the upper aqueous phase was recovered. The aqueous phase was re-centrifuge to remove any residual cell particles. Then, the aqueous phase was filtered and transferred to HPLC vials and crimp-sealed. Each sample was extracted in triplicates. HPLC analysis was performed on a Hitachi Auto Sampler L-2200 Elite LaChrom chromatograph coupled to a Refractive Index (RI) detector. The injections were of 20 µL and the flow rate of constant 0.5 mL min^{-1} at 60 °C. The used column was a Rezex RCM monosaccharide Ca^{2+} (8%) and the mobile phase was water. Sugar concentrations were determined by comparison of the peak area with established calibration curves of each compound. The determined concentrations of all compounds are expressed always on a dry weight basis.

2.6 - Grapevine SWEET and ERD6l *in silico* analysis

Grapevine *SWEET* genes were identified by performing a Blastp analysis against the *Vitis vinifera* newly grapevine proteome annotation, VCost.v3 (Canaguier et al., 2017), on the URGI website (https://urgi.versailles.inra.fr/blast/) using each *Arabidopsis thaliana* SWEET protein as a query and an E-value of 1.00^{E-04} as threshold. A similar approach was used for grapevine *ERD6l* gene identification. *Arabidopsis* ERD6l proteins were used as a query to perform Blastp against the *V. vinifera* newly grapevine proteome annotation, VCost.v3 (Canaguier et al., 2017), the GENOSCOPE 12x annotation proteins database

and the NCBI grapevine protein database. Only genes which encoded to a protein with 12 transmembrane domains were considered (Annex figure 2). Moreover, correct gene structure was manually assessed with confirmation of exon expression in several RNAseq experiments available in the NCBI database (Annex Table 3). Topology predictions were performed with TOPCONS software http://topcons.cbr.su.se/ (Tsirigos et al., 2015). Protein identity of the studied proteins was performed in the website http://imed.med.ucm.es/Tools/sias.html. Phylogenetic analysis were performed with the MEGA7 software. Protein sequence alignment was performed using MUSCLE software and phylogenetic tree was constructed with the Maximum Likelihood method based on the JTT matrix-based (Jones et al., 1992) with 100 bootstrap replicates. Promoter analysis was performed using the 2 Kb upstream sequence of each gene (except some genes) in the PLACE website (PLAnt Cis acting regulatory DNA Elements database (PLACE: http://www.dna.affrc.go.jp/PLACE/index.html).

2.7 - *VvSWEET7*, *VvSWEET15* and *VvERD6l13* molecular cloning and construction of destination plasmids

The putative sugar transporter genes, *VvSWEET7*, *VvSWEET15* and *VvERD6l13* were cloned by Gateway® technology. Primers pairs, designed with the *att*B sequences (Annex Table 1) for site-specific recombination with the entry plasmid pDONR221, were used for PCR amplification of the target genes. Subsequently, recombination of the *att*B-containing target genes with the entry plasmid was performed using the BP clonase enzyme. The target genes carried in the entry plasmid were then recombined by the LR clonase enzyme into the pH7WGF2 plasmid (containing the *egfp* gene) for sub-cellular localization and into the pYES-DEST52 plasmid for heterologous expression in yeast. All constructs were confirmed by sequencing.

2.8 - Sub-cellular co-localization studies in tobacco leaves

The N-terminally fused constructs *pH7WGF2-VvSWEET7-GFP*, *pH7WGF2-VvSWEET15-GFP* and *pH7WGF2-VvERD6l13-GFP* were introduced in *Agrobacterium tumefaciens* strain EHA105 and transient transformation of tobacco leaves (*Nicotiana benthamiana*) was performed according to Sparkes et al. (2006). Transformed *Agrobacterium* cells were inoculated overnight in liquid LB medium, with the appropriate antibiotic selection, up to the exponential-stationary phase and then diluted to OD_{600nm} = 0.1 with infiltration buffer (50 mM MES pH 5.6, 2 mM Na_3PO_4, 0.5% glucose, 100 µM acetosyringone). Cells were then incubated until the culture reached an OD_{600nm} = 0.2. Leaves of three different four-week-old tobacco plants were infiltrated with the *Agrobacterium* culture and, after 2 days, discs of the infected leaves were observed at the scanning confocal microscope (Leica TCS SP5IIE-Leica Microsystems). Data stacks were analyzed and projected using ImageJ 1.42m software (http://rsb.info.nih.gov/ij/). The plasma membrane

marker used was the plasma membrane aquaporin AtPIP2;1 C-terminally fused to the fluorescent protein mCherry (AtPIP2;1-mCherry construct) (Nelson et al., 2007). This plasma membrane marker was co-expressed with either GFP-VvSWEET7, GFP-VvSWEET15 or GFP-VvERD5l13 constructs allowing the observation of their co-localization at the plasma membrane.

2.9 - Heterologous expression of VvSWEET7, VvSWEET15 and VvERD6l13 in *Saccharomyces cerevisiae*

The *S. cerevisiae* mutant strain EBY.VW4000 (Wieczorke et al., 1999) was used in this study to functionally characterize VvSWEET7, VvSWEET15 and VvERD6l13. This strain does not have the capacity to transport monosaccharides and sucrose due to multiple mutations in sugar-sensing and sugar transporter genes. The yeast was grown on rich medium supplemented with maltose (1% yeast extract, 2% peptone, 2% maltose). After transformation by the lithium acetate method (Gietz and Woods, 2002), with the constructions *pYES-DEST52-VvSWEET7* or *pYES-DEST52-VvSWEET15* or *pYES-DEST52-VvERD6l13* the yeast was grown in basic selective medium (0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulfate, 2% (w/v) carbon source) supplemented with maltose 2% (w/v) and without uracil for URA3-based selection. For control, the yeast cells were transformed with the empty vector.

2.10 - Measurement of proton pumping activity of the yeast plasma membrane ATPase

EBY.VW4000 yeast cells, transformed with *pYES-DEST52-VvSWEET7* and control ones (harboring the empty vector) were washed with deionized water and suspended in water (20 mg mL^{-1}) at room temperature under stirring during 3 h to induce starvation. For each experiment, 15 mg (D. W.) of yeast cell suspensions grown until OD_{600nm} = 0.8 was placed in a water-jacketed chamber in a total volume of 5 mL of non-buffered water. The suspension was mixed with a magnetic stirrer and the temperature-regulated circulating water was at 30 °C. Changes in pH were detected with a combination electrode (PHC-4000-8 RadioMeter) attached to a sensitive pH meter (PHM82 Standard pH Meter) and recorder (KIPP & ZONEN) with scale expander, as described by Serrano (1980). A concentration of 45 mM of different sugars (glucose, fructose, galactose and sucrose) were used to activate the proton pump. Calibration was performed through the addition of 100 nmol HCl to the cell suspension. For each proton pumping activity analysis, four experimental repetitions were performed, each one consisting of an independent VvSWEET7-overexpressing and control yeast growth and a subsequent sugar-induced pH variation analysis.

2.11 - Transport studies in *S. cerevisiae* with radiolabeled sugars

EBY.VW4000 yeast cells, transformed with *pYES-DEST52-VvSWEET7* or *pYES-DEST52-VvSWEET15* or *pYES-DEST52-VvERD6l13* (empty pYES-DEST52 for control) were grown in basic selective medium supplemented with 2% maltose, at 30 °C on a rotatory shaker at 220 rpm up to the exponential-stationary phase. To induce the expression of the target genes, the culture was washed twice in ice-cold sterile water and cultivated in fresh basic selective medium supplemented with 2% galactose for at least 4 h. Then, the cells were harvested by centrifugation and washed twice with ice-cold sterile distilled water and suspended in sterile water.

To estimate the initial uptake rates of radiolabeled sugars, 30 μL of cell suspension were mixed with 15 μL of 100 mM KH_2PO_4 buffer at pH 5.0 in 1.5 mL microcentrifuge tubes. After 2 min of pre-incubation at 30 °C in a thermoblock, the reaction was initiated by the addition of a volume of up to 15 μL of an aqueous solution of radiolabeled substrate. Depending on the desired radiolabeled substrate final concentration, different radiolabeled substrate solutions with different specific activities were used. For a final concentration of 7.5-50 mM of glucose (D-[^{14}C] glucose) or fructose (D-[^{14}C] fructose) a solution with a specific activity of 150 dpm nmol^{-1} was used. To determine sucrose initial transport rates, an aqueous solution of radiolabeled sucrose ([^{14}C] sucrose) with a specific activity of 500 (for final concentrations between 7.5 and 50 mM) or 250 dpm nmol^{-1} (for final concentrations between 75 and 125 mM) was used. To study the uptake rate of 3-*O*-Methyl-D-Glucose (3-OMG), a radiolabeled solution of 6.41x10^5 dpm nmol^{-1} (for final concentrations between 5.6 and 150 μM), 1000 dpm nmol^{-1} (final concentration of 1 mM) or 50 dpm nmol^{-1} (final concentration of 50 mM) was used. Potential competitive inhibitors or CCCP (carbonyl cyanide m-chlorophenylhydrazone) were added to the reaction mixture before the addition of the radiolabeled sugar for transport specificity and energetics assessment, respectively. After 3 min, the reaction was stopped by dilution with 1 mL of ice-cold water. Accumulation studies were performed during 20, 40 and 60 min. Then, cells were washed twice with ice-cold water and 1 mL of scintillation fluid added for complete cell membrane disruption and radioactivity measurements. The radioactivity was then measured in a scintillation counter (Packard Tri-Carb 2200 CA). D-[^{14}C] glucose (287 mCi mmol^{-1}), D-[^{14}C] fructose (316 mCi mmol^{-1}), 3-*O*-Methyl-D-Glucose (289 mCi mmol^{-1}) and [^{14}C] sucrose (592 mCi mmol^{-1}) were obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). For every radiolabeled sugar transport experiment, three independent experimental repetitions, thus, three biological replicates, each one consisting of an independent gene of interest-overexpressing and respective control yeast growth and subsequent radiolabeled sugar uptake were performed. Also, each experimental repetition was performed with internal triplicates.

2.12 - Statistical analysis

To test if the data were normally distributed, the Shapiro-Wilk normality test was used, while the homogeneity of variances was confirmed using Bartlett's tests using Prism v. 6 (GraphPad Software, Inc.). Subsequently, the results were statistically verified by analysis of variance tests (one-way ANOVA) or Student's t-test using Prism v. 6 (GraphPad Software, Inc.). Post-hoc multiple comparisons were performed using the HSD Tukey test. Throughout the results, different letters denote statistical differences between columns and are presented in a progressive order from the highest to the lowest value and asterisks indicate statistical significance.

Chapter 3
Results

Parts of the work presented in this chapter has been published in:

Breia, Richard; Conde, A; Pimentel, D; Conde, C; Fortes, AM; Granell, A; Gerós, H. (2020) VvSWEET7 is a mono- and disaccharide transporter up-regulated in response to *Botrytis cinerea* infection in grape berries. *Frontiers in Plant Science*, (in press) DOI: 10.3389/fpls.2019.01753.

Conde, A; Neves, A; **Breia, Richard**; Pimentel, D; Dinis, L; Bernardo, S; Correia, C; Cunha, A; Gerós, H; Moutinho-Pereira, J. (2018) Kaolin particle film application stimulates photoassimilate synthesis and modifies the primary metabolome of grape leaves. *Journal of Plant Physiology*, v. 223, p. 47-56.

Conde, A; Soares, F; **Breia, Richard**; Gerós, H. (2018) Postharvest dehydration induces variable changes in the primary metabolism of grape berries. *Food Research International*, v. 105, p. 261-270.

3.1 - Grapevine SWEET family *in silico* analysis

As referred to in the introduction, the grapevine SWEET family was identified by Lecourieux et al. (2014) and Chong et al. (2014) in the Genoscope 12x annotation (Adam-Blondon et al., 2011; Adam-Blondon et al., 2014). Here, we identified the *VvSWEET* family in the newly grapevine genome annotation, VCost.v3 (Canaguier et al., 2017). This family is composed by 17 members (Table 3.1) and all genes encode proteins predicted to have 7 TMD (Annex figure 1). Regarding gene structure, all *VvSWEET* genes assemble in a 6 exons/5 introns configuration, except *VvSWEET7* which has one less exon/intron pair. Members of this family are evenly distributed in the *Vitis* genome and only *VvSWEET17b* and *VvSWEET17c* are located in tandem. Almost all members are predicted to localize in the plasma membrane, however VvSWEET2b and VvSWEET17a are predicted to localize at the tonoplast, VvSWEET11 and VvSWEET12 at the endoplasmic reticulum and VvSWEET2a at the chloroplast membrane. This family shows high identity, between 78% (VvSWEET17b and VvSWEET17c) and 44% (VvSWEET2a and VvSWEET17a) (Table 3.2). Phylogenetic analysis shows that *VvSWEET* members clearly separate in the classic four clades (Figure 3.1): clade I – *VvSWEET1*, *VVSWEET2a*, *VvSWEET2b* and *VvSWEET3*; clade II – *VvSWEET4*, *VvSWEET5a*, *VvSWEET5b* and *VvSWEET7*; clade III – *VvSWEET9*, *VvSWEET10*, *VvSWEET11*, *VvSWEET12* and *VvSWEET15*; clade IV – *VvSWEET17a*, *VvSWEET17b*, *VvSWEET17c* and *VvSWEET17d*.

A phylogenetic tree was constructed using SWEET family members of *Arabidopsis*, tomato (climacteric fruit model) and grapevine (Figure 3.2). Proteins from clade I and clade II are highly conserved between the studied species, as shown by the strong support of branches separating the proteins groups. Grapevine SWEET members appeared to be underrepresented in clade III, having 5 members against *Arabidopsis* 7 and tomato 13, however in clade IV, *Vitis* has 4 members while other species only have 2.

Moreover, to analyze the promoter region of *VvSWEET* genes, a search in the PLACE database (Higo et al., 1999) was performed (Table 3.3 and Annex Table 1). We identified a 2 kb promoter region for each *VvSWEET* member, except in *VvSWEET17b* that contains a shorter promoter sequence (523 bp) due to the presence of another ORF. *VvSWEETs* promoter sequences revealed several biotic stress related cis-acting elements, such as WRKY71OS, GT1GMSCAM4 or WBOXATNPR1. These cis-acting elements are evenly distributed between all *VvSWEETs* promoter regions. GT1GMSCAM4, which is related with both pathogen defense and salt stress is more abundant in the promoter region of *VvSWEET* genes of clade I, and HSELIKENTACIDICPR1, another pathogen-responsive element, is only present in the promoter region of *VvSWEET3*.

Likewise, sugar-responsive elements were detected. PYRIMIDINEBOXOSRAMY1A and WBOXHVISO1 were detected in all the *VvSWEETs* promoter sequences. The sucrose responsive cis-elements SURE1STPAT21 and SURE2STPAT21 were scarcely found, being mainly present in the promoter region of

clade I *VvSWEETs*. The promoters with more sugar-responsive elements were those of *VvSWEET2b*, *VvSWEET4* and *VvSWEET17d*.

Additionally, regulatory elements responsive to abiotic stress were also identified. The element MYCCONSENSUSAT, which is related with drought, cold and ABA responses was detected multiple times in the promoter regions of *VvSWEETs*, mainly in clade III *SWEETs*. Other cis-acting elements, such as GT1GMSCAM4 are also abundant. The promoter regions of *VvSWEET2a*, *VvSWEET11* and *VvSWEET17a* were the most enriched with this type of cis-acting elements. The elements ABREATCONSENSUS and CRTDREHVCBF2 were found only in *VvSWEET2a* promoter and LTREATLTI78 in *VvSWEET17d*.

Few cis-acting elements responsive to hormones were also found in the promoters of *VvSWEET* genes, especially responsive to gibberellins and ABA, and none was common to all the analyzed sequences. Contrarily, several elements present in only one or two *VvSWEET* promoter sequences were detected, such as ATHB6COREAT, GARE2, ABREMOTIFAOSOSEM or SBOXATRBCS. The promoter region of *VvSWEET11* is particularly enriched with hormone responsive cis-acting elements.

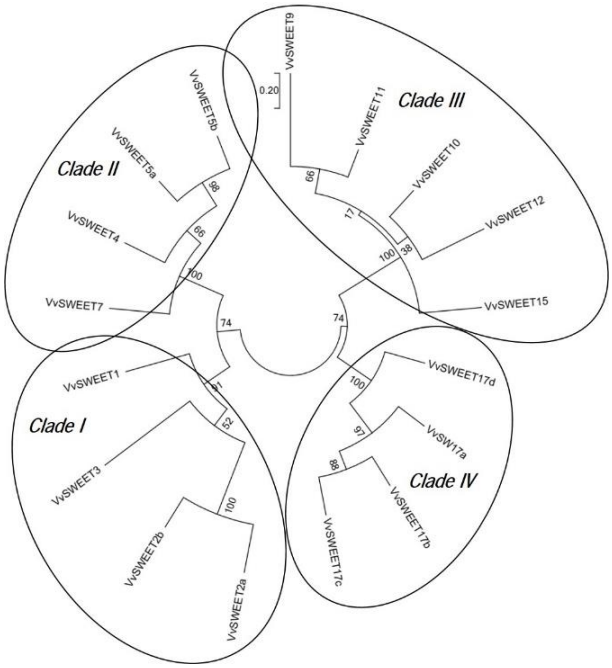

Figure 3.1 - Phylogenetic analysis of grapevine SWEET proteins. The different clades are highlighted by ellipses. Bootstrap values based on 100 replicates are indicated beside the nodes. Evolutionary analyses end tree drawn in MEGA7 and analysis performed with the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992).

Table 3.1 – Grapevine *SWEET* family. Gene name, Genoscope 12X ID, VCost.v3 ID, chromosome, chromosome position, strand, exon number, open reading frame base pairs, coding sequence base pairs, protein amino acids, trans-membrane domains, sub-cellular localization, theoretical protein molecular weight (KDa), theoretical isoelectric point (PI) and UniProt ID.

Gene name	GENOSCOPE 12X ID	VCost.v3 ID	Chr.	Position	Strand	Exon	ORF (bp)	CDS (bp)	AA	TMD	LOC	MW KDa	PI	UniProt ID
VvSWEET1	GSVIVT01010015001	Vitvi18g01215	18	13476577..13478521	-	6	1945	747	248	7	PM	27.352	9.64	E0CQM5
VvSWEET2a	GSVIVT01014088001	Vitvi19g00024	19	278703..280610	-	6	1908	708	235	7	CHL	26.117	9.30	E0CS05
VvSWEET2b	GSVIVG01021317001	Vitvi10g00679	10	7519923..7522199	+	6	2277	699	232	7	VC	25.777	8.84	D7TJV0
VvSWEET3	GSVIVT01028713001	Vitvi16g01984	16	21023964..21025567	+	6	1604	750	249	7	PM	28.036	9.13	E0CUJ5
VvSWEET4	GSVIVT01032489001	Vitvi14g01783	14	27825543..27827843	-	6	2301	765	254	7	PM	27.899	9.36	D7TWR3
VvSWEET5a	GSVIVG01007779001	Vitvi17g00791	17	9249346..9250571	+	6	1226	705	234	7	PM	26.160	9.43	D7SHU1
VvSWEET5b	GSVIVG01007777001	Vitvi17g00793	17	9294434..9295807	+	6	1603	708	236	7	PM	26.200	9.41	A5ANZ5*
VvSWEET7	GSVIVG01019601001	Vitvi02g00181	2	1670769..1672706	-	5	1938	738	260	7	PM	28.845	9.62	D7TVL5
VvSWEET9	GSVIVG01026399001	Vitvi04g01075	4	15791258..15792899	+	6	2119	837	279	7	PM	31.500	9.06	F6HI37*
VvSWEET10	GSVIVG01008595001	Vitvi17g00070	17	682720..684274	+	6	1645	903	300	7	PM	33.955	7.63	D7SH34
VvSWEET11	GSVIVT01010993001	Vitvi07g00250	7	2750242..2751583	-	6	1342	831	276	7	ER	31.004	9.30	F6HQ15
VvSWEET12	GSVIVG01008597001	Vitvi17g00069	17	678130..680069	+	6	1940	852	283	7	ER	31.699	9.34	F6GT01
VvSWEET15	GSVIVG01000938001	Vitvi01g01719	1	23092272..23093619	-	6	1348	870	289	7	PM	32.149	9.08	P0DKJ5
VvSWEET17a	GSVIVG01035138001	Vitvi05g00013	5	124236..126407	+	6	2172	702	233	7	VC	25.392	7.73	D7SYD6
VvSWEET17b	GSVIVT01031172001	Vitvi14g00148	14	1533798..1535425	-	6	1,628	717	238	7	PM	26.473	6.90	F6I4N9*
VvSWEET17c	GSVIVT01031172001	Vitvi14g00147	14	1525182..1527210	-	6	2029	714	237	7	PM	26.266	6.29	F6I4N9*
VvSWEET17d	GSVIVG01031170001	Vitvi14g00149	14	1542825..1544958	-	6	2134	897	298	7	PM	32.764	9.68	A5BI99

* Partial UniProt protein sequence

Table 3.2 – Protein identity of grapevine SWEET proteins. Performed in http://imed.med.ucm.es/Tools/sias.html.

	VvSW1	VvSW2a	VvSW2b	VvSW3	VvSW4	VvSW5a	VvSW5b	VvSW7	VvSW9	VvSW10	VvSW11	VvSW12	VvSW15	VvSW17a	VvSW17b	VvSW17c	VvSW17d
VvSW1	100%	54.46%	54.74%	53.22%	50.8%	52.99%	51.06%	53.62%	46.77%	47.17%	47.58%	49.19%	47.58%	54.93%	51.68%	51.89%	54.03%
VvSW2a		100%	71.98%	54.04%	51.91%	47.86%	48.51%	51.48%	47.65%	45.95%	48.08%	48.51%	47.23%	44.2%	45.95%	45.53%	47.23%
VvSW2b			100%	51.29%	50.86%	52.58%	52.15%	52.15%	46.98%	46.98%	47.41%	50%	46.55%	46.98%	49.13%	47.41%	50.43%
VvSW3				100%	48.99%	50.42%	50.63%	48.99%	47.79%	46.98%	46.98%	48.59%	48.99%	51.07%	52.52%	47.67%	45.38%
VvSW4					100%	69.23%	68.08%	64.96%	48.42%	48.03%	44.48%	52.36%	48.42%	58.36%	55.04%	55.69%	53.93%
VvSW5a						100%	79.91%	68.37%	44.87%	51.7%	47.43%	52.13%	51.28%	55.79%	54.7%	56.83%	59.82%
VvSW5b							100%	66.38%	47.23%	47.65%	46.38%	50.63%	49.36%	52.36%	51.48%	52.34%	58.29%
VvSW7								100%	44.23%	47.69%	45%	49.23%	43.84%	54.93%	54.2%	54.85%	50.76%
VvSW9									100%	59.62%	60.07%	51.43%	52.87%	55.36%	57.98%	56.96%	50.71%
VvSW10										100%	64.44%	62.22%	61.85%	52.78%	54.2%	56.54%	49.25%
VvSW11											100%	55.55%	57.09%	51.93%	52.1%	53.58%	44.29%
VvSW12												100%	56.94%	54.07%	52.52%	54.85%	46.18%
VvSW15													100%	51.5%	50.42%	51.47%	42.9%
VvSW17a														100%	70.81%	70.81%	70.81%
VvSW17b															100%	78.05%	66.8%
VvSW17c																100%	68.77%
VvSW17d																	100%

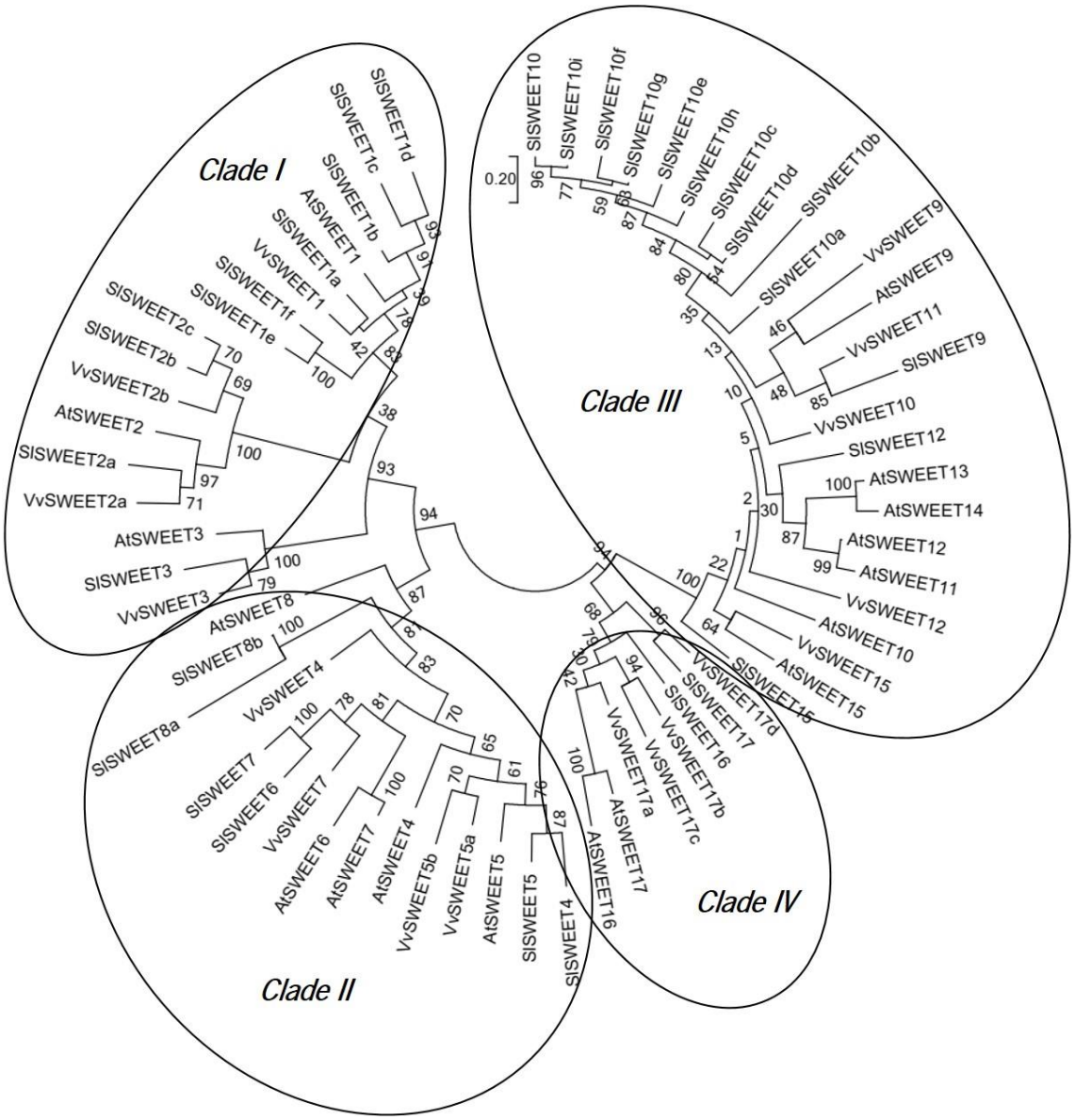

Figure 3.2 - Phylogenetic analysis of *Vitis vinifera*, *Solanum lycopersicum* and *Arabidopsis thaliana* SWEET proteins. The different clades are highlighted by ellipses. Bootstrap values based on 100 replicates are indicated beside the nodes. Evolutionary analyses end tree drawn in MEGA7 and analysis performed with the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992).

Table 3.3 – Representative cis-acting elements identified in the promoter sequence of *VvSWEET* genes via PLACE (Higo et al., 1999). Cis-element name, sequence motifs, signaling pathway and number of copies on the promoter are shown.

Cis-acting elements	Sequence	Response	SW1	SW2a	SW2b	SW3	SW4	SW5a	SW5b	SW7	SW9	SW10	SW11	SW12	SW15	SW17a	SW17b	SW17c	SW17d
CATATGGMSAUR	CATATG	Auxin	0	0	0	0	0	2	2	0	0	4	2	0	2	2	0	0	4
GAREAT	TAACAAR	Hormone regulation	2	4	1	2	3	0	4	2	2	0	2	2	0	0	1	2	4
GT1GMSCAM4	GAAAAA	Pathogen defense: salt stress	15	10	17	9	6	3	12	5	7	6	3	4	4	7	1	3	4
MYBCORE	CNGTTR	Drought	1	1	4	4	1	1	3	7	3	6	4	2	4	7	1	3	1
MYBGAHV	TAACAAA	Gibberellin; sugar repression	1	3	0	1	3	0	4	2	1	0	2	2	2	0	1	2	4
MYCCONSENSUSAT	CANNTG	Drought, cold, ABA	2	4	20	22	8	10	16	18	12	26	30	22	20	28	0	12	20
OSE2ROOTNODULE	CTCTT	Nodule; root	8	6	6	12	4	7	10	9	4	2	7	3	5	5	2	7	2
PYRIMIDINEBOXOSRAMY1A	CCTTTT	Gibberellin; sugar repression	1	2	11	2	2	3	2	0	2	3	2	4	3	4	2	2	4
SBOXATRBCS	CACCTCCA	Sugar-responsive; ABA	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	**1**
SEBFCONSSTPR10A	YTGTCWC	Pathogenesis-related	1	1	0	2	0	2	1	0	0	0	3	1	2	2	0	0	2
SREATMSD	TTATCC	Sugar repression	1	1	3	2	6	0	2	1	3	0	0	0	0	0	0	0	4
SURE2STPAT21	AATACTAAT	Sucrose responsive	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
TATCCAOSAMY	TATCCA	Sugar; Hormone regulation	0	0	1	2	5	3	0	2	1	1	1	0	0	2	0	1	1
WBOXATNPR1	TTGAC	Pathogen defense	2	3	1	4	1	2	2	2	3	1	6	8	2	3	1	2	1
WBOXHVISO1	TGACT	Sugar-responsive	5	3	4	4	2	3	2	5	3	1	5	4	0	3	1	1	6
WRKY71OS	TGAC	Pathogen defense; gibberellin	7	7	11	12	5	8	6	11	7	10	14	14	11	14	2	6	14

3.2 - Effect of elicitation of grape suspension-cultured cells with *Botrytis cinerea* on the expression profile of sugar transporters

The expression profile of different *Vitis vinifera* sugar transporter genes, including *VvSWEETs*, was analyzed by Real-Time PCR in heterotrophic suspension-cultured cells of grape berry pulp (CSB). Suspension-cultured cells are a useful model to study sugar transport and its regulatory mechanisms as it allows the control of all the parameters regarding cell culture environment and the application of biotic and abiotic stress agents. These models have already provided important insights in different plant physiologic mechanisms (Graham et al., 1994; Roitsch and Tanner, 1994; Ehness and Roitsch, 1997; Oliveira et al., 2002; Çakir et al., 2003; Conde et al., 2006; Conde et al., 2007; Conde et al., 2011).

Cells were cultivated in liquid culture medium with 2 % (w/v) sucrose as the sole carbon and energy source, and the growth together with the sugar content in the medium were monitored during 10 days (Figure 3.3). Results showed that 10 days after subculture the cultures approached the maximum population, as previously observed (Conde et al., 2006), and that after 7 days external sugar (mostly glucose and fructose, as previously observed – Conde et al., 2006) was almost depleted from the culture medium. In agreement, the steady-state transcript levels of expression of *VvCwINV* (Figure 3.4) were high at the initial phase of growth, diminishing afterwards.

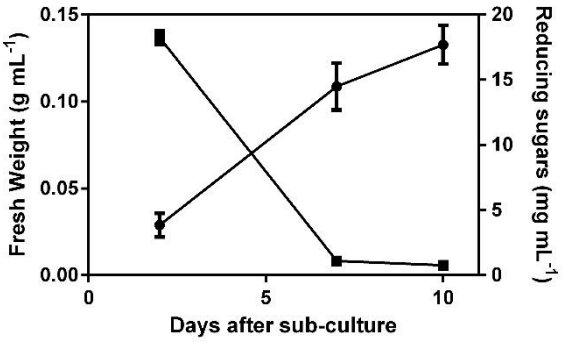

Figure 3.3 - Fresh weight and reducing sugar concentration in grape suspension cultured cells (CSB cells) cultivated in mineral medium supplemented with an initial concentration of 2% sucrose.

Figure 3.4 shows that the monosaccharide transporter *VvHT3* was highly expressed during the initial growth phase and the expression of the tonoplast monosaccharide transporter 1 (*TMT1*) peaked 7 days after subculture. The expression of the sucrose transporter *VvSUC11* peaked 7 days after subculture, while *VvSUC12* and *VvSUC27* transcript levels were more abundant at day 2.

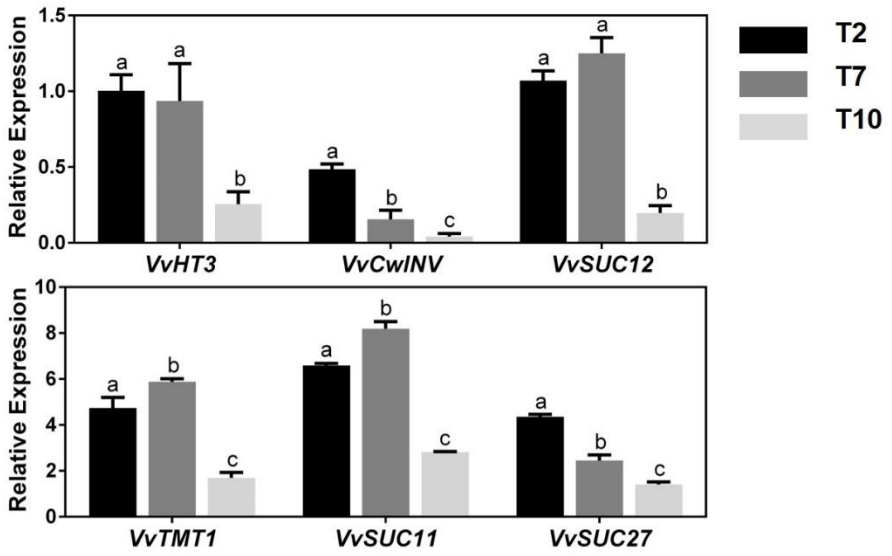

Figure 3.4 – Expression profile of prominent members of the *MFS* super-family genes and a cell-wall invertase, performed by real-time PCR in CSB suspension cultured cells, sampled after 2 (■), 7 (■) and 10 (■) days after subculture. Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample T2 *VvHT3*, which was set to 1. For all experimental conditions tested, three independent laboratorial analyses were performed on adequately composite CSB suspension cultured cell samples. Values are the mean ± SD. Letters indicate statistical significance (one-way ANOVA with Tukey's post-test).

The expression pattern of *VvSWEET* genes is shown in figure 3.5. From all the studied genes, the steady-state transcript levels of *VvSWEET2a*, *VvSWEET2b,* and especially *VvSWEET4,* were the more abundant, while the levels of *VvSWEET10*, *VvSWEET11* and *VvSWEET15* transcripts were low. The expression of nearly all *VvSWEET* genes (*VvSWEET1*, *VvSWEET2a*, *VvSWEET2b*, *VvSWEET4*, *VvSWEET7*, *VvSWEET15* and *VvSWEET17d*) was highest 7 days after subculture, but the expression of *VvSWEET11* peaked at the final sampling time.

The effect of *Botrytis* elicitation on the expression of the above-referred sugar transporters is depicted in figure 3.6. As referred to in the Materials and Methods section, suspension-cultured cells were cultivated with an initial sucrose concentration of 2% and then elicited with mycelium extract of *B. cinerea* for 48 h. *Botrytis* elicitation shifted the expression profile of different *VvSWEET* genes. Thus, *VvSWEET10*, *VvSWEET11* and *VvSWEET17d* were strongly down-regulated, while *VvSWEET1* was completely repressed. Contrarily, *VvSWEET2a* and *VvSWET15* were slightly up-regulated and *VvSWEET7* gene expression was strongly up-regulated, by 15-fold.

Regarding the effect of *Botrytis* on the expression of the classical members of MFS family, results showed that both the vacuolar transporter *TMT1* and *VvHT3* were up-regulated, while the effect of the elicitation on the SUC transporters was not statically significant.

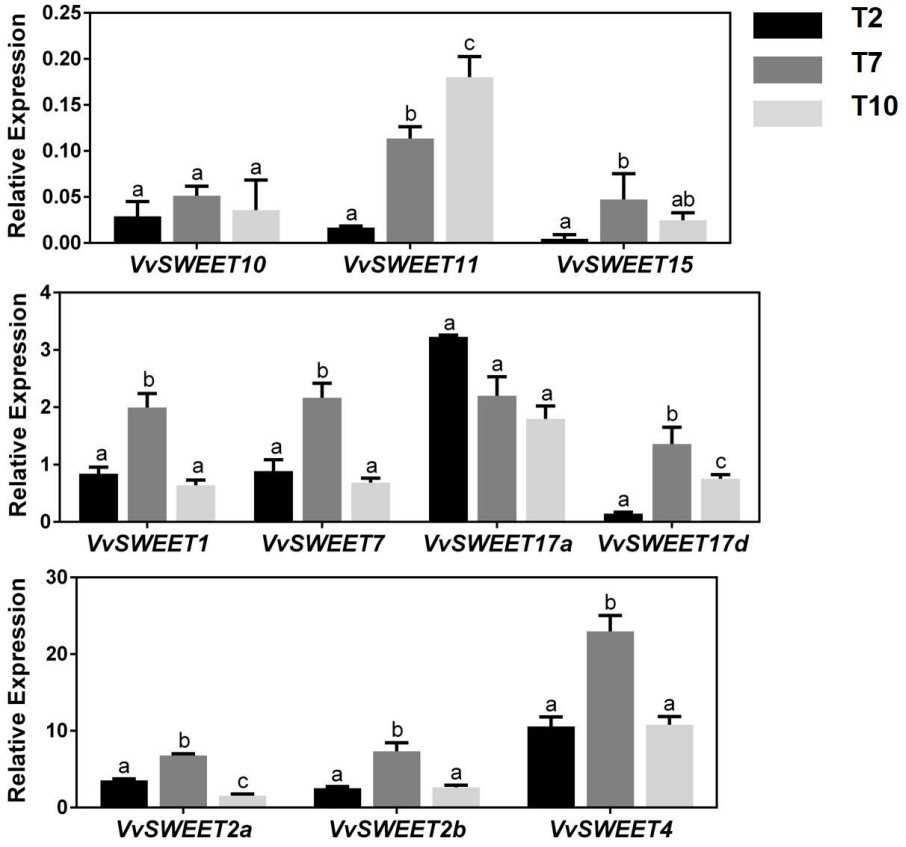

Figure 3.5 – Expression profile of *VvSWEET* members, performed by real-time PCR in CSB suspension cultured cells, sampled after 2 (■), 7 (■) and 10 (■) days after subculture. Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample T2 *VvHT3* (from figure 3.4), which was set to 1. For all experimental conditions tested, three independent laboratorial analyses were performed on adequately composite CSB suspension cultured cell samples. Values are the mean ± SD. Letters indicate statistical significance (one-way ANOVA with Tukey's post-test).

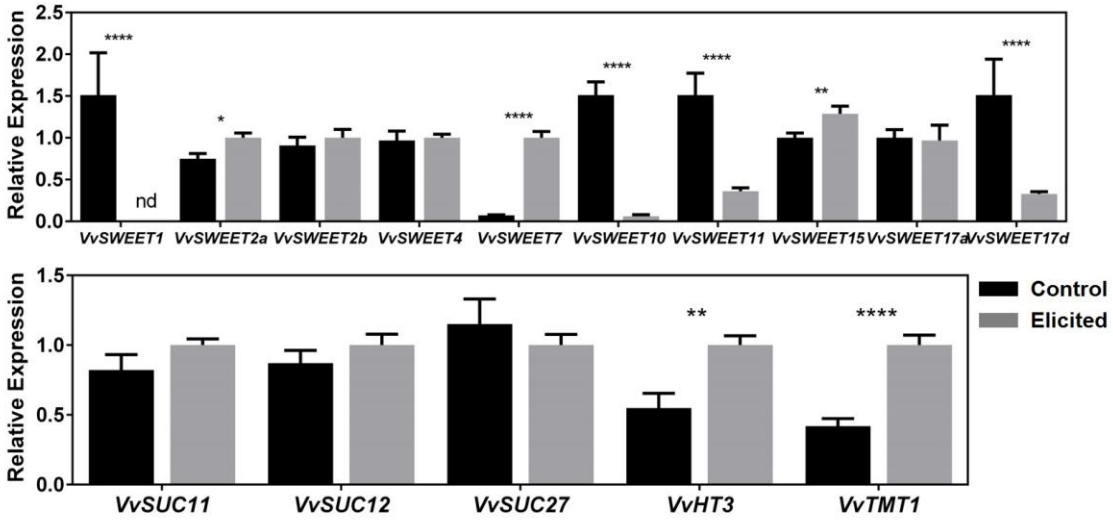

Figure 3.6 – Expression profile of several grapevine sugar transporters, performed by real-time PCR in CSB suspension cultured cell, sampled at the middle exponential phase and elicited 48 h with *Botrytis* mycelia

(■), or mock conditions (■). Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample with the lowest expression level, which was set to 1. For all experimental conditions tested, three independent laboratorial analyses were performed on adequately composite CSB suspension cultured cell samples. Values are the mean ± SD. Asterisks indicate statistical significance (one-way ANOVA with Tukey's post-test; *P<0.05; **P<0.01; ***P<0.001; **** P < 0.0001).

3.3 - Effect of the infection by *Botrytis cinerea* and *Erysiphe necator* on the expression of grapevine sugar transporters in field conditions

As referred to in the Introduction, the study - in field conditions - of the effects of the grapevine infection by the necrotrophic pathogen *B. cinerea* and by the biotrophic one *E. necator* on the expression profile of different sugar transporters was conducted in collaboration with the group headed by Prof. Ana Fortes from Universidade de Lisboa, who performed the infections in the field.

As reported in Materials and Methods, grape berries (cv. Trincadeira) were inoculated with a conidial suspension of *B. cinerea* at the early green stage (peppercorn-size; EL29) and collected at three different developmental stages - EL32-green, EL35-veraison and EL38-fully mature. Berry clusters showed clear symptoms of *Botrytis* infection at the green (EL32), veraison (EL35) and fully mature stages (EL38) (Figure 3.7a, b), and the infection was confirmed by amplification by qPCR of specific fungal genomic DNA (Coelho et al., 2019). Similarly, grape berries with strong visual signs of naturally occurring *E. necator* infection were sampled at green (EL32) and veraison (EL35) stages (Figure 3.7c). Frozen samples were cordially sent to Universidade do Minho were the expression profile of several sugar transporters was evaluated by real-time PCR.

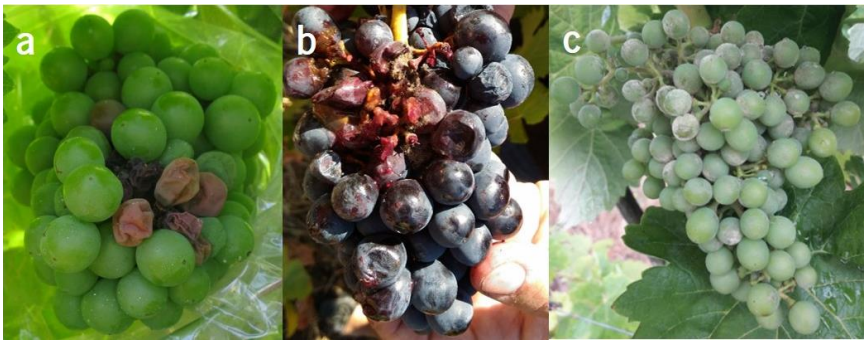

Figure 3.7 - Bunches of grapes (cv. Trincadeira) infected with *Botrytis* at EL32 (a) and EL38 (b) and of cultivar "Carignan" infected with *E. necator* at EL32 (c).

Figure 3.8 shows that distinctive expression patterns along grape berry development were observed for each *VvSWEET* gene. While the transcript levels of *VvSWEET11* and *VvSWEET15* increased along development, the expression of *VvSWEET1*, *VvSWEET2b*, *VvSWEET4*, *VvSWEET7* and *VvSWEET17a*

decreased from green to mature stage. The transcript levels of *VvSWEET2a*, *VvSWEET10* and *VvSWEET17d* peaked at veraison. In each stage, gene expression in whole berries was compared between control non-infected grapes (solid bars in figure 3.8) and *B. cinerea*–infected berries (striped bars in figure 3.8). As can be seen, *B. cinerea* infection up-regulated *VvSWEET2a* and *VvSWEET7* expression at the green stage and *VvSWEET15* expression at the mature stage. Contrarily, *Botrytis* infection down-regulated *VvSWEET17a* expression at the green stage, *VvSWEET10* and *VvSWEET17d* expression at the veraison stage and *VvSWEET11* expression at the mature stage. Interestingly, down-regulation of *VvSWEET* genes occurred specifically in the developmental stages where the gene was most expressed in normal conditions.

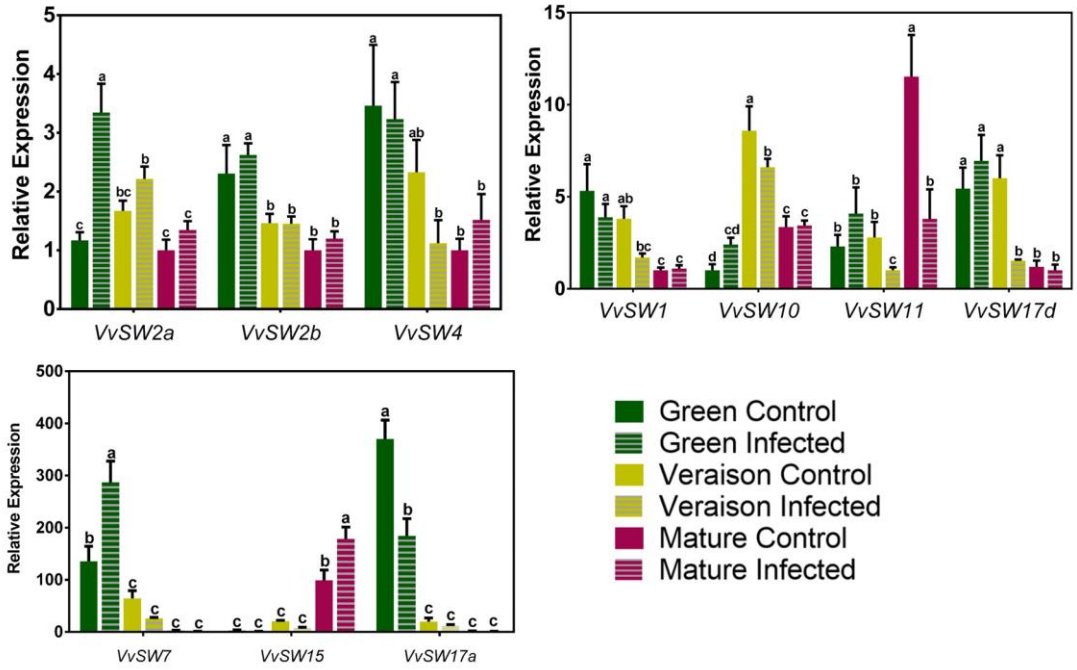

Figure 3.8 - Expression profile of *VvSWEET* genes that are expressed in the grape berry, performed by real-time qPCR in *Botrytis*-infected (striped bars) and control (solid bars) berries, collected at three different developmental stages (green, veraison and mature). Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample with the lowest expression level, which was set to 1. For each of the three biological replicates, after RNA extractions and cDNA synthesis, an independent qPCR analysis was performed with internal triplicates. Letters indicate statistical significance (one-way ANOVA with Tukey's post-test).

The effect of *B. cinerea* infection on the expression profile of the classical members of MFS family *VvSUC11*, *VvSUC12*, *VvSUC27*, *VvHT3* and *VvTMT1* is shown in figure 3.9. As previously shown (Afoufa-Bastien et al., 2010), *VvSUC11* is mostly expressed in mature berries, while the transcript levels of *VvSUC12*, *VvSUC27* and *VvHT3* are more abundant at the green stage. *VvTMT1* expression peaked at veraison. From all studied genes only *VvHT3* was responsive to *B. cinerea* infection, which caused a 3-fold up-regulation at the mature stage. Moreover, when the concentration of glucose and fructose in berries at

the fully mature stage (EL38) was determined by HPLC (Figure 3.10), only a small decrease in both sugars in response to infection became apparent, but statistically not significant.

The effect of *E. necator* infection on the expression profile of sugar transporter genes along two developmental phases is depicted in figure 3.11 (VvSWEETs) and in figure 3.12 (monosaccharide and disaccharide transporters). The steady-state transcript levels of *VvSWEET2a*, *VvSWEET4*, *VvSWEET10*, *VvSWEET11*, *VvSWEET15* and *VvSWEET17d* increased from green to the veraison stage, while the expression of *VvSWEET1*, *VvSWEET2b*, *VvSWEET7* and *VvSWEET17a* was down-regulated in response to infection.

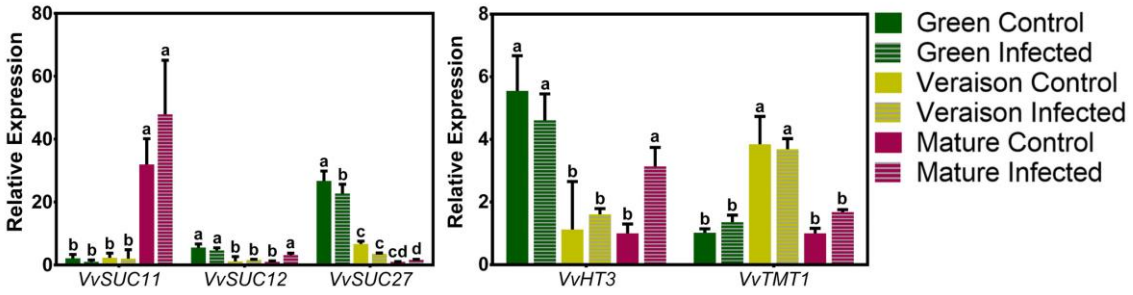

Figure 3.9 - Expression profile of prominent members of the *MFS* super-family genes, highly expressed in the grape berry, and performed by real-time PCR in *Botrytis*-infected (striped bars) and control (solid bars) berries, collected at three different developmental stages (green, veraison and mature). Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample with the lowest expression level, which was set to 1. For each of the three biological replicates, after RNA extractions and cDNA synthesis, an independent qPCR analysis was performed with internal triplicates. Letters indicate statistical significance (one-way ANOVA with Tukey's post-test).

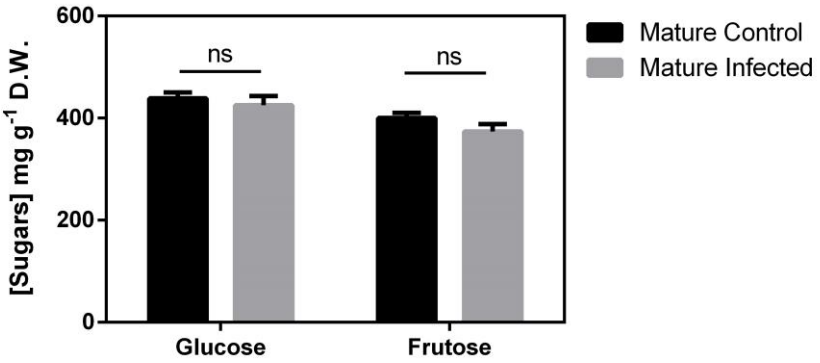

Figure 3.10 – Effect of *B. cinerea* infection on the concentration of glucose and fructose in grape berries at the mature stage (EL38). Sugars were quantified by HPLC and values are the mean ± SD of three independent experiments. Ns stands for not statistically significant (one-way ANOVA with Tukey's post-test);

When gene expression was compared between non-infected grapes (solid bars in figure 3.11) and *E. necator*–infected berries (striped bars in figure 3.11) one may conclude that, in general, *VvSWEET*s were more responsive to *E. necator* than to *Botrytis* infection. *E. necator* infection slightly up-regulated *VvSWEET7* gene expression in green berries, but a small down-regulation of *VvSWEET2a*, *VvSWEET17a* and

VvSWEET10 was observed. However, *VvSWEET2b* and *VvSWEET4* suffered a strong down-regulation in response to infection. At the veraison stage, *VvSWEET15* transcript levels increased by almost 2-fold in response to infection, while *VvSWEET4, VvSWEET10*, *VvSWEET11* and *VvSWEET17d* were strongly down-regulated. Overall, *VvSWEETs* transcripts abundance was negatively affected by *E. necator* infection, especially during the veraison stage.

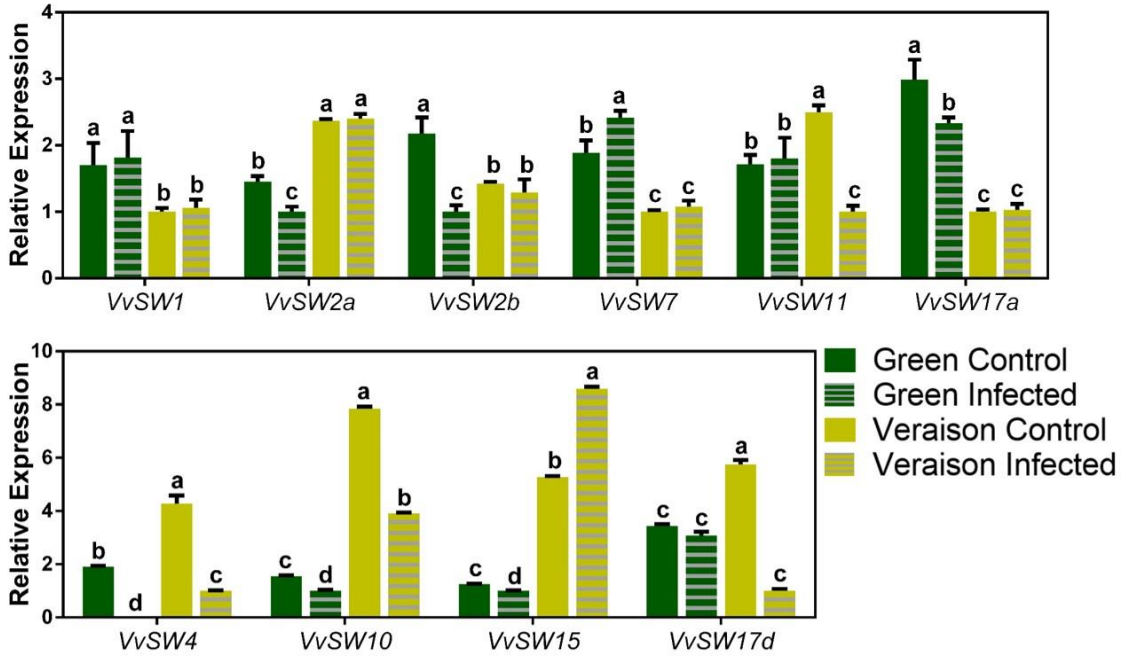

Figure 3.11 – Expression profile of *VvSWEET* genes performed by real-time PCR in *E. necator*-infected (striped bars) and control (solid bars) berries, collected at two different developmental stages (green and veraison). Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample with the lowest expression level, which was set to 1. For each of the three biological replicates, after RNA extractions and cDNA synthesis, an independent qPCR analysis was performed with internal triplicates. Letters indicate statistical significance (one-way ANOVA with Tukey's post-test).

The members of the MFS family *VvSUC11*, *VvSUC12*, *VvSUC27* and *VvTMT1* followed an expression pattern similar to that in samples from the Trincadeira cultivar (Figure 3.12). Contrarily to previously described results (Figure 3.9), the gene expression pattern of *VvHT3* was slightly different, increasing its expression from green to veraison stage. *E. necator* infection modified the gene expression of almost all studied genes. In infected green berries, *VvSUC12* and *VvSUC27* gene expression was down-regulated while *VvTMT1* gene expression was slightly up-regulated. At the veraison stage, the infection caused a down-regulation of *VvSUC27* and *VvHT3* expression. Interestingly, *VvSUC27* expression was down-regulated in both developmental phases in response to infection.

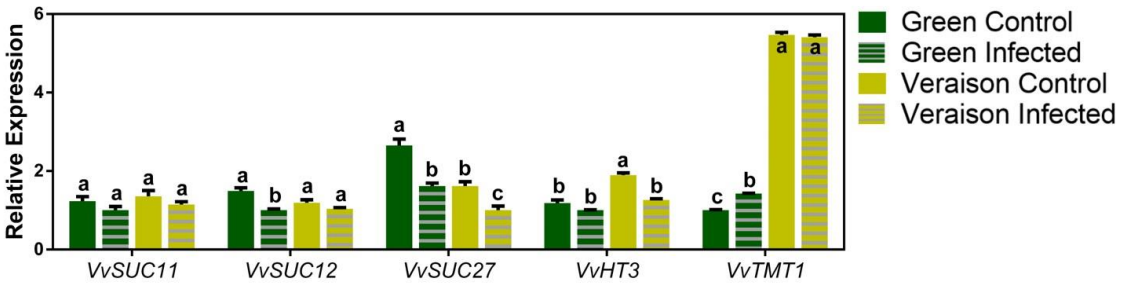

Figure 3.12 – Expression profile of prominent members of the *MFS* super-family genes, performed by real-time PCR in *E. necator*-infected (striped bars) and control (solid bars) berries, collected at two different developmental stages (green and veraison). Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample with the lowest expression level, which was set to 1. For each of the three biological replicates, after RNA extractions and cDNA synthesis, an independent qPCR analysis was performed with internal triplicates. Letters indicate statistical significance (one-way ANOVA with Tukey's post-test).

3.4 - Response of key grape berry *VvSWEET*s to drought, kaolin and berry dehydration

As referred to in the Introduction, we took advantage from the studies conducted by Conde et al. (2015), aimed to evaluate the effect of different irrigation treatments on the grapevine to evaluate how different water-deficit stress conditions affect the expression of *VvSWEETs*. As referred to in Material and Methods section, these field studies were conducted in a commercial vineyard in Estremoz and the vines of the cv. Tempranillo were subjected to different irrigation treatments (FI – Full irrigation and NI – Non irrigation). Grape berries were sampled at three different developmental stages (green, veraison and mature) and immediately frozen in liquid N_2 and cordially sent to Universidade do Minho to evaluate *VvSWEET* expression by qPCR (Figure 3.13).

Overall, during grape berry development, *VvSWEETs* gene expression followed a trend similar to the one reported in figure 3.8. Figure 3.13 shows that, in general, *VvSWEET* genes were down-regulated in response to drought stress at different developmental stages but at the green stage the down-regulation of *VvSWEETs* was more consistent. During this stage *VvSWEET1*, *VvSWEET4*, *VvSWEET7*, *VvSWEET17a* and *VvSWEET17d* were down-regulated in grapes from drought-affected grapevines. *VvSWEET2b* gene expression was also down-regulated at the veraison stage and *VvSWEET15* at the mature stage. Results showed that only *VvSWEET10 and VvSWEET11* were up-regulated in response to water scarcity at veraison and mature stages, respectively.

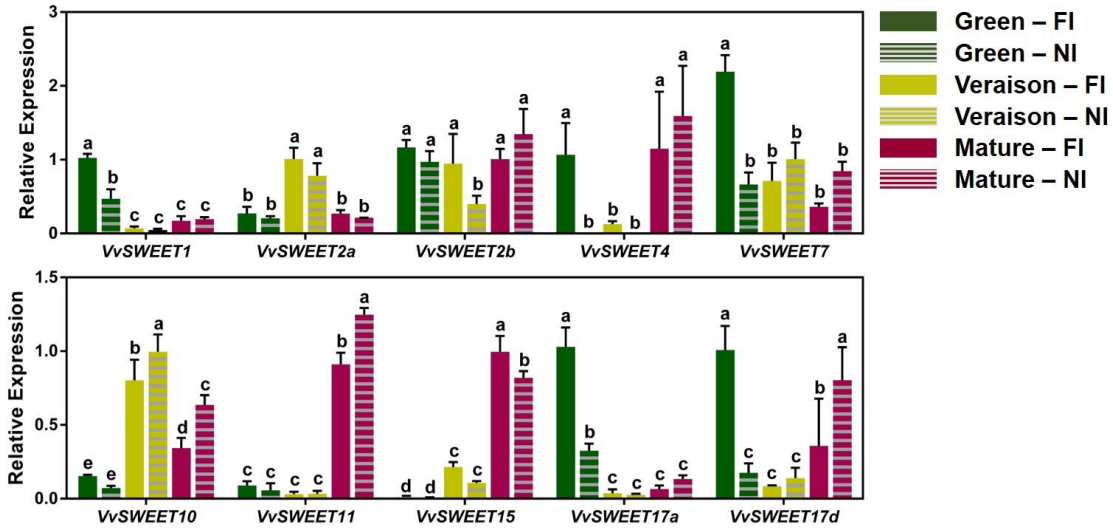

Figure 3.13 - Expression profile of *VvSWEET* genes performed by real-time qPCR in grape berries sampled at three different developmental stages (green, version and mature) from grapevines (cv. Tempranillo) under different irrigation treatments, FI (Full irrigation – solid bars) and NI (Non irrigation – striped bars). Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample with the lowest expression level, which was set to 1. For each of the three biological replicates, after RNA extractions and cDNA synthesis, an independent qPCR analysis was performed with internal triplicates. Letters indicate statistical significance (one-way ANOVA with Tukey's post-test).

As mentioned in section 1.4 – research objectives - in the context of the ongoing field studies performed by our group in collaboration with the group headed by Prof. José Moutinho Pereira from Universidade de Trás-os-Montes e Alto Douro, aimed to evaluate the effect of chemically inert mineral Kaolin in the protection of grapevine from extreme drought, high irradiance and high temperatures, we addressed the hypothesis that kaolin treatment affects the molecular mechanisms of sugar transport in mature leaves, in particular the expression of key *VvSWEET* transporters. As shown in figure 3.14 results were very consistent regarding the response of *VvSWEETs* to kaolin application, because all the key genes selected (*VvSWEET1*, *VvSWEET4* and *VvSWEET11*) were up-regulated in response to the application of this sunscreen. To address the hypothesis that sugar metabolism is changed in berries subjected to a dehydration process normally utilized to produce raisins and sweet and fortified wines (up to 11 days at 50 °C), we also evaluated the expression of these sugar transporters. This work was performed in the context of a broader project developed by our group aimed to study the modifications that postharvest dehydration induces in the primary metabolism of grape berries of cv. Sémillon (Conde et al., 2018b).

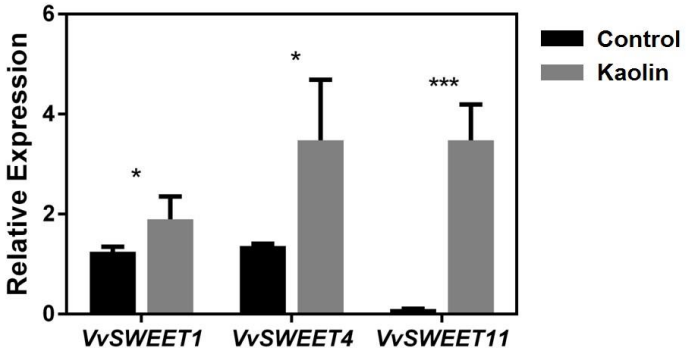

Figure 3.14 - Expression profile of *VvSWEET1*, *VvSWEET4* and *VvSWEET11* performed by real-time qPCR in grapevine leaves collected two weeks after application (2 WAA) from Kaolin-treated (■) and control (■) vines. Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample with the lowest expression level, which was set to 1. For all experimental conditions tested, three independent laboratorial analyses were performed on adequately composite grape berry samples. Values are the mean ± SD. Asterisks indicate statistical significance (one-way ANOVA with Tukey's post-test; *P<0.05; **P<0.01; ***P<0.001; **** P < 0.0001).

As can be seen in figure 3.15, *VvSWEET11* suffered a strong, somewhat unexpected, up-regulation (up to 200-fold) 5 days after incubating the bunches at 50 °C, while the expression of *VvSWEET15* increased 3-fold. Strikingly, after 11 days, when the berries are almost completely dehydrated the expression of *VvSWEET11* remained very high.

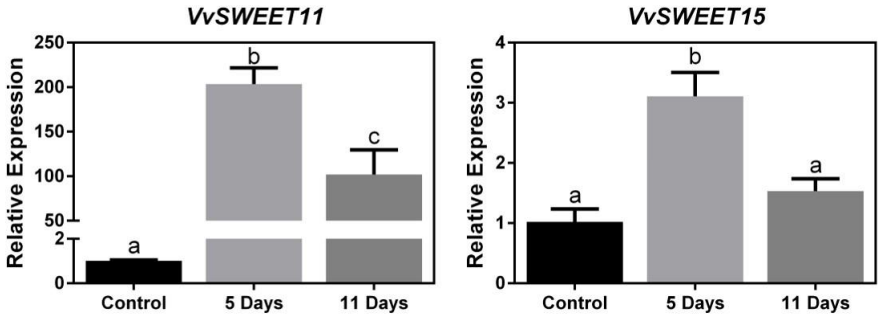

Figure 3.15 - Expression profile of *VvSWEET11* and *VvSWEET15* performed by real-time qPCR in grape berries subjected to 5 and 11 days of postharvest dehydration and without treatment (control). Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample with the lowest expression level, which was set to 1. For each of the three biological replicates, after RNA extractions and cDNA synthesis, an independent qPCR analysis was performed with internal triplicates. Values are the mean ± SD. Different letters denote statistical differences between columns (one-way ANOVA with Tukey's post-test)

3.5 - Sub-cellular localization and functional characterization of VvSWEET7 and VvSWEET15

VvSWEET7 (*Vitvi02g00181*) and *VvSWEET15* (*Vitvi01g01719*) were selected to study their function and sub-cellular localization, because, as previously shown, they are highly expressed in the grape berry and up-regulated in response to *Botrytis* and *E. necator* infection.

The phylogenetic tree depicted in figure 3.16 was constructed with VvSWEET7 (Figure 3.16a) and VvSWEET15 (Figure 3.16b) homologues from other plant species. It is evident that VvSWEET7 is phylogenetically closer to SlSWEET6, whereas VvSWEET15 is phylogenetically closer to AtSWEET15.

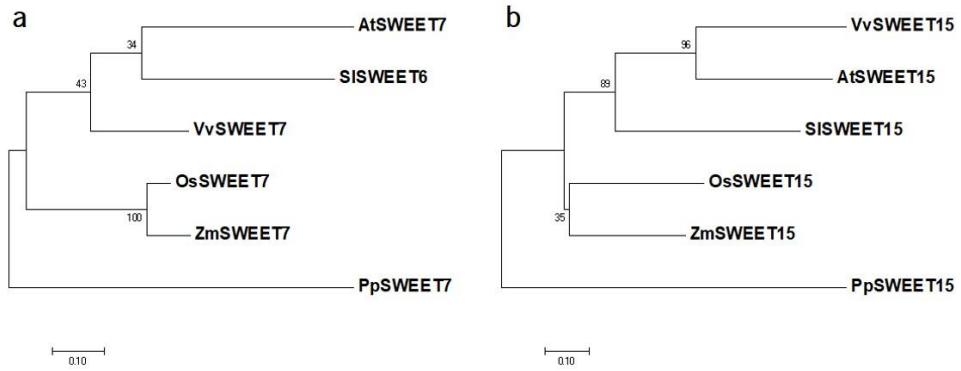

Figure 3.16 – Phylogenetic tree comparing SWEET7 (a) and SWEET15 (b) proteins from different plant species. Sequences from *V. vinifera* (Vv), *S. lycopersicum* (Sl), *A. thaliana* (At), *Oryza sativa* (Os), *Zea mays* (Zm) and *Physcomitrella patens* (Pp). Bootstrap values based on 100 replicates are indicated beside the nodes. Evolutionary analyses end tree drawn in MEGA7 and analysis performed with the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992).

To assess the sub-cellular localization of VvSWEET7 and VvSWEET15, the corresponding VvSWEET7-GFP and VvSWEET15-GFP fusion proteins were transiently expressed in *Nicotiana benthamiana* epidermal cells. Co-localization experiments with the fusion protein AtPIP2.1-RFP, an aquaporin targeted to the plasma membrane, revealed that both VvSWEET7 and VvSWEET15 localize to the plasma membrane (Figure 3.17).

To study the function of VvSWEET7 as a putative plasma membrane sugar transporter, the hxt-null yeast strain EBY-VW4000 was transformed with *pYES-DEST52*, containing the cloned *VvSWEET7* cDNA under the control of the galactose-inducible *GAL1* promoter. The first evidence for the involvement of a sugar transport system was provided from the studies of the P-type ATPase activity after the addition of different sugars to suspensions of VvSWEET7-transformed cells (Figure 3.18). As can be seen, a clear acidification signal was recorded after addition of glucose, fructose or sucrose to yeast cells harboring the construct *pYES-DEST52-VvSWEET7* that was not observed in suspensions of yeast cells transformed with the empty vector. The acidification signal after the addition of galactose was less evident. These results suggested that VvSWEET7 is capable to transport both mono- and disaccharides that, once inside the cells, are catabolized into ATP which activates the plasma membrane proton-pump.

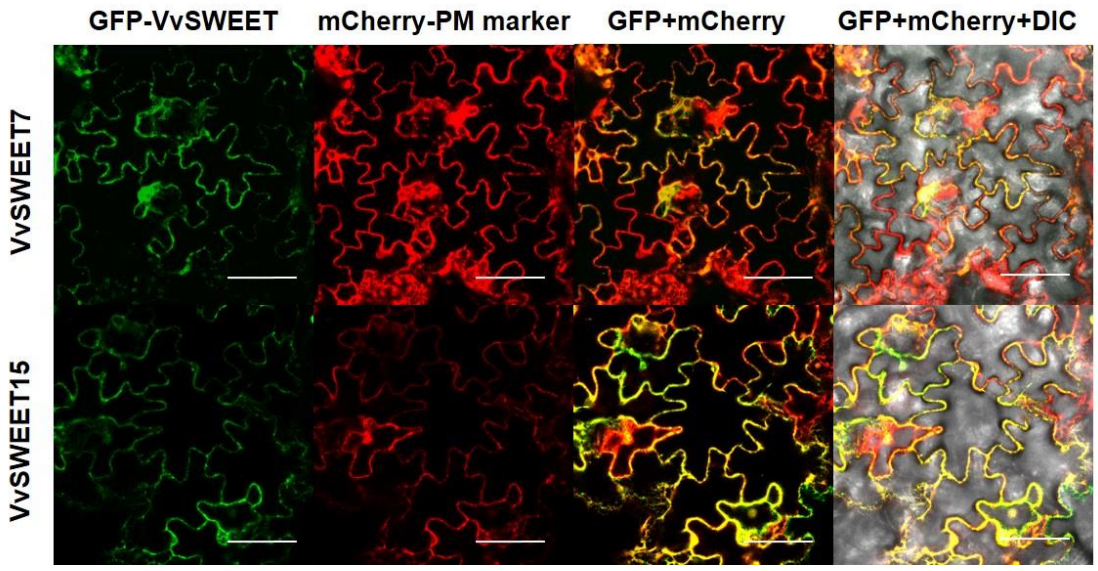

Figure 3.17 - Sub-cellular localization of VvSWEET7 and VvSWEET15 in tobacco leaves. AtPIP2;1-RFP was used as a plasma membrane marker (Nelson et al., 2007). Both GFP-VvSWEET7 and GFP-VvSWEET15 localize to the plasma membrane of leaf epidermis cells, as demonstrated by the fluorescence signal observed by confocal microscopy, co-localized with the positive control AtPIP2;1-mCherry. Bar = 100 µm.

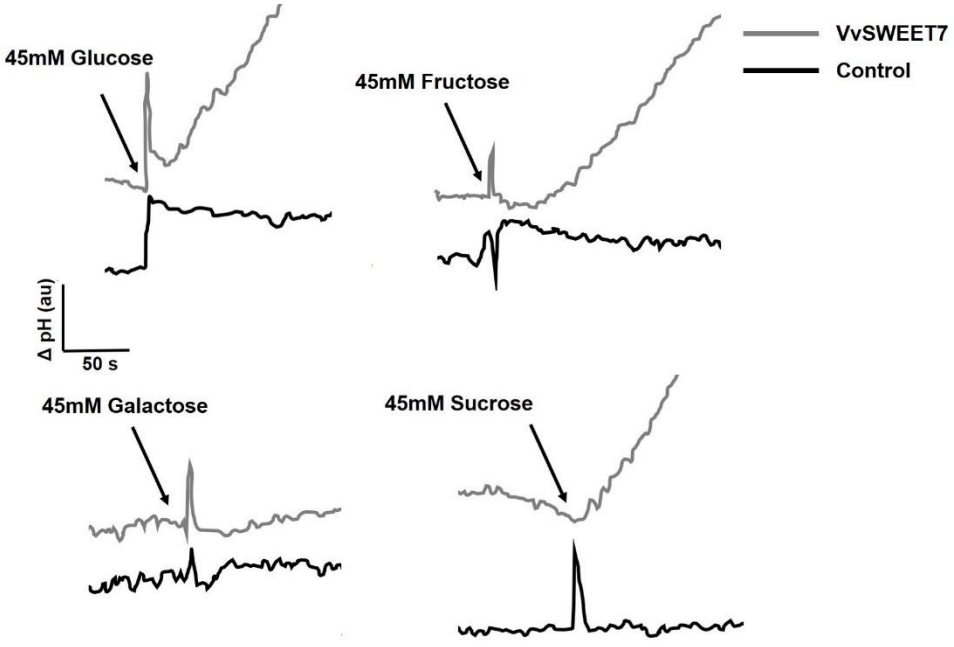

Figure 3.18 - Representative experiments of the activation of the plasma membrane H^+-ATPase in suspensions of VvSWEET7-expressing EBY.VW4000 yeast cells and controls (empty vector) induced by mono- and disaccharides. For each proton pumping activity analysis, four experimental repetitions were performed, each one consisting of an independent VvSWEET7-overexpressing and control yeast growth and a subsequent sugar-induced pH variation analysis. All illustrations are representative of 4 different replicates.

The uptake of radiolabeled substrates was also performed in yeast cells harboring the construct *pYES-DEST52-VvSWEET7* and in cells harboring the empty vector (control cells). Initial results showed that the uptake of 5.6 μM non-metabolizable glucose analog 3-*O*-methyl-alpha-D glucopyranoside in *pYES-DEST52-VvSWEET7* transformed cells was 0.02 nmol 3-*O*-methyl-D-[U-^{14}C]Glc mg D.W.$^{-1}$ min^{-1}, while in control cells a basal value was obtained (0.002 nmol 3-*O*-methyl-D-[U-^{14}C]Glc mg D.W.$^{-1}$ min^{-1}), clearly demonstrating that VvSWEET7 is a functional monosaccharide transporter.

As shown in figure 3.19, both the initial uptake rates of 7.5-50 mM D-[^{14}C]-glucose and 7.5-125 mM [^{14}C]-sucrose followed Michaelis-Menten kinetics, suggesting carrier-mediated transport for both substrates. The kinetic parameters were as follows: K_m, 15.42 mM glucose and V_{max}, 7.4 nmol glucose mg D.W.$^{-1}$ min^{-1} and K_m, 40.08 mM sucrose and V_{max} 15.12 nmol sucrose mg D.W.$^{-1}$ min^{-1} (Figure 3.19A and 3.19B). Moreover, the addition of 50 μM of the protonophore carbonyl cyanide m-chlorophenyl hydrazine (CCCP) did not inhibit the uptake of 25 mM D-[^{14}C]-glucose, at pH 5.0, suggesting the transport mechanism was not dependent on the proton gradient (Figure 3.19C).

To assess the substrate specificity of VvSWEET7, the uptake rate of 25 mM of D-[^{14}C]-glucose was determined in the presence of putative competitive inhibitors, such as other monosaccharides, disaccharides and polyols, at a concentration 20-fold higher the K_m value for D-[^{14}C]-glucose uptake (Figure 3.19D). Fructose inhibited radiolabeled glucose uptake by 79%, galactose by 56%, sucrose by 47%, mannitol by 29% and sorbitol by 38%. These results suggest that VvSWEET7 has a broad transport capacity, including for sugar-alcohols. Attempts were also made to assess the ability of EBY-VW4000 cells expressing VvSWEET15 to transport sugars (glucose, fructose and sucrose), but results were unsuccessful (data not shown), suggesting that VvSWEET15 may not have such a function.

Further studies were performed to assess the transport capacity of VvSWEET7. The glucose analog 3-*O*Methyl-D-Glucose (3-OMG) was used to study the accumulative capacity of the transport system because this sugar is not intracellularly phosphorylated by hexokinase and, consequently, cannot be further metabolized, thus accumulating inside the cells. Results showed that at low initial concentration ranges of 20-160 μM (Figure 3.20A) and 0.2-2 mM (Figure 3.20B) the transport was not saturable (followed first-order kinetics), supporting above results performed with D-[^{14}C]-glucose that VvSWEET7 mediates low-affinity transport. However, surprisingly, after 60 min of incubation (extracelular pH = 5.0), 3-OMG accumulated intracelullarly by 10-fold (initial 3-OMG concentration = 5.6 μM) and nearly 4-fold (initial 3-OMG cocentrations = 1 mM) (Figure 3.20C). These results were obtained assuming an intracellular volume = 130 μm^3, reported in the literature for this yeast strain (Bryan et al., 2010). Also remarkably, 50 μM of the protonophore carbonyl cyanide m-chlorophenyl hydrazine (CCCP), substantially inhibited 3-OMG accumulation (Figure 3.20D). Altogether, these results suggest that, at least in a low-substrate concentration range, this transport system may depend on the transmembrane proton-motive force. This assumption is supported by the observation that the external pH affected the initial velocities 5.6 μM 3-OMG uptake: the

uptake was higher at lower external pHs when the transmembrane electrochemical proton gradient is higher. The confirmation that VvSWEET7 can operate as an active transporter at low substrate concentration and as a facilitated diffusion at the millimolar range, and thus behaving as a dual-affinity transport system, would shift the way the scientific community looks to these transporters, but so far the results are still exploratory, and experimental artifacts should not be ruled out.

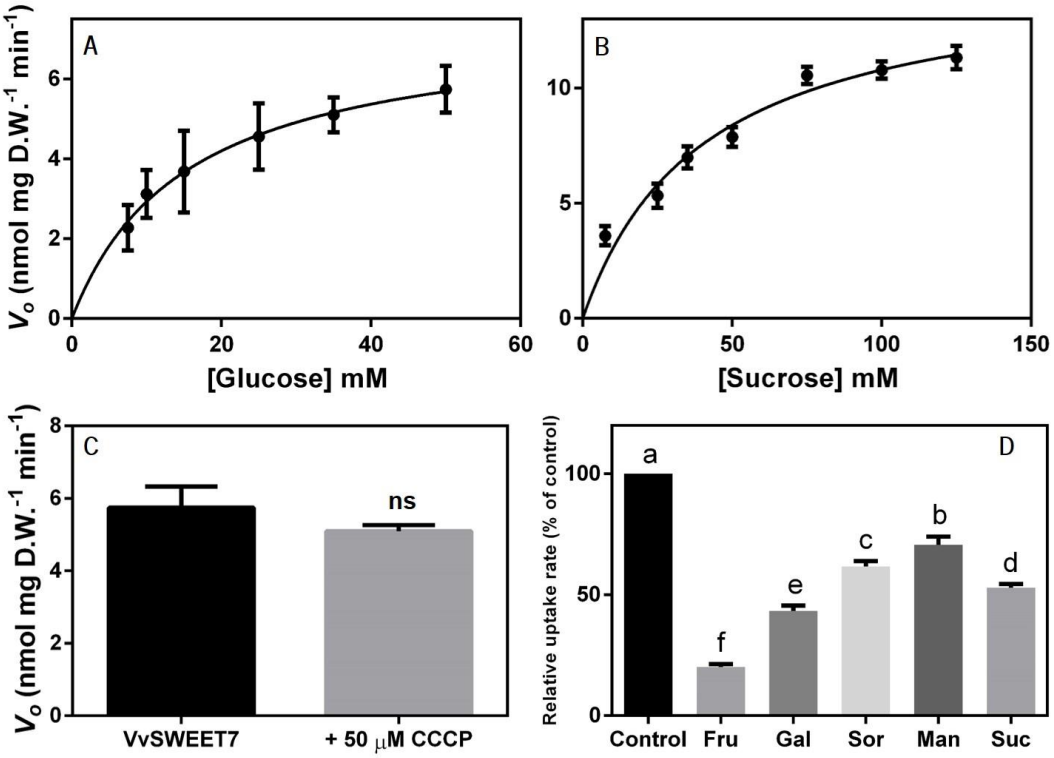

Figure 3.19 - Concentration dependence of the initial uptake rates of D-[^{14}C] glucose (A) and [^{14}C] sucrose (B) in VvSWEET7-expressing EBY.VW4000 cells at pH 5.0. (C) Effect of 50 μM of the protonophore CCCP (carbonyl cyanide m-chlorophenylhydrazone) on VvSWEET7-mediated uptake of 25 mM D-[^{14}C] glucose. (D) Competitive inhibition of VvSWEET7-mediated glucose uptake by 500 mM fructose, galactose, sorbitol, mannitol or sucrose. For each experimental condition, values are the mean ±SD of 3 independent experiments. Each experimental repetition was performed with internal triplicates. Letters indicate statistical significance and "ns" indicate non statistical significance (one-way ANOVA with Tukey's post-test).

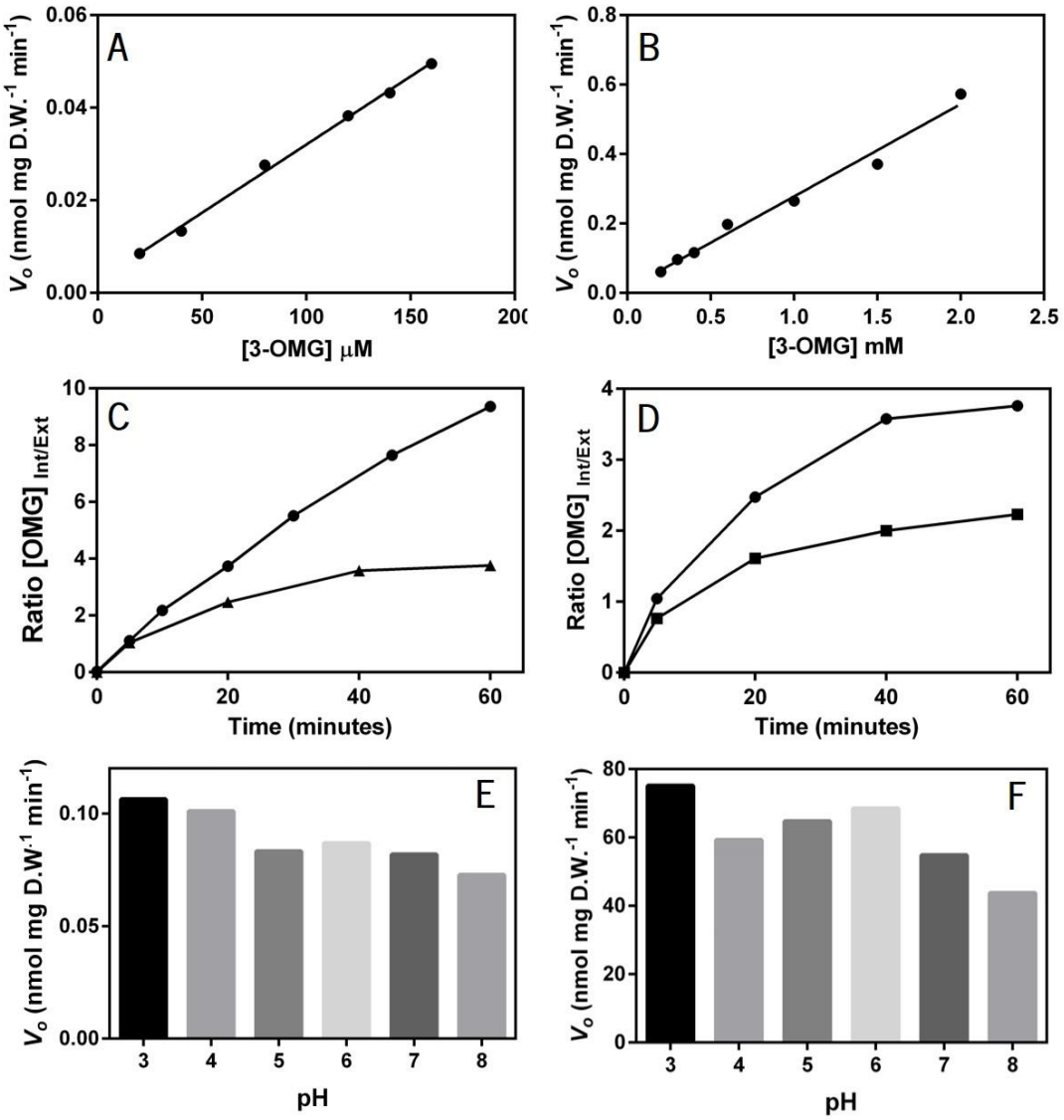

Figure 3.20 - Concentration dependence of the initial uptake rates of 3-*O*-methyl-D-[U-^{14}C]Glc at two distinct concentrations ranges [20-160 μM] (A) and [0.2-2 mM] (B) in VvSWEET7-expressing EBY.VW4000 cells at pH 5.0. (C) Accumulation of labeled 3-OMG, at pH 5.0, at the initial substrate concentration of (●) 5.6 μM and (▲) 1 mM. (D) Accumulation of radiolabeled 3-OMG at the initial extracellular concentration of 1 mM in the absence (●) or in the presence (■) of 50 μM of CCCP. Effect of the pH in the uptake rate of radiolabeled 3-OMG at initial concentration of 5.6 μM (E) and 50 mM (F). All values are one independent uptake experiment.

3.6 - Grapevine Early-Response to Dehydration 6-like (ERD6l) family *in silico* analysis

The grapevine Early-Reponse to Dehydration 6-like (ERD6l) family was previously identified by Afoufa-Bastien et al. (2010). Twenty-two ORFs showing strongest similarity with the 19 AtERD6-like proteins were identified, but various ORFs corresponded to partial sequences in which either the beginning or the end of the protein were not clearly identified. In the present study, using as queries the protein sequences of the

Arabidopsis ERD6l family, BLAST searches were performed against the new grapevine genome annotation, VCost.v3 (Canaguier et al., 2017), the GENOSCOPE 12x annotation and the NCBI database. Only genes which encoded to a protein with 12 transmembrane domains were considered (Annex figure 2). Moreover, correct gene structure was manually assessed with confirmation of exon expression in several RNAseq experiments available in the NCBI database (Annex Table 3).

A total of 18 *VvERD6l* genes with confirmed Pfam conserved domains (*Sugar_tr PF00083.24 and MFS_1 PF07690.16*) were identified (Table 3.4), all encoding for a 12 TMD protein (Annex figure 2). The *VvERD6l* genes are distributed only between 5 chromossomes, the majority being located at the chromossome 14. In this chromossome, two tandem groups exist, being one formed by *VvERD6l1*, *VvERD6l2*, *VvERD6l3*, *VvERD6l4*, *VvERD6l5*, *VvERD6l6*, *VvERD6l10*, *VvERD6l15* and *VvERD6l16*, and another by *VvERD6l7*, *VvERD6l8* and *VvERD6l9* . In chromossome 5, *VvERD6l11* and *VvERD6l12* are also located in tandem. The ORFs of the *VvERD6l* genes range from 1317 bp to 1809 bp in length and encode polypeptides that range from 439 aa to 602 aa in length. The predicted subcellular localization of the VvERD6l proteins is mostly to the plasma membrane, however the subcellular localization of two of the members to the tonoplast (VvERD6l2 and VvERDl613). Regarding gene stucture, *VvERD6l* genes vary between 16 and 20 exons, however most of the members assemble in a 18 exon/17 intron configuration.

This family shows a high identity percentage between its members, varying between 92% (VvERD6l11 and VvERD6l12) and 37% (VvERD6l7 and VvERD6l17) (Table 3.5). Phylogenetic analysis shows that this family clearly separates from the other depicted sugar transporter members (Figure 3.21) and separate in four different clades: clade I – VvERD6l1; VvERD6l7, clade II – VvERD6l8; VvERD6l10, clade III VvERD6l11 to VvERD6l16 and clade IV – VvERD6l17 and VvERD6l18. Also, 12 loci form 6 sister pairs. Moreover, a phylogenetic tree was constructed using the ERD6l members from *Arabidopsis*, tomato and grapevine (Figure 3.22). The separation of the four previously identified groups is evident. Clades II, III and IV includes transporters from all the species and are highly conserved between species. Nonetheless, clade IV members subdivide in sub-groups of members from only one species.

Moreover, to analyze the promoter region of *VvERD6l* genes, a search in the PLACE database (Higo et al., 1999) was performed (Table 3.6 and annex table 2). We identified a 2 kb promoter region for each of *VvERD6l* member except for *VvERD6l5*, *VvERD6l9* and *VvERD6l10*, whose promoter sequence is shorter (883 bp, 628 bp and 820 bp, correspondingly) due to the presence of another ORF.

VvERD6l gene promoter sequences revealed several sugar-responsive related cis-acting elements, including PYRIMIDINEBOXOSRAMY1A, TATCCAOSAMY and TATCCAYMOTIFOSRAMY3D. The promoter region of some members is richer in this class of cis-acting elements than others, ranging from 3 (*VvERD6l6*) to 20 (*VvERD6l11*). The most abundant element is PYRIMIDINEBOXOSRAMY1A, appearing in the promoter region of all *VvERD6l* genes. It is also the most abundant cis-acting element in the promoter region of

VvERD6l13. Two sucrose-responsive elements, SURE1STPAT21 and SURE2STPAT21, are scarcely present in few promoter sequences, nevertheless they are both present in the promoter region of *VvERD6l13*.

Furthermore, *VvERD6l* promoters are also enriched in pathogenesis related cis-acting elements. GT1GMSCAM4 and WRKY71OS are the more abundant pathogen-related cis-acting elements. GT1GMSCAM4, which also is responsive to salt stress, appears in all *VvERD6l* members, particularly in *VvERD6l4* promoter region. The promoter sequence of *VvERD6l16* is richer in pathogen related cis-acting elements than *VvERD6l6.*

Several abiotic-related elements were also identified. The most abundant is MYCCONSENSUSAT, which is responsive to drought and cold stresses, appearing 224 times in all the *VvERD6l* promoters. The salt stress responsive element GT1GMSCAM4 was also detected multiple times. Other drought responsive elements were also identified, as DRE2COREZMRAB17 and DRECRTCOREAT, however in lower numbers and in fewer gene promoter regions. Furthermore, cis-elements which have been found in the promoter region of early-responsive to dehydration genes are also present in the promoter of *VvERD6l* members, such as ABRELATERD1 and ACGTATERD1, however they are in the promoter sequence of only some genes, such as *VvERD6l6* and *VvERD6l8*. The genes with more cis-acting elements responsive to abiotic stress are *VvERD6l3* and *VvERD6l7*.

Additionally, several cis-acting elements responsive to different hormones, such as gibberellins, ABA or auxins were also identified. The most abundant element is CATATGGMSAUR, which is responsive to auxins. This sequence was detected in all promoter regions, being more abundant in *VvERD6l3* and *VvERD6l4*. ABA-responsive elements are also abundant, like MYB1AT. GARE2OSREP1 and RYREPEATVFLEB4, which are ABA and GA-responsive elements, appear in the promoter sequence of a single gene. Among all the genes analyzed, *VvERD6l4* is the one whose promoter region is richer in hormone-responsive elements.

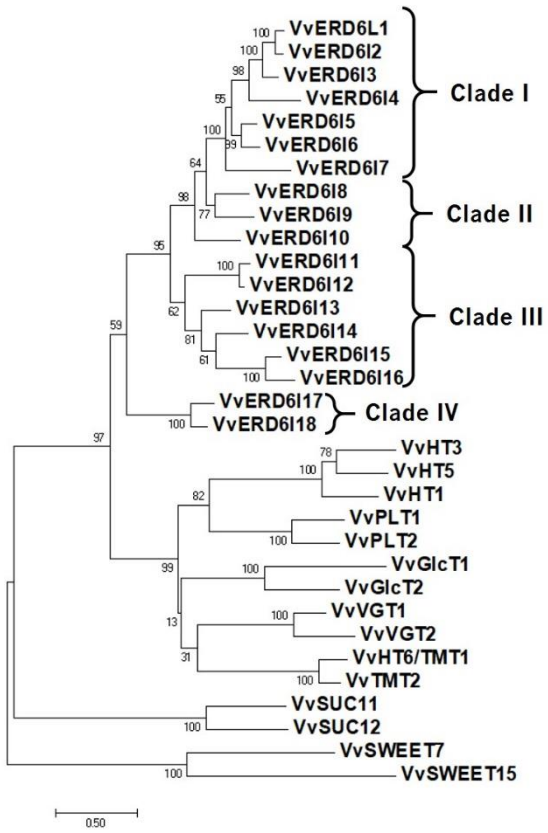

Figure 3.21 - Phylogenetic analysis of grapevine ERD6l proteins. The different clades are highlighted by brackets. Bootstrap values based on 100 replicates are indicated beside the nodes. Evolutionary analyses end tree drawn in MEGA7 and analysis performed with the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992).

Table 3.4 – Grapevine *ERD6-like* family. Gene name, GenBank ID, Genoscope 12X ID, VCost.v3 ID, chromosome, strand and chromosome position, open reading frame base pairs, exon number, coding sequence base pairs, protein amino acids, trans-membrane domains, predicted sub-cellular localization, theoretical protein molecular weight (KDa), theoretical isoelectric point (PI).

Gene Name	GenBank ID	GENOSCOPE 12x ID	VCost_v3 ID	Chr	Position	ORF (bp)	Exon	CDS (bp)	AA	TMD	LOC	MW KDa	PI	
VvERD6-like 1	LOC104881446	GSVIVG01022029001	Vitvi14g00310	14	(-) 4110119..4114550	4432	17	1515	504	12	PM	54,78	6,03	a
VvERD6-like 2	LOC100247058	GSVIVG01022026001	Vitvi14g00311	14	(-) 4155593..4159582	3990	17	1452	483	12	VC	52,62	5,71	
VvERD6-like 3	LOC100267582	GSVIVG01022030001	Vitvi14g02582	14	(-) 4098676..4102575	3900	18	1473	490	12	PM	52,50	5,47	
VvERD6-like 4	LOC100241924	GSVIVG01022025001	Vitvi14g00312	14	(-) 4161656..4166339	4684	16	1257	418	12	PM	46,36	5,69	b
VvERD6-like 5	LOC100245278	GSVIVG01022032001	Vitvi14g02580	14	(-) 4086918..4090600	3683	18	1425	474	12	PM	51,31	5,1	
VvERD6-like 6	LOC104881445	GSVIVG01022031001	Vitvi14g02581	14	(-) 4091477..4096205	4729	18	1431	476	12	PM	51,23	5,45	
VvERD6-like 7	LOC100854088	GSVIVG01022033001	Vitvi14g00308	14	(-) 4064867..4073298	7989	18	1443	481	12	PM	52,68	6,4	a
VvERD6-like 8	LOC100265873	GSVIVG01022034001	Vitvi14g00305	14	(-) 4039419..4044725	5307	18	1467	488	12	PM	52,12	5,19	
VvERD6-like 9	LOC100253784	GSVIVG01022035001	Vitvi14g00304	14	(-) 4034741..4038791	4051	18	1467	488	12	PM	52,05	5,45	
VvERD6-like 10	LOC104881447	GSVIVG01022024001	Vitvi14g00314	14	(-) 4170827..4175849	4948	18	1476	491	12	PM	52,79	5,1	
VvERD6-like 11	LOC100263082	GSVIVG01017845001	Vitvi05g01870	5	(+) 3978962..3984862	5769	18	1521	506	12	PM	55,69	6,27	b
VvERD6-like 12	LOC100240820	GSVIVG01017844001	Vitvi05g01869	5	(+) 3968917..3972241	3325	18	1570	488	12	PM	53,51	6,2	
VvERD6-like 13	LOC100261307	GSVIVG01017836001	Vitvi05g00377	5	(+) 3900557..3903993	2958	18	1317	439	12	VC	47,15	8,54	
VvERD6-like 14	LOC100263109	GSVIVG01011047001	Vitvi07g00207	7	(-) 2358570..2363154	4585	18	1473	490	12	PM	52,65	5,48	
VvERD6-like 15	LOC100264207	GSVIVG01022022001	Vitvi14g02586	14	(-) 4185846..4190504	4659	18	1416	471	12	PM	50,63	6,89	c
VvERD6-like 16	LOC104881444	GSVIVT01022023001	Vitvi14g02585	14	(-) 4176644..4185717	9074	20	1809	602	12	PM	65,52	7,07	d
VvERD6-like 17	LOC100263259	GSVIVG01018949001	Vitvi04g01302	4	(-) 18797709..18804373	6665	18	1461	486	12	PM	52,61	8,34	
VvERD6-like 18	LOC100266019	GSVIVG01009719001	Vitvi18g00970	18	(-) 10757270..10762351	5082	18	1461	486	12	PM	52,8	9,02	

a-GenBank correct annotation; b-Vcost_v3 correct annotation; c-Manually curated sequence; d-Genoscope correct annotation.

Table 3.5 – Protein identity of grapevine ERD6-like proteins. Performed in http://imed.med.ucm.es/Tools/sias.html.

	ERD6l1-	ERD6l2	ERD6l3	ERD6l4	ERD6l5	ERD6l6	ERD6l7	ERD6l8	ERD6l9	ERD6l10	ERD6l11	ERD6l12	ERD6l13	ERD6l14	ERD6l15	ERD6l16	ERD6l17	ERD6l18
ERD6l1	100%	90.26%	80.61%	65.78%	67.51%	68.69%	54.67%	55.53%	54.91%	54.17%	45.63%	47.54%	52.84%	48.16%	45.85%	40.47%	40.32%	41.76%
ERD6l2		100%	80.33%	63.39%	66.66%	67.22%	54.88%	56.31%	55.69%	55.07%	45.96%	46.37%	51.7%	48.03%	46.28%	42.65%	40.57%	41.82%
ERD6l3			100%	66.26%	69.62%	68.69%	55.92%	58.4%	57.37%	55.71%	48.57%	49.38%	53.53%	50.81%	49.46%	44.48%	42.59%	44.44%
ERD6l4				100%	60.52%	60.04%	50.23%	52.39%	52.63%	52.39%	43.77%	45.21%	45.21%	45.93%	43.77%	41.38%	40.66%	39.71%
ERD6l5					100%	84.17%	60.97%	64.55%	64.13%	61.6%	50%	50%	54.44%	53.16%	49.89%	46.41%	42.19%	43.45%
ERD6l6						100%	60.08%	63.86%	63.02%	60.29%	51.26%	50.84%	54.66%	51.68%	49.89%	46%	40.96%	42.85%
ERD6l7							100%	54.46%	53.63%	50.93%	43.86%	43.65%	46.24%	42.2%	43.52%	39.08%	37.21%	38.25%
ERD6l8								100%	68.03%	61.06%	50.4%	50%	60.82%	52.86%	56.26%	51.63%	42.38%	42.59%
ERD6l9									100%	61.06%	47.74%	47.95%	57.63%	52.25%	54.56%	48.77%	43.2%	43%
ERD6l10										100%	50.5%	51.43%	56.94%	52.44%	54.35%	48.06%	41.56%	42.59%
ERD6l11											100%	92.62%	60.13%	55.51%	49.68%	45.25%	40.74%	40.12%
ERD6l12												100%	61.95%	57.58%	50.95%	47.33%	41.56%	40.74%
ERD6l13													100%	70.61%	61.73%	60.59%	46.46%	45.55%
ERD6l14														100%	63.26%	58.97%	45.47%	43.82%
ERD6l15															100%	82.59%	46.7%	44.79%
ERD6l16																100%	41.35%	40.53%
ERD6l17																	100%	80.65%
ERD6l18																		100%

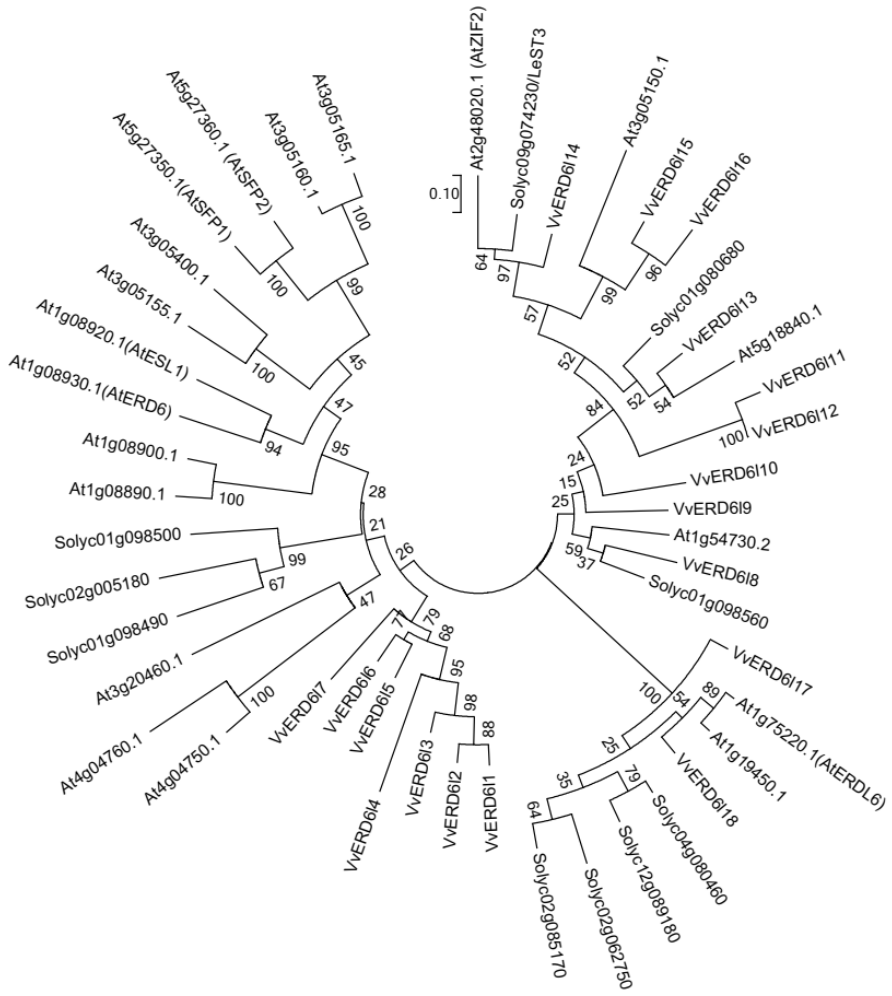

Figure 3.22 - Phylogenetic analysis of *V. vinifera*, *S. lycopersicum* and *A. thaliana* ERD6l proteins. Bootstrap values based on 100 replicates are indicated beside the nodes. Evolutionary analyses end tree drawn in MEGA7 and analysis performed with the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992).

Table 3.6 – Representative cis-acting elements identified in the promoter sequence of *VvERD6l* genes via PLACE (Higo et al., 1999). Cis-element name, sequence motifs, signaling pathway and number of copies on the promoter are shown. Asterisks indicate shorter promoter regions.

Cis-acting elements	Sequence	Response	ERD6l1	ERD6l2	ERD6l3	ERD6l4	ERD6l5*	ERD6l6	ERD6l7	ERD6l8	ERD6l9*	ERD6l10*	ERD6l11	ERD6l12	ERD6l13	ERD6l14	ERD6l15	ERD6l16	ERD6l17	ERD6l18
CATATGGMSAUR	CATATG	Auxin	1	6	13	15	1	7	6	7	2	8	4	5	7	4	7	7	6	7
GAREAT	TAACAAR	Hormone regulation	3	2	0	2	1	2	2	4	0	0	2	1	1	1	1	0	3	2
GT1GMSCAM4	GAAAAA	Pathogen defense; salt stress	1	6	13	15	1	7	6	7	2	8	4	5	7	4	7	7	6	7
MYBCORE	CNGTTR	Drought	2	3	6	3	0	2	8	0	4	0	3	4	1	3	2	6	0	6
MYBGAHV	TAACAAA	Gibberellin; sugar repression	1	2	0	2	1	1	2	3	0	0	2	0	1	0	1	0	1	2
MYCCONSENSUSAT	CANNTG	Drought; cold; ABA	12	8	12	12	16	4	20	6	8	14	8	14	14	18	16	16	12	14
OSE2ROOTNODULE	CTCTT	Nodule; root	6	6	8	1	2	12	9	8	0	4	3	4	4	5	3	2	3	5
PYRIMIDINEBOXOSRAMY1A	CCTTTT	Gibberellin; sugar repression	4	2	5	3	1	2	2	5	2	3	2	3	7	1	2	3	6	5
SBOXATRBCS	CACCTCCA	Sugar-responsive; ABA	0	0	0	0	0	0	2	0	0	0	0	0	0	0	1	0	**0**	0
SEBFCONSSTPR10A	YTGTCWC	Pathogenesis-related	2	1	2	2	0	2	2	0	0	2	0	3	0	1	0	2	0	2
SREATMSD	TTATCC	Sugar repression	1	1	1	0	1	0	0	2	0	0	1	0	3	0	1	2	2	0
SURE2STPAT21	AATACTAAT	Sucrose responsive	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
TATCCAOSAMY	TATCCA	Sugar; Hormone regulation	1	1	1	2	0	0	2	0	1	0	4	0	1	0	0	1	1	0
WBOXATNPR1	TTGAC	Pathogen defense	5	3	3	1	0	0	3	1	3	2	5	6	3	4	3	7	4	4
WBOXHVISO1	TGACT	Sugar-responsive	8	1	2	2	0	0	5	2	2	2	5	5	3	6	3	7	0	1
WRKY71OS	TGAC	Pathogen defense; gibberellin	16	7	12	10	1	1	15	7	6	8	18	11	9	13	11	17	7	9

3.7 - Sub-cellular localization and functional characterization of VvERD6l13

The analysis of the expression profile of *VvERD6l* members in the microarray database produced by Agudelo-Romero and co-workers (2015), led us to select for further studies the putative grapevine sugar transporter *VvERD6l13* because it was the most up-regulated gene of this family in response to *Botrytis* infection.

To determine the sub-cellular localization of VvERD6l13, the corresponding VvERD6l13-GFP fusion protein was transiently expressed in *N. benthamiana* epidermal cells. Co-localization studies with the fusion protein AtPIP2.1-RFP, an aquaporin targeted to the plasma membrane, revealed that VvERD6l13 localize in the plasma membrane, contrarily to its predicted sub-cellular localization (Figure 3.23).

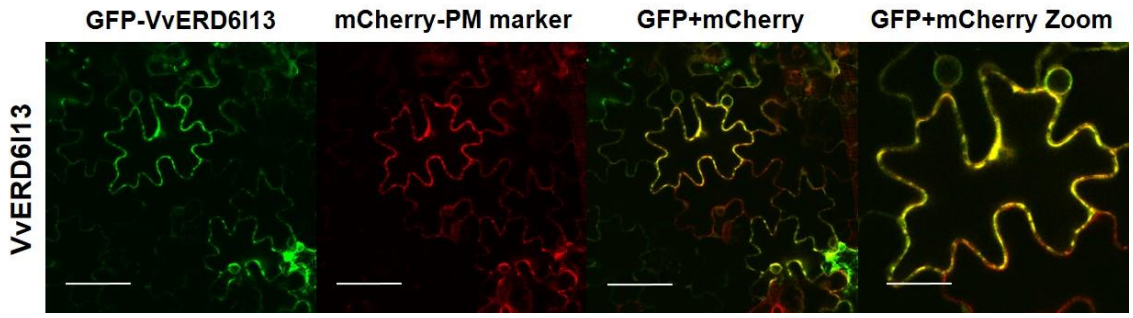

Figure 3.23 - Sub-cellular localization of VvERD6l13 in tobacco leaves. AtPIP2;1 was used as a plasma membrane marker (Nelson et al., 2007). GFP-VvERD6l13 localize to the plasma membrane of leaf epidermis cells, as demonstrated by the fluorescence signal observed by confocal microscopy, co-localized with the positive control AtPIP2;1-mCherry. Bar = 100 µm / 200 µm in the zoom in box.

To study the function of VvERD6l13 as a putative plasma membrane sugar transporter, the *hxt-null* yeast strain EBY-VW4000 was transformed with *pYES-DEST52*, containing the cloned *VvERD6l13* cDNA under the control of the galactose-inducible *GAL1* promoter. The first evidence for the involvement of a sugar transport system came from uptake studies of 50 mM of radiolabeled sucrose (Figure 3.24A). Similar experiments with radiolabeled glucose and fructose showed no differences between transformed cells and control cells, thus suggesting that VvERD6l13 is a disaccharide transporter.

The initial uptake rates of 7.5-50 mM [^{14}C] sucrose by VvERD6l13-expressing EBY.VW4000 cells were evaluated at pH 5.0 to estimate the kinetic parameters of VvERD6l13 (Figure 3.24B). Results showed a Michaelis-Menten kinetics, supporting carrier-mediated transport, with a K_m = 33.28 mM sucrose and V_{max} = 0.85 nmol sucrose mg D.W.$^{-1}$ min^{-1}.

Furthermore, the addition of 50 µM of CCCP substantially inhibited the uptake of 50 mM [^{14}C]-sucrose, at a pH 5.0, suggesting that the transport mechanism is dependent on the H^+ gradient (Figure 3.24C), and, therefore, a secondary active symport mechanism is involved. In agreement, when the

transport experiments were performed at lower external pH (pH=3) (thus, at a higher transmembrane proton gradient) the initial uptake rates of 50 mM [^{14}C] sucrose were higher than at pH = 5 (Figure 3.24D).

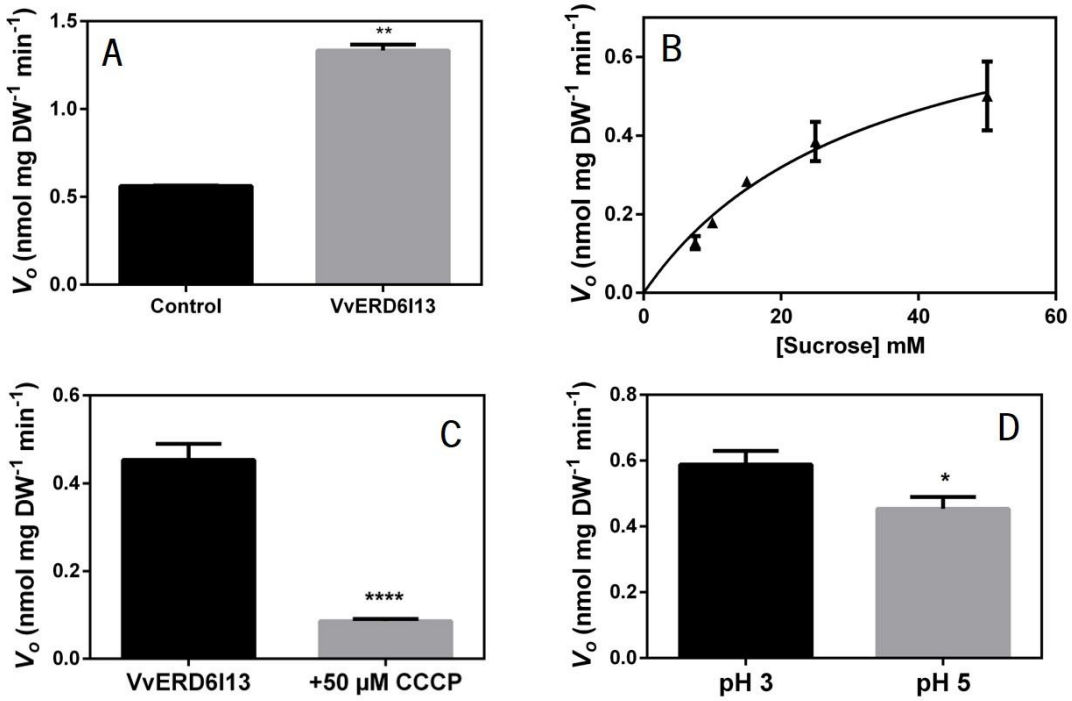

Figure 3.24 – (A) Initial uptake rates of 50 mM of [^{14}C] sucrose in pYES-DEST52-empty (control) and VvERD6I13-expressing EBY.VW4000 cells at pH 5.0. (B) Concentration dependence of the initial uptake rates of [^{14}C] sucrose in VvSWEET7-expressing EBY.VW4000 cells at pH 5.0. (C) Effect of 50 µM of the protonophore CCCP (carbonyl cyanide m-chlorophenylhydrazone) on VvERD6I13-mediated uptake of 50 mM [^{14}C] sucrose. (D) Effect of the pH in the uptake rate of 50 mM [^{14}C] sucrose. For every experimental condition, all values are the mean ±SD of 3 independent experimental. Also, each experimental repetition was performed with internal triplicates. Asterisks indicate statistical significance (one-way ANOVA with Tukey's post-test; *P<0.05; **P<0.01; ***P<0.001; **** P < 0.0001).

3.8 - Effect of the infection by *Botrytis cinerea* and *Erysiphe necator* on the expression of *VvERD6I13* and tissue-specific expression

To study the organ-specific expression of the sucrose transporter *VvERD6I13*, total RNA was isolated from cv. Vinhão leaves, canes, roots, flowers and grape berries at three developmental stages (green, veraison and mature). As shown in figure 3.25, *VvERD6I13* transcripts were detected in all sampled tissues, but were more abudant in the roots and leaves. In this cultivar, *VvERD6I13* gene expression did not change significantly during berry development, contrarily to the results obtained in cv. Trincadeira (see below).

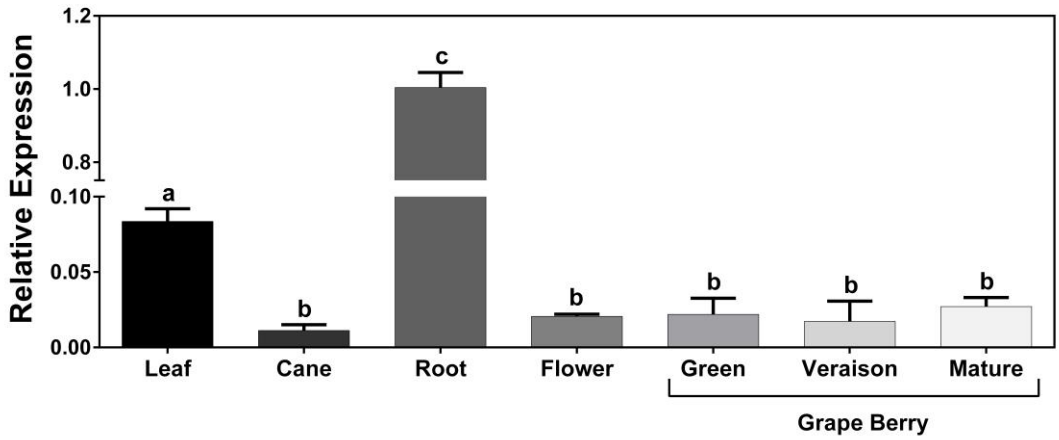

Figure 3.25 – Expression profile of *VvERD6l13*, performed by real-time PCR in different tissues of the grapevine cv. Vinhão. Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample with the lowest expression level, which was set to 1. For each of the three biological replicates, after RNA extractions and cDNA synthesis, an independent qPCR analysis was performed with internal triplicates. Different letters indicate statistical significance between columns (one-way ANOVA with Tukey's post-test).

In the context of the work in collaboration previously reported (section 3.3), *VvERD6l13* expression was studied in berries of cv. Trincadeira in response to *Botrytis*-infection (Figure 3.26). *VvERD6l13* expression was detected in all developmental stages mainly at maturation, when the steady-state thanscript levels were mostly affected (up-regulated) by *Botrytis* infection.

The expression profile of *VvERD6l13* was also studied in grape berries infected with the causal agent of powdery mildew, *E. necator* (Figure 3.26). Grape berries with strong visual signs of naturally occurring *E. necator* infection were sampled at green (EL32) and veraison (EL35) stages. In agreement with the results obtained in cv. Trincadeira, the expression of *VvERD6l13* increased from green to veraison stages and was up-regulated in response to *E. necator* infection.

Furthermore, the effect of *B. cinerea* on the expression profile of *VvERD6l13* was studied using CSB suspension cultured cells. Using the same procedure as reported in the section 3.2, cells were elicited with a mycelium extract of *B. cinerea* during 48 h. As seen in figure 3.27 *VvERD6l13* was up-regulated by 4-fold in response to *Botrytis* elicitation.

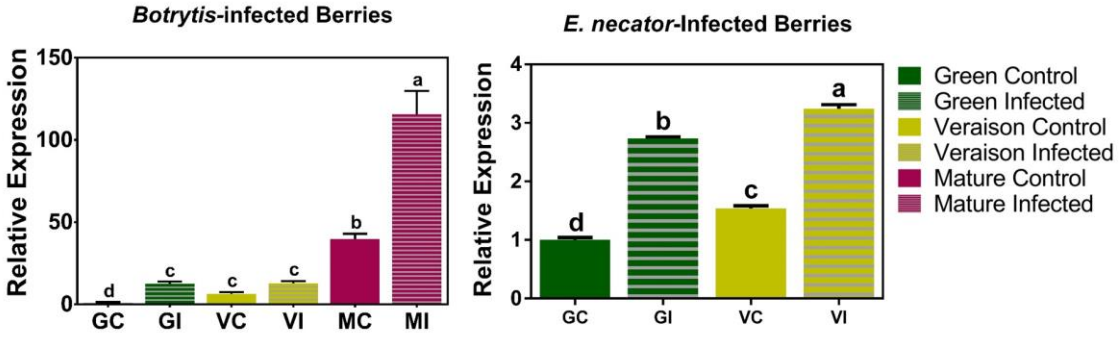

Figure 3.26 - Expression profile of *VvERD6l13*, performed by real-time qPCR in *Botrytis*-infected (striped bars) and control (solid bars) berries, collected at three different developmental stages (green, veraison and mature). Gene expression of *VvERD6l13* was also studied in *E. necator*-infected (striped bars) and control (solid bars) berries at two different developmental stages (green and veraison). Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample with the lowest expression level, which was set to 1. For each of the three biological replicates, after RNA extractions and cDNA synthesis, an independent qPCR analysis was performed with internal triplicates. Different letters indicate statistical significance between columns (one-way ANOVA with Tukey's post-test).

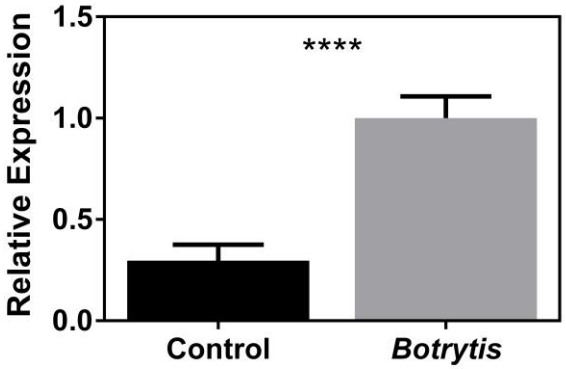

Figure 3.27 – Expression profile of *VvERD6l13*, performed by real-time PCR in CSB suspension cultured cell, sampled at the middle exponential phase and elicited 48 hours with *Botrytis* mycelia (■), or mock conditions (■). Relative expression for each gene was calculated by the Bio-Rad® CFX Manager 2.0 Software and was determined against the sample with the lowest expression level, which was set to 1. For all experimental conditions tested, 3 independent analyses were performed on adequately composite CSB suspension cultured cell samples. Values are the mean ± SD. Asterisks indicate statistical significance (one-way ANOVA with Tukey's post-test; *P<0.05; **P<0.01; ***P<0.001; **** P < 0.0001).

Chapter 4
Discussion

4.1. The way MSTs, SUCs and VvSWEETs cooperate in sugar transport is still puzzling

While in the majority of plants, the *SWEET* family comprises 20 members (Eom et al., 2015), we identified 17 members in the new versions of the grapevine genome assembly and annotation (VCost.v3) (Canaguier et al., 2017), in line with previous studies with the 12X.v0 version of the grapevine reference genome (Lecourieux et al., 2014; Chong et al., 2014). Our results showed that most *VvSWEET* genes assemble in a 6 exons/5 introns configuration, as previously observed in other plant species (Patil et al., 2015). Other configurations have been identified, as in the tomato SWEET family where the exon number ranges between eight and five (Feng et al., 2015). We found that *VvSWEET* genes are uniformly distributed in the chromosomes and that only *VvSWEET17b* and *VvSWEET17c* are in tandem, but other sister pairs were also found, such as *VvSWEET5a* and *VvSWEET5b*, which suggests the occurrence of gene tandem duplication. Tandem duplication has been also observed in *SWEET* genes of soybean, rice and cucumber (Patil et al., 2015; Hu et al., 2017). A previous study proposed *VvSWEET17b* and *VvSWEET17c* as a single gene, identifying it as a 14-TMD extraSWEET (Patil et al., 2015), but this assumption resulted from an error in the 12x Genoscope annotation.

The *in silico* analysis for cis-acting elements of the present study revealed different motifs related with biotic and abiotic stress responses and with sugar and hormone regulation, which was later confirmed by the observation that *VvSWEETs* were extremely responsive to pathogen attack and abiotic stress, as discussed below. Accordingly, as thoroughly described in the Introduction, the expression of *SWEETs* transporters has been reported to be regulated by pathogens as well as by abiotic factors such as cold, drought and high salinity.

The expression of classical disaccharide and monosaccharide transporters is regulated by external sugar concentration (William et al., 2000). Different studies in our group have strengthened this conclusion, both in grapevine (Conde et al., 2006) and in other perennial plants like olive (Conde et al., 2007), but the regulation of the expression of *SWEET* genes by sugars remains so far less explored. Several sugar-responsive elements present in the promoter region of classical disaccharide and monosaccharide transporters were also detected in the promoter region of *VvSWEET* genes (Afoufa-Bastien et al., 2010). In the present study, we observed that the sucrose sensing-related element SURE2STPAT21, previously found in the promoter region of grapevine sucrose transporters (*VvSUCs*) (Afoufa-Bastien et al., 2010), is indeed present in the promoter of *VvSWEET2a* and *VvSWEET7*, which suggests that *VvSWEETs* can effectively sense sucrose availability.

The expression pattern of *VvHT3* and *VvTMT1* in grape cells suspensions cultivated in mineral medium supplemented with sucrose is in line with the results obtained in our group by Conde et al. (2006). Besides, *VvSUC12* was barely expressed and *VvSUC11* and *VvSUC27* expression decreased along sucrose consumption. These results suggest that sucrose (or the monosaccharides resulting from sucrose

hydrolysis) effectively regulates the expression of *VvSUC* transporters that are presumably involved in sucrose uptake in cooperation with VvHTs that incorporate the monosaccharides released soon after sucrose hydrolysis (Conde et al., 2006).

In what concerns the expression studies of *VvSWEETs*, our results showed that Clade III *SWEET* transporters (putative sucrose transporters) were scarcely expressed in grape cell cultures (*VvSWEET10*, *VvSWEET11* and *VvSWEET15*). However, although the steady-state transcript levels of these *VvSWEETs* were low it is likely that, at least some of them, are effectively regulated by sugar levels in the medium and are involved in sugar uptake because they have been characterized as low-affinity, high capacity transporters.

One important issue that deserves attention in the context of these expression studies is the possible role of VvSWEETs in the non-saturable (diffusion-like component) uptake of sugars that has been observed in cultured cells of grapevine (Conde et al., 2006) and *Olea europaea* (Conde et al., 2007) and in many other cell models (see Conde et al., 2007). The nature of this diffusive glucose transport was studied in detail in our group with *O. europaea* cell cultures (Conde et al., 2007) because in glucose-sufficient (3%, w/v) cells the diffusive-type transport represents the major mode of uptake, while in sugar-starved cells saturating transport is induced (Oliveira et al., 2002; Conde et al., 2007). At that time, we proposed that the low-affinity, high-capacity, diffusional component of glucose uptake occurred through a channel-like structure regulated by intracellular protonation and phosphorylation/dephosphorylation. One year later the Frommer's group described the linear uptake of sucrose in roots of *Arabidopsis* (Chaudhuri et al., 2008) and cited our above report to support their findings. Soon after, the same group discovered and characterized the SWEET transporters (Chen et al., 2010). Therefore, it is highly plausible that the *VvSWEETs* expressed in the grape cell suspensions could account for the low-affinity component of sugar uptake that superimposes the activity of proton-dependent active transport systems of glucose and sucrose (Conde et al., 2006; Conde et al., 2007). Yet, additional experiments will be necessary to validate this hypothesis.

4.2. Botrytis cinerea and *Erysiphe necator* induce a transcriptional reprogramming of *VvSWEET* genes in grape berries and grape cultured cells

To address one of the major goals of the present PhD project, we studied the expression profile of different grapevine sugar transporters in response to grape berry infection with a necrotrophic pathogen - *Botrytis cinerea* (causal agent of gray mould) – and a biotrophic one - *Erysiphe necator* (causal agent of powdery mildew). Overall, we observed that *E. necator* infection caused more pronounced modifications in *VvSWEET* gene expression than *Botrytis* infection. As the lifestyle of *E. necator* is more dependent on the living host, it is likely that it massively modulates plant metabolism (Doehlemann et al., 2017), secreting for that purpose a vast array of effectors (Hogenhout et al., 2009). But, as observed in other reports, the pattern

of expression of sugar transporters in response to fungal attack is rather complex, which difficult the proposal of a consistent theory about the role of each member during the progress of the infection.

In the present study, we observed that *VvSWEET2a* and *VvSWEET7* were significantly up-regulated in *Botrytis*-infected green grape berries. We also observed that *VvSWEET7* was up-regulated in grapevine suspension cultured cells 48 h after elicitation with *Botrytis* mycelium extract, and that in berries infected with *E. necator* the steady-state transcript levels of *VvSWEET7* and *VvSWEET15* were also high. Altogether, our results suggest that VvSWEET7 is a key player in the interactions between plant and fungal pathogens. In a previous study in grapevine leaves, *VvSWEET2a* and *VvSWEET7* were up-regulated in response to *Botrytis* infection, and *VvSWEET3*, *VvSWEET4* and *VvSWEET17* expression was also enhanced 72 h after inoculation with *E. necator* (Chong et al., 2014).

The transcriptional reprograming of the expression of *SWEET* genes in response to *Botrytis* and *E. necator* infection has also been reported in other plant species. The *Arabidopsis AtSWEET4*, *AtSWEET15* and *AtSWEET17* (Chen et al., 2010) and tomato *SlSWEET15* (Asai et al., 2016) were up-regulated by *Botrytis* infection. Other fungal pathogens such as the biotroph *Golovinomyces cichoracearum* and mycorrhizal fungus as *Rhizophagus irregularis* (Ferrari et al., 2007; Chen et al., 2010; Manck-Götzenberger and Requena, 2016), as well as bacterial pathogens (Chen et al., 2010), are also known to modulate host *SWEET* gene expression. TAL effectors of *Xanthomonas oryzae* pv. *oryzae* induce rice *OsSWEET11*, *OsSWEET13* and *OsSWEET14* expression (Chen et al., 2010; Streubel et al., 2013; Zhou et al., 2015). Cassava *MeSWEET10a* and citrus *CsSWEET1* are also induced by *Xanthomonas* (Cohn et al., 2014; Hu et al., 2014). Other bacteria including *Pseudomonas syringae* induced several *AtSWEET* genes in infected leaves of *Arabidopsis* (Chen et al., 2010).

The induction of *SWEET* genes by pathogens has been linked to their survival using the sugars of the apoplast secreted by SWEET transporters (Chen et al., 2010; Cohn et al., 2014). However, this assumption has been contradicted by some studies. For instance, in *Arabidopsis* roots *AtSWEET2* gene expression was induced more than 10-fold during *Pythium* infection but *atsweet2*-knockout mutants were more susceptible to infection (Chen et al., 2015a). Also, *IbSWEET10* expression was significantly up-regulated in sweet potato infected with *Fusarium oxysporum* and overexpression of the gene improved host resistance (Li et al., 2017b). It has been proposed that sugar remobilization can trigger signaling cascades that activate defense mechanisms in plants (Gebauer et al., 2017), which may explain the above results. In agreement, it has been reported, that the induction of sucrose and glucose transport observed after pathogen attach may feed defense related plant secondary metabolism (Xiao et al., 2000; Morkunas et al., 2005; Solfanelli et al., 2006; Dao et al., 2011; Kim and Hwang, 2014; Tonnessen et al., 2014).

From the above-referred controversy, *VvSWEET2a* and *VvSWEET7* expression observed in the present study can be either induced by the fungal attack to promote the infection or may help plant defense against

the fungus, but the second hypothesis seems more plausible because green grape berries are known to be relatively resistant to *Botrytis* attack, as previously reported (Goetz et al., 1999).

In the present study, we observed that from the five genes coding for secondary active transporters highly expressed in the grape berry (*VvSUC11, VvSUC12, VvSUC27, VvHT3 and VvTMT1*) (Lecourieux et al., 2014), only *VvHT3* was up-regulated at the mature stage in response to *Botrytis* infection. Accordingly, we observed that *VvHT3* was also up-regulated in grape cell cultures elicited with a *Botrytis* extract. It was reported that this putative hexose transporter gene is the most expressed member of the *VvHT* family in the mature berry (Afoufa-Bastien et al., 2010). Therefore, it is tempting to speculate that *VvHT3* could be induced as a defense mechanism to retrieve sugar accumulated in the apoplast in response to infection, thus limiting its progression, but the possibility that *VvHT3* may help fungal infection cannot be discarded. An increase of glucose uptake by *Pinus pinaster* suspension-cultured cells was observed after 12 h elicitation by *B. cinerea* spores (Azevedo et al., 2006). Similarly, *Arabidopsis* cells co-cultured with *B. cinerea*, showed enhanced glucose and fructose uptake rates. In this system, several sugar transporter genes, including *AtSTP1*, *AtSTP4*, and *AtSTP13* were induced upon elicitation (Veillet et al., 2017). In *Arabidopsis* plants infected by bacteria, hexose/H^+ symporters were also induced and it was speculated that they could counteract SWEET-mediated sugar secretion provoked by the infection (Fotopoulos et al., 2003; Yamada et al., 2016). In this model, the sugar transporter AtSTP13 effectively competes with bacteria for extracellular sugars as its uptake activity is enhanced by its phosphorylation after the interaction with the flagellin receptor AtFLS2 and its co-receptor receptor kinase 1 AtBAK1 (Yamada et al., 2016).

In berries infected by *E. necator* only *VvTMT1* was up-regulated. VvTMT1 is a tonoplast monosaccharide transporter, thus the observed up-regulation can increase sugar sequestration in the vacuole.

Intriguingly, our results showed that while some of the *SWEET* members are consistently up-regulated, as observed above, many others are down-regulated in response to *Botrytis (VvSWEET10*, *VvSWEET11*, *VvSWEET17a* and *VvSWEET17d)* and *E. necator (VvSWEET2a*, *VvSWEET2b*, *VvSWEET4*, *VvSWEET10, VvSWEET11*, *VvSWEET17a* and *VvSWEET17d)* infection. Only a recent study reported the down-regulation of *SWEET* genes upon infection (Asai et al., 2016). In *Botrytis*-infected cotyledons 21 of the 31 tomato *SWEET* genes were down-regulated, including the tomato *VvSWEET10*, *VvSWEET11*, *VvSWEET17a* and *VvSWEET17d* homologues. Therefore, the physiological role of the simultaneous up- and down-regulation of different sugar transporter genes in response to infection, as observed in the present study, is still puzzling. It was proposed that pathogen-promoted down-regulation of SWEET genes disrupts various signaling defense pathways (Berger et al., 2006, Sade et al., 2013). In fact, *Botrytis* may silence *Arabidopsis* and tomato genes involved in immunity by producing and translocating sRNAs that hijack the host RNAi machinery (Weiberg et al., 2013).

Recently it was proposed that fructose content is a major marker of tomato stem defense to *B. cinerea* (Lecompte et al., 2017), thus it is tempting to speculate that the observed down-regulation of *VvSWEET17a* and *VvSWEET17d* during infection, together with the up-regulation of *VvTMT1*, could limit fructose outflow from the vacuole increasing the grape berry relative fructose and then boosting grapevine defense against infection. The previous observation that *VvSWEET17a* and *VvSWEET17d Arabidopsis* homologues were fructose specific uniporters - playing a key role in facilitating fructose transport across the tonoplast to maintain cytosolic fructose homeostasis (Chardon et al., 2013; Guo et al., 2014) - supports the above hypothesis. Furthermore, clade IV tomato SWEETs were also strongly down-regulated during *Botrytis* infection (Asai et al., 2016), as reported above.

4.3. The expression profile of the *VvSWEETs* is also affected by abiotic stress, including drought

As referred to in the Introduction, we took advantage from the studies conducted by Conde et al. (2015), aimed to evaluating the effect of different irrigation treatments on the grapevine, to evaluate how different water-deficit stress conditions affect the expression of *VvSWEETs*. Results showed that, while the majority of *SWEET* genes were down-regulated, *VvSWEET10* and *VvSWEET11* were up-regulated. In other plant species, transcript abundance of *SWEET* transporters was also modified during drought. In *Arabidopsis*, banana and *Camellia sinensis* several *SWEET* transporters were induced under drought stress (Miao et al., 2017; Wang et al., 2018). The vacuolar *SWEET* transporter *AtSWEET16* and *CsSWEET16* were down-regulated in water scarcity conditions (Klemens et al., 2013; Wang et al., 2018). Again, the formulation of a coherent theory about a general role of SWEET transporters during drought stress response is difficult due to the observed diversity of expression patterns of these genes in response to water shortage. It has been reported that water scarcity drastically limits crop productivity and quality, by reducing photosynthesis and plant carbon assimilation (Moutinho-Pereira et al., 2004; Chaves et al., 2009), so it is somewhat expected that these alterations in the sugar metabolism could modify the expression of different sugar transporters to maintain cellular homeostasis, as previously discussed (Yamada et al., 2010; Yamada et al., 2011; Schulz et al., 2011; Frost et al., 2012; Osakabe et al., 2013; Osakabe et al., 2014; Gong et al., 2015), but the role of a specific gene is rather difficult to identify.

Our results also showed that in kaolin treated leaves the sucrose transporters *VvSUC12* and *VvSUC27* and a few *SWEET* transporters were up-regulated. Remarkably, the putative sucrose transporter *VvSWEET11* was strongly up-regulated (up to 18-times). Kaolin is an inert mineral that reflects ultraviolet and infrared radiations, lowers canopy temperatures and stomatal conductance under non-limiting soil moisture conditions, and protects photosystem II structure and function (Glenn et al., 2010; Dinis et al., 2016a, 2016b, 2018). Different reports show consistent data regarding the effect of kaolin in the alteration of the total soluble solids content, among other metabolic changes (Shellie and Glenn, 2008; Ou et al.,

2010; Song et al., 2012; Shellie, 2015; Conde et al., 2016). The *Arabidopsis AtSWEET11*, homologous to *VvSWEET11* that in the present study was strongly up-regulated in response to kaolin, mediates sucrose efflux from the mesophyll cells to the apoplast for phloem loading (Chen et al., 2012), thus, together with our data, it appears that kaolin stimulates sucrose transport capacity within the leaves improving source-to-sink transport of sucrose (Conde et al., 2018a).

The hypothesis that sugar metabolism is changed in berries subjected to dehydration normally used to produce raisins and sweet and fortified wines, was clearly supported by the observation that a strong transcriptional reprogramming of sugar transporter genes was observed after postharvest dehydration. As reported in the Results section, the most striking result was the sudden up-regulation of *VvSWEET11* up to 200-fold 5 days after incubation of the bunches at 50 °C. The physiological meaning of these strong modifications in gene expression (together with grapevine aquaporins, among other genes) (Conde et al., 2018b) is far from being understood. Yet, some modifications in gene expression were somewhat expected like those in *VvCwINV* that resulted in the increase of compatible solutes but, intriguingly, the total amounts of glucose and fructose were not significantly changed (Conde et al., 2018b). Given the putative bidirectional transport capacity of SWEET transporters it is likely that they could play a role in the redistribution of the sugars inside the grape tissues or even between different intracellular compartments.

The literature is relatively scarce on this subject, but an improvement in sugar capacity was observed during a transcriptional profiling on postharvest withering of Corvina cv. grapes, where a putative sucrose transporter-like protein and the *VvHT5* hexose transporter were up-regulated (Hayes et al., 2007; Zamboni et al., 2008). Moreover, it is reported that post-harvest dehydration processes might strongly influence important primary and secondary metabolic pathways of grape berry cells (Costantini et al., 2006; Schreiner and Huyskens-Keil, 2006; Rizzini et al., 2009), but further studies are necessary to correlate gene expression to the observed modifications of these metabolic pathways.

4.4 - VvSWEET7 is a mono- and disaccharide low-affinity, high capacity transporter localized in the plasma membrane

Clear-cut co-localization experiments with GFP-tagged proteins, revealed that VvSWEET7 and VvSWEET15 are plasma membrane-bound. Both *VvSWEET7* and *VvSWEET15* were heterologously expressed in an *hxt-null S. cerevisiae* mutant strain to study their function as sugar transporters. Our results were particularly relevant because so far only two grapevine SWEETs (VvSWEET4 and VvSWEET10) have been characterized (Chong et al., 2014; Zhang et al., 2019b), but in our study we performed a very complete kinetic analysis in yeast cells. The yeast expressing VvSWEET7 restored the capacity to transport glucose and, remarkably, sucrose. The protonophore CCCP did not inhibit sugar transport capacity of VvSWEET7, suggesting the involvement of a proton-independent facilitated diffusion, in line with previous reports (Review

by Chen et al., 2015b). Competitive inhibition experiments showed that fructose, mannitol and sorbitol also inhibited glucose transport, suggesting that, besides mono- and disaccharides, VvSWEET7 may mediate the transport of polyols. The kinetic analysis showed that the affinity of VvSWEET7 was in the millimolar range, in line with previous reports (Chen et al., 2010; Chen et al., 2012).

Unambiguous results were obtained regarding the capacity of VvSWEET7 (clade II) to transport sucrose. AtSWEET16 (clade IV) also mediates the transport of both mono- and disaccharides (Klemens et al., 2013). The behavior of these two SWEET transporters contradicts previous observations that clade I and II and IV prefer hexoses while clade III are sucrose transporters (Chen et al., 2015b; Eom et al., 2015), however it should be noted that so far few SWEETs have been functionally characterized.

The possible polyol transport capacity evidenced by VvSWEET7 is so far unique in the SWEET family. In plants, other proteins from different families have been assigned to this function. The polyol transporter AtPLT5, localized in the plasma membrane of *Arabidopsis*, is able to actively transport a broad-spectrum of substrates such as sorbitol, xylitol, erythritol or glycerol, but also different hexoses, such as glucose and pentoses including ribose, tetroses and a sugar acid (Klepek et al., 2005; Reinders et al., 2005). Similarly, in our group we have characterized VvPLT1 (VvPMT5) as a polyol transporter that is competitively inhibited by monosaccharides (Conde et al., 2015).

The observed broad range of transported substrates as well as its high expression in the green stage suggests that VvSWEET7 may play an important role in sugar partitioning during fruit development. At the green fruit stage, sucrose is predominantly translocated to the berry mesocarp cells via plasmodesmata (Zhang et al., 2006); however, apoplasmic transport through VvSWEET7 may be also involved. The *Arabidopsis* VvSWEET7 homologue functions as a glucose transport and is expressed mainly in the flower and seed (Chen et al., 2010) and the cucumber CsSWEET7b transports glucose and, to a minor degree, mannose and galactose (Li et al., 2017a). Interestingly, the tomato VvSWEET7 homologue (SlSWEET6) is also strongly regulated during the early phases of tomato fruit development (Shammai et al., 2018).

Another challenging hypothesis, but more speculative, is that SWEETs, at least some, can behave as dual-affinity transporters, by mediating proton-dependent active (concentrative) transport at low substrate concentration ranges. As reported, SWEETs have been characterized as catalyzing diffusive, non-concentrative, transport (Chen et al., 2010; Chen et al., 2012; Guo et al., 2014; Lin et al., 2014) in spite of the fact that some physiological functions of SWEET transporters actually require accumulative capacity (Eom et al., 2015; Jeena et al., 2019). So, in the present study, to address the hypothesis that VvSWEET7 can mediate concentrative transport, the uptake of the non-metabolizable glucose analog 3-*O*-Methyl-D-Glucose (3-OMG) was tested. As shown in the Results section, 3-OMG accumulated intracelullarly by up to 10-fold and the accumulation ratio was inhibited by the protonophore CCCP. Therefore, the conclusion that VvSWEETs could mediate dual-affinity transport (H^+-dependent transport at low substrate concentrations and

facilitated diffusion at millimolar sugar concentrations), and thus display a biphasic kinetics, is scientifically very relevant, but further experiments are needed to validate it and exclude possible experimental artifacts.

Indeed, the recent breakthrough discoveries of transport systems assigned with atypical functions provide evidence for complexity in membrane transport biochemistry (reviewed by Conde et al., 2010). The nitrate transporter CHL1 in *Arabidopsis*, for long considered as a low-affinity transport system, mediates, in fact, a dual-affinity uptake displaying K_m values of 50 μM and 4 mM, respectively, for the high-affinity and low-affinity components (Liu et al., 1999). More recently, it was shown that the change between the two modes of transport activity of CLH1 is mediated by a strict post-translational regulation involving phosphorylation/dephosphorylation of the threonine residue 101 (Liu and Tsay, 2003). According to the authors, when phosphorylated, CHL1 functions as a high-affinity transporter, whereas, when dephosphorylated, it acts as a low-affinity nitrate transporter. Interestingly, an obvious feature characteristic of eukaryotic SWEETs is their long cytosolic C terminus, which may act as a binding-site for other proteins (e.g., regulatory components) (Chen et al., 2015b) that mediate post-translational regulation. Thus, this fascinating area of research is still wide open.

In the present study we were not able to demonstrate the transport capacity of VvSWEET15 with a similar approach used to characterize VvSWEET7, despite the *Arabidopsis* ortholog AtSWEET15 being well characterized as a sucrose transporter (Chen et al., 2012). AtSWEET15 appears to be involved in the remobilization of carbohydrates in senescent leaves as its expression increases by 22-fold during senescence (Quirino et al., 1999). Also, it regulates cell viability under high salinity (Seo et al., 2011) and is also involved, along with AtSWEET11 and AtSWEET12, in the sugar efflux required for seed filling (Chen et al., 2015c). In tomato, SlSWEET15 is not well characterized but showed a similar expression pattern to its grapevine homologue along fruit development (Shammai et al., 2018), being more expressed in the mature stage. To functionally characterize VvSWEET15, further experiments are therefore necessary, including, for instance, the utilization of a different heterologous expression cell model. But we cannot discard the possibility that VvSWEET15 is not a true sugar transporter, but rather a sugar sensing protein without transporting functions.

4.5 - VvERD6l13 is a sucrose transporter localized at the plasma membrane up-regulated in grape berries infected by fungi

In the present study, the grapevine Early-Response to Dehydration 6-like (*VvERD6l*) family, with 18 members, were thoroughly identified in different databases, but twenty-two members were identified by Afoufa-Bastien et al. (2010). Previously, six of the identified ORFs corresponded to partial sequences in which either the beginning or the end of the protein was not clearly identified. In this work, we considered only those genes predicted to encode a 12 transmembrane domains protein. The exons of each gene were

carefully checked in different RNAseq experiments available in the NCBI database (Annex table 3). As shown in the results section, almost all the members are likely localized to the plasma membrane, however two of them are predicted to localize in the tonoplast (VvERD6l2 and VvERDl613). In *Arabidopsis*, all of the functionally characterized ERD6l transporters were localized in the tonoplast (Yamada et al., 2010; Poschet et al., 2011; Klemens et al., 2014). As expected, the phylogenetic analysis showed that the VvERD6l family is more closelly related with monosaccharide transporters sub-families than with the SUC and SWEET families. This family forms one of the largest sugar transporter subfamilies in *Vitis*, as in *Arabidopsis* (Büttner, 2007). The majority of the ORFs are located at the chromossome 14 and 12 *loci* form 6 sister pairs, which suggests they were formed by tandem duplications during evolution (Johnson et al., 2006; Afoufa-Bastien et al., 2010).

Several cis-acting elements were detected in the promoter region of *VvERD6l* genes. In particular, sugar-responsive elements were identified, suggesting that the expression of these genes is regulated by sugar. The *Arabidopsis* gene *ERDl6* is repressed under conditions which require glucose accumulation and expressed under glucose starvation (Poschet et al., 2011). Of note, two sucrose-responsive elements were found in the promoter region of *VvERD6l13*, being one of them the cis-acting element SURE2STPAT21, which was previously detected only in the promoter regions of *VvSUC* genes (Afoufa-Bastien et al., 2010). Regulatory elements related with both biotic and abiotic stress and responsive to different hormones were also identified. In *Arabidopsis*, *ERD6l* genes are strongly induced by dehydration (Kiyosue et al., 1994). *AtESL1* expression is also induced after ABA treatment (Yamada et al., 2010), *AtERDl6* by wounding (Poschet et al., 2011) and *AtERD6* by drought, high salinity and cold temperatures (Kiyosue et al., 1998; Yamada et al., 2010). The tomato *LeST3* gene expression was increased during plant colonization by the arbuscular mycorrhizal (AM) fungi *Glomus mosseae* or *Glomus intraradices* and by pathogen attack by *Phytophthora parasitica* (García-Rodríguez et al., 2005).

As referred to in the Results section, taking into account previous results showing that *VvERD6l13* was strongly up-regulated during *Botrytis* infection (Agudelo-Romero et al., 2015), we decided to further characterize this gene. Our observation that VvERD6l13-GFP clearly localizes at the plasma membrane was somewhat unexpected because *in-silico* analysis predicted a tonoplast localization, much like all other so far characterized ERD6l sugar transporters (Yamada et al., 2010; Poschet et al., 2011).

The heterologous expression of this gene in *hxt-null* yeast mutant strain clearly suggested that the encoding protein mediates H^+-dependent sucrose transport with relatively low affinity (K_m = 33 mM). This finding is particularly relevant because so far members of SUC/SUT family were only known to transport sucrose with a relatively low K_m. VvSUC11 and VvSUC12 are characterized as intermediate affinity sucrose transporters (K_m of 0.9 mM and 1.4 mM, respectively) (Ageorges et al., 2000; Manning et al., 2001), while VvSUC27 is a low affinity sucrose transporter (K_m = 8–10 mM; Zhang et al., 2008). Therefore, up to date,

VvERD6l13 is the H^+/sucrose symporter with the lowest substrate affinity so far characterized in the grapevine.

Up to now, only three members of this family were functionally characterized in *Arabidopsis*. AtESL1 was characterized as a low affinity facilitated diffusion monosaccharide transporter, with a K_m =102.2 mM glucose (Yamada et al., 2010). AtERDl6 was initially characterized as a vacuolar glucose exporter, as suggested by the increased vacuolar glucose concentration in plants lacking AtERDl6 (Poschet et al., 2011), but more recent results suggested that AtERDl6 is an H^+ dependent glucose exporter (Klemens et al., 2014). AtERDl10 mediates the active transport of glucose also driven by the proton gradient (Klemens et al., 2014).

Remarkably, in the present study we observed that, unlike its already characterized *Arabidopsis* homologues, VvERD6l13 is a disaccharide transporter. Phylogenetically this transporter is closer to other monosaccharide transporters, such as VvHT1 (Vignault et al., 2005) or VvTMT1 (Zeng et al., 2011) than to sucrose transporters, like VvSUC11 (Ageorges et al., 2000). Nevertheless, some members of the TMT sub-family, which belongs to the monosaccharide transporter family, also transport sucrose. The *Arabidopsis* AtTMT1 and AtTMT2 appear to transport glucose and fructose (Wormit et al., 2006), as well as sucrose (Schulz et al., 2011). In sugar beet, the tonoplast localized protein BvTST2.1 is a sucrose-specific transporter (Jung et al., 2015) and in watermelon, ClTST2 is a vacuole-localized sucrose and hexoses transporter (Ren et al., 2018).

In the present study we also found that *VvERD6l13* is strongly up-regulated in grape berries infected with *Botrytis* or *E. necator*, as well as, in grape cells 48 h after elicitation with *Botrytis* extracts. The tomato *VvERD6l* homologue, *LeST3,* was also up-regulated during pathogen infection. *LeST3* gene expression was increased in leaves of plants infected with the root pathogen *Phytophthora parasitica* or the arbuscular mycorrhizal (AM) fungi *Glomus mosseae* or *Glomus intraradices*. These results suggest that LeST3 plays a role in the transport of sugars from source tissues towards the infected or colonized sink tissue (García-Rodríguez et al., 2005). Interestingly, LeST3 is phylogenetically more closely related to VvERD6l13 than to other so far characterized ERD6l proteins.

Previous results showed that the hexose transporter *VvHT5* is strongly up-regulated during *E. necator* and *P. viticola* infection (Hayes et al., 2010) while in the present study we observed a similar pattern for *VvHT3* (see above), so both transporters may play a key role by retrieving hexoses from the apoplast upon infection. If this is the case, we hypothesize that VvERDl13, as a secondary active sucrose symporter, may play a similar function, by retrieving apoplasmic sucrose at a higher transport rate due to its lower affinity, in complementarity with higher affinity transporters, to limit the progression of the infection.

Results also showed that VvERDl13 may play an important role in roots where its expression is very high. It may mediate the uptake of sucrose from the apoplast by root sink cells after its unloading from phloem through SWEET transporters or H^+/sucrose co-transporters (SUC) (Carpaneto et al., 2005).

Chapter 5
Conclusions and Future Perspectives

5.0 – Conclusions and Future Perspectives

From a climate perspective, agriculture is extremely vulnerable to climate change as most crop systems have been optimized to fit a given climate niche allowing for economically sustainable quality and production. As in the past, today's wine production occurs over relatively narrow geographical and climatic ranges. Wine grapes also have relatively large cultivar differences in climate suitability, further limiting some wine grapes to even smaller areas that are climatically appropriate for their cultivation. These narrow niches for optimum quality and production put the cultivation of wine grapes at greater risk from both short-term climate variability and long-term climate changes than other more broadacre crops (Reviewed by Jones, 2006). Several biotic agents also represent serious threats for grapevine and its derived industries and the degree of susceptibility to each pathogen, although this depends on the varieties. Major diseases can have a fungal (powdery mildew, botrytis, black rot, etc.), oomycete (downy mildew), bacterial (pierce disease), phytoplasmic (bois-noir, flavescence doree) or viral (court-nou., leafroll, etc.) origin (Reviewed by Grimplet, 2016). Some stress responses have been studied at the whole plant or organ physiology level for decades. Now the genome sequence provides the opportunity to examine the involvement of any individual gene or gene network in grapevine stress response and to predict their effects on berry quality (Reviewed by Grimplet, 2016).

During infection, pathogens cause profound metabolic and transcriptomic modifications in their host. Sugar metabolism and mobilization are specially affected during the infection process and we hypothesized in the present study that SWEET transporters are important players during this clash in grapevine. Therefore, one of the main objectives of this thesis was to evaluate the impact that fungal pathogens - a necrotroph (*Botrytis cinerea*) and a biotroph (*Erysiphe necator*) - have on the expression profile of several grapevine sugar transporters, mainly on the SWEET family members. As referred to above, we benefitted from previous work performed by our collaborator Prof. Ana Fortes (Agudelo-Romero et al., 2015) as well from the healthy and infected grape berries sampled by our collaborators from Universidade de Lisboa. Globally, we observed that infection of grape berries with *Botrytis* and *E. necator* promoted an ample transcriptional reprogramming of the expression of several SWEET genes. Particularly, infection with the biotrophic pathogen *E. necator* induced more pronounced modifications than with the necrotrophic pathogen *Botrytis*. Indeed, as observed before (Doehlemann et al., 2017), as the lifestyle of a biotroph depends on its living host, this class of pathogens generally induced massive changes in their host. Thus, the answer to the first question raised in the present project "i) Does *B. cinerea* or *E. necator* infection induce transcriptional reprogramming of *VvSWEETs* in the grape berry?" is unequivocally "Yes".

Interestingly, both up-regulation and down-regulation of *VvSWEETs* was observed. Genes such as *VvSWEET7* or *VvSWEET15* were strongly up-regulated in infected grapes, while other genes such as *VvSWEET10*, *VvSWEET11* were down-regulated. These complex patterns of expression were already

observed in other plants, being the proposal of a coherent explanation a difficult task. As reported before, the induction of plant SWEET transporters by pathogens has been linked with the ability of pathogens to obtain host-derived sugars for nutrition, however, other reports describe contradictory evidences. In fact, up-regulation of *VvSWEET* genes can help in the remobilization of sugars, which can trigger signaling cascades that activate defense mechanisms, or feed defense related plant secondary metabolisms. Similarly, SWEET repression can be related with plant defense mechanisms, suppressing sugar leakage and starving the pathogen, or with the pathogen ability to suppress sugar translocation to disrupt various signaling defense pathways. Conclusively, sugar metabolism and mobilization are important players that decide the fate of the ongoing battle between plant and pathogen during the infection process. However, despite recent advances, the metabolic signatures and their regulatory nodes, which decide the susceptibility or resistance responses, remain poorly understood. This is indeed an excellent topic for future research.

Our group has been focused on the study of the biochemical and molecular mechanisms of grape berry ripening and how fruit development is influenced by both abiotic and biotic stress conditions, taking advantage from combination of different biological models (*in vitro* cell cultures, fruiting cuttings, potted and field-grown vines) and different classical molecular and biochemical analysis, and, more recently, omics approaches. The collaborations we have set with recognized national and international research groups paved the way for the present thesis project, particularly focused on the specific role of the newly identified SWEET transporters in grapevine-environment interactions and grapevine stress biology. We are presently continuing our collaboration with Ana Fortes group in the context of a newly launched FCT-funded scientific project whose major goal is to investigate the transcriptome and metabolome of *V. vinifera* (susceptible species) and *V. rupestris* grapes (resistant species) in response to infection with *E. necator*. In particular we aim to identify candidate genes involved in resistance and susceptibility that might be used in future in breeding programs or alternatively in gene editing. This project is also expected to provide important and useful information that can help viticulturists to improve the current management practices that are heavily reliant on the use of fungicides which pose health and environmental impacts.

In plants, sugars have different biological roles, including as nutrients, osmotic regulators and signaling molecules. In fruits, in general, sugar concentration is a critical parameter for quality, but in grapevine, fruit sugars have an additional pivotal role in wine quality. Thus, the study of the mechanisms and regulation of sugar transport and compartmentation in grapevine, and particularly in the grape berry, is of great importance. In the present study, we also explored the possible regulation of grapevine SWEET transporters by external sugar concentrations both by *in silico* analysis and experimental approaches using grapevine suspension cell cultures where external sugar could be easily adjusted. We observed that the promoter region of several *SWEETs* genes has numerous sugar regulatory cis-acting elements and, accordingly, the steady-state transcript levels of key *SWEET*s were regulated by external sugar, confirming our hypothesis. However, much work remains to be done on this subject. For instance, it is highly plausible

that the *VvSWEETs* expressed in the grape cell suspensions account for the first order kinetics we demonstrated before in our group (Conde et al., 2006; Conde et al., 2007).

Another objective we aimed to address in the present project was to evaluate if drought stress could regulate the expression of *VvSWEETs* in grape berry tissues. It is well known that, in grapevine, detrimental effects caused by drought are generally associated with limitations on photosynthesis, which negatively affects plant carbon and assimilation. Therefore, as SWEET transporters are key players in sugar mobilization, it was reasonably plausible to speculate that they could indeed be affected by water deprivation. Taking advantage from the collaboration we set with Prof. Manuela Chaves at ITQB, who provided us grape samples from vines subjected to different deficit irrigation treatments, we observed that the majority of *SWEET* genes were down-regulated in response to drought stress, while *VvSWEET10* and *VvSWEET11* were up-regulated. Future experiments are necessary to evaluate the specific role of each *VvSWEETs* under drought. A complex pattern of sugar transporters expression was also observed during the dehydration of grape berries, a procedure normally used to produce raisins and sweet and fortified wines. As reported in the Introduction, this work was part of a broader project that aimed to study the effects of the dehydration process in the primary metabolism of grape berries (Conde et al., 2018b). Remarkably, we observed that *VvSWEET11*, a putative sucrose transporter, was up-regulated up to 200-fold. So, what should be the role of such a transporter when no significant changes in total sugar levels were observed during dehydration? This question is of utmost scientific interest, but additional work is necessary to address it. One can speculate that SWEET transporters can play a role in the redistribution of sugars inside grape berry tissues or between different intracellular compartments.

When we were challenged by the group of José Moutinho Pereira from UTAD to evaluate the biochemical and molecular responses of grapevine to the application in the field of the radiation-reflecting inert mineral kaolin, we hypothesized that Kaolin particle film application could stimulate carbohydrate metabolism and thus reprogramming the expression of *VvSWEETs*. In the present study we indeed confirmed that sucrose transporters, such as *VvSUC12* and *VvSUC27*, and a few putative sucrose transporting *VvSWEETs* were up-regulated in kaolin treated leaves which, suggest that kaolin stimulates sucrose transport capacity within leaves, improving source-to-sink transport of sucrose.

Our results suggested that VvSWEET7 and VvSWEET15 are likely to play important roles during fruit development and ripening, as well in the response to pathogen attack, as they are strongly expressed at the green and mature stage, respectively, and were clearly up-regulated in response to infection. This observation claimed for further studies in order to functionally characterize these putative sugar transporters and to study their sub-cellular localization. Clear-cut results with GFP fusion proteins showed that both VvSWEETs are plasma membrane-bound transporters. Also, the overexpression of VvSWEET7 in an *hxt-null* yeast strain clearly showed that this protein is indeed a high-capacity, low-affinity mono- and disaccharide transporter. For the first time we characterized a protein able to transport both disaccharides and

monosaccharides. Remarkably, our results suggested that VvSWEET7 could also transport other substrates, like sugar-alcohols. This substrate plasticity of VvSWEET7 deserves further investigation in a near future, in particular to understand details of protein structure/function relationships, which is a big scientific challenge.

The recent breakthrough discoveries of transport systems assigned with atypical functions provide evidence for complexity in membrane transport biochemistry. Some channels are far from being simple pores creating hydrophilic passages for solutes and can, unexpectedly, act as enzymes, or mediate high-affinity uptake, and some transporters are surprisingly able to function as sensors, channels or even enzymes. Furthermore, numerous transport studies have demonstrated complex multiphasic uptake kinetics for organic and mineral nutrients (Reviewed by Conde et al., 2010). We have tackled this scientific enigma before (Conde et al. 2006; Conde et al. 2007), but in the present study we have also observed that VvSWEET7 mediates concentrative, proton-dependent transport at low external sugar concentrations, which suggests that VvSWEET7 can have dual-affinity properties: a H^+-dependent transport at low substrate concentrations and a facilitated diffusion at millimolar concentrations. This hypothesis is so far rather speculative and controversial because SWEET transporters have been characterized as bi-directional uniporters (Chen et al., 2015b), however their transport properties have been studied only in the mM concentration range. Future research will bring new hints that may validate our observations.

To the best of our knowledge this is the first study reporting the functional characterization of a SWEET transporter in fruits, although, as reported elsewhere, other pioneering studies have already addressed the role of key SWEET genes highly expressed in roots, stems, leaves and nectary tissue.

We were not able to demonstrate that VvSWEET15 is a functional sugar transporter, so in future studies we need to exclude the possibility that the protein is incorrectly folded or is mistargeted to the plasma membrane of the yeast. In the case that one of those possibilities is confirmed, transport experiments could then be performed using a different heterologous expression system. In any case, one cannot exclude that VvSWEET15 is a transmembrane protein without transport function, functioning as a sugar sensing protein, much like SNF3 and RGT2 in yeast.

Moreover, to better understand the physiological role of SWEET transporters in the grapevine, we resorted to plant genetic engineering techniques, taking advantage from the expertise of Prof. Antonio Granell (UPV) in plant genetic manipulation. At UPV, different genetic engineering techniques were performed to further understand the physiological role in planta of VvSWEET7 and VvSWEET15. In a first approach, we heterologously expressed in Micro-TOM tomato a reporter gene (YFP) under the control of the promoter of VvSWEET7 and VvSWEET15 to evaluate in what tissues our genes are expressed, giving important hints about its physiological role. For that, the plasmid *p3Ω2_PROVvSW7:YFP:KanR*, that express a reporter gene (YFP) under the control of our GOI promoter was constructed. This plasmid was then inserted in a suitable mutant *Agrobacterium* strain (LBA4404) and the transformed *Agrobacterium* was used

to transform cotyledons of Micro-Tom that were then cultivated in appropriate solid medium. The objective was to promote *calli* regeneration from the transformed cotyledons that could regenerate to a fully developed plant.

VvSWEET7 and VvSWEET15 were also overexpressed in tomato Micro-Tom plants as a second approach to evaluate their function in planta. The plasmids *p3ΩΩ2:PROM2x35S:VvSW7:KanR* and *p3ΩΩ2:PROM2x35S:VvSW15:KanR,* which over-express our GOI was constructed and inserted in a mutant *Agrobacterium* strain (LBA4404) that was used to transform Micro-Tom tomato cotyledons. Moreover, plasmids to express our GOIs with specific gene promoters were also constructed, namely the tomato specific promoter TFM5 that allows the expression of the gene in green tomatoes and the promoter E8 that allows the expression of the gene in mature fruits (Santino et al., 1997).

The third strategy we tested at UPV was the silencing of tomato SWEETs homologues to VvSWEET7 (SlSWEET6) and VvSWEET15 (SlSWEET15) by the CRISP-Cas9 which could allow us to better understand their physiological role through the loss of their function. Recently, genetic modification using CRISPR–Cas9 editing tools has been exploited efficiently in various plant species, such as *Arabidopsis*, tobacco, rice, sorghum, maize, wheat, poplar, tomato, soybean, petunia, and citrus (Jia and Wang, 2014; Fan et al., 2015; Woo et al., 2015; Osakabe et al., 2016; Song et al., 2016; Zhang et al., 2016; Osakabe et al., 2018). A plasmid with the necessary components to gene knock-out by CRISP-Cas9 was constructed (*p3αα1_SlSW6:hCAS9:KanR* and *p3αα1_SlSW15:hCAS9:KanR)* and introduced in a particular *Agrobacterium* mutant strain that was used to transform Micro-TOM tomato cotyledons. The three above referred strategies started at the UPV take a significant period of time to yield the expected results but promising results were already obtained. For instance, we have successfully obtained transformed plantlets (Figure 5.1).

In the present project, the so far scarcely studied family of sugar transporters, the *Early-Response to Dehydration 6-like* (*VvERD6l*) were extensively analyzed. In the context of these studies, 18 members were identified in the grapevine genome and *in silico* analysis showed that both abiotic and biotic stress *cis-acting* elements are abundant in their promoter regions. We observed that this family forms one of the largest sugar transporter families in grapevine, thus it would be scientifically relevant to functionally characterize these proteins. It is known that members of *ERD6l* are up-regulated in response to dehydration in *Arabidopsis* (Kiyosue et al., 1994), but in the present study we observed that VvERD6l13 was also strongly up-regulated in response to infection with *Botrytis* and *E. necator* thus these proteins seem also responsive to biotic stress.

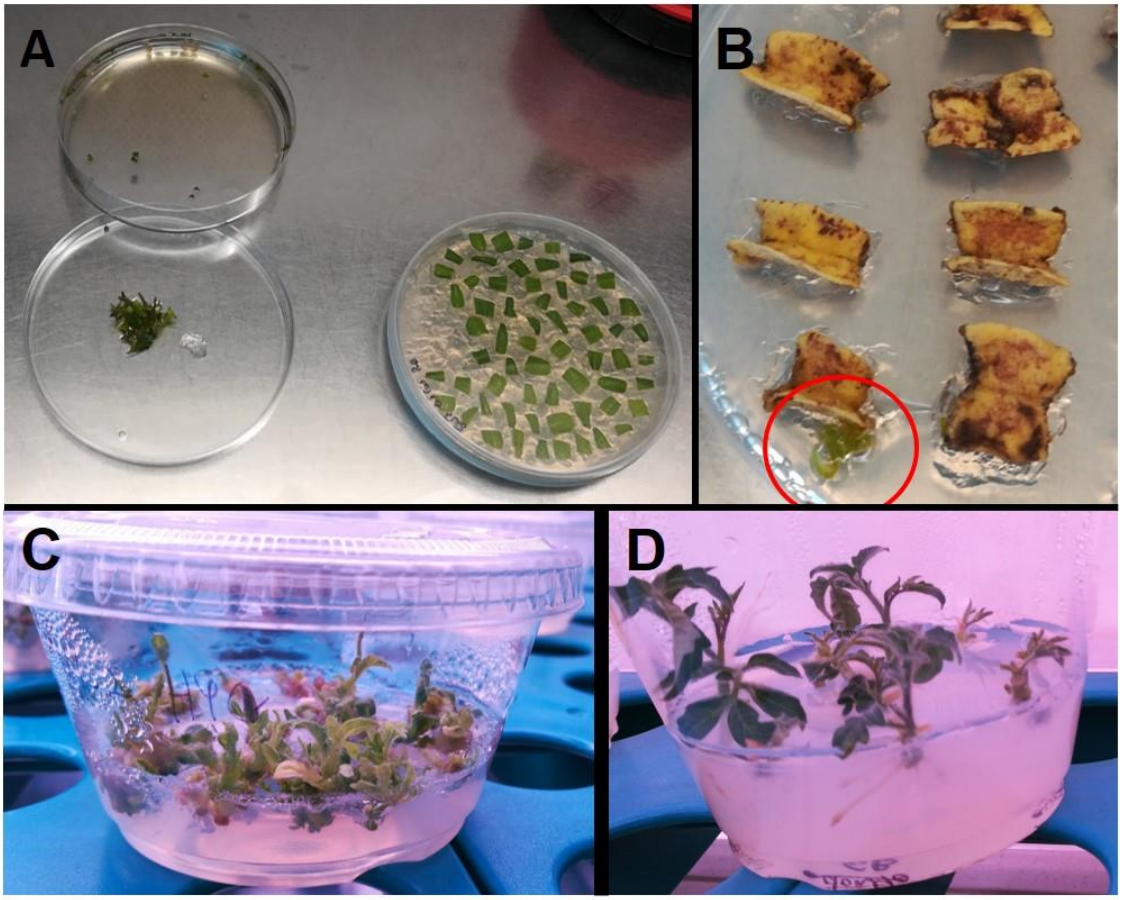

Figure 5.1 - Steps in the transformation of tomato cv. Micro-Tom with *Agrobacterium*. (A) Transformation of dissected cotyledons explants with *Agrobacterium* mutant lines carrying the construct *promVvSWEET7:YFP:KanR*. (B) Calli induction from the transformed cotyledon explant (highlighted). (C) Shoot elongation. (D) Rooting and plant development.

To address the hypothesis that that VvERD6l13 a true sugar transporter of the plasma membrane in grapevine the cloned gene was heterologously expressed in an *hxt-null* mutant strain of *S. cerevisiae* and the transport of radiolabeled sugars was evaluated. Results showed that VvERD6l13 is a plasma-membrane bound H^+-dependent, low affinity, sucrose transporter. Because so far only members of SUC/SUT family are known to transport sucrose this result is particularly relevant.

Because *VvERD6l13* was highly up-regulated by both *Botrytis* and *E. necator* this gene may have a protective physiological role during infection. As pathogens can induce the leakage of sugars from the host (by the action of pathogen-recruited plant SWEET transporters) to the invading space, VvERD6l13 can help retrieving the lost sugars back to the cell, limiting growth and development of the pathogen and thus limiting the progression of the infection. Although this hypothesis is so far rather speculative, its confirmation could be scientifically relevant.

Future work is aimed to characterize other key members of this sugar family and to study VvERD6l13 in more detail. Similar approaches to those used to study VvSWEET7 and VvSWEET15 in Micro-TOM tomato mutant lines are in progress.

Recent advances show that specialized plant membrane transporters can be used to enhance yields of staple crops, increase nutrient content and increase resistance to key stresses, including salinity and pathogens. This research topic has been updated very nicely by Schroeder et al. (2013). As stressed by the authors, transport proteins are key targets for improving the efficiency with which plants take up and use water and nutrients. These proteins transport mineral nutrients and control drought tolerance but are also essential for moving sucrose to where it is needed. Advances driven by physiology, genetics and biophysics over the past 20 years have dramatically improved our understanding of the molecular basis of plant nutrition and how plants respond to stress. Next-generation sequencing is leading to an understanding of how the natural genetic diversity of plant membrane transporters can be exploited for agriculture, whether by marker-assisted breeding or through genetic engineering (reviewed by Schroeder et al., 2013). Indeed, the fascinating world of plant sugar transport can have huge agronomic applications. For instance, different grapevine orientation and agricultural practices can modify the expression profile of different sugar transporters, which, in turn, induce modifications of the chemical composition of grape berries and, thus, their quality and wine properties. Thus, the study of grapevine sugar transporters, may, ultimately, result in gains in grapevine productivity and quality and in plant tolerance to biotic and abiotic stress. Therefore, basic science can assist the improvement of agricultural practices, like canopy management and irrigation, and the design of stress mitigation strategies, including the foliar spraying with exogenous substrates or hormones.

Chapter 6
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5.0 – References

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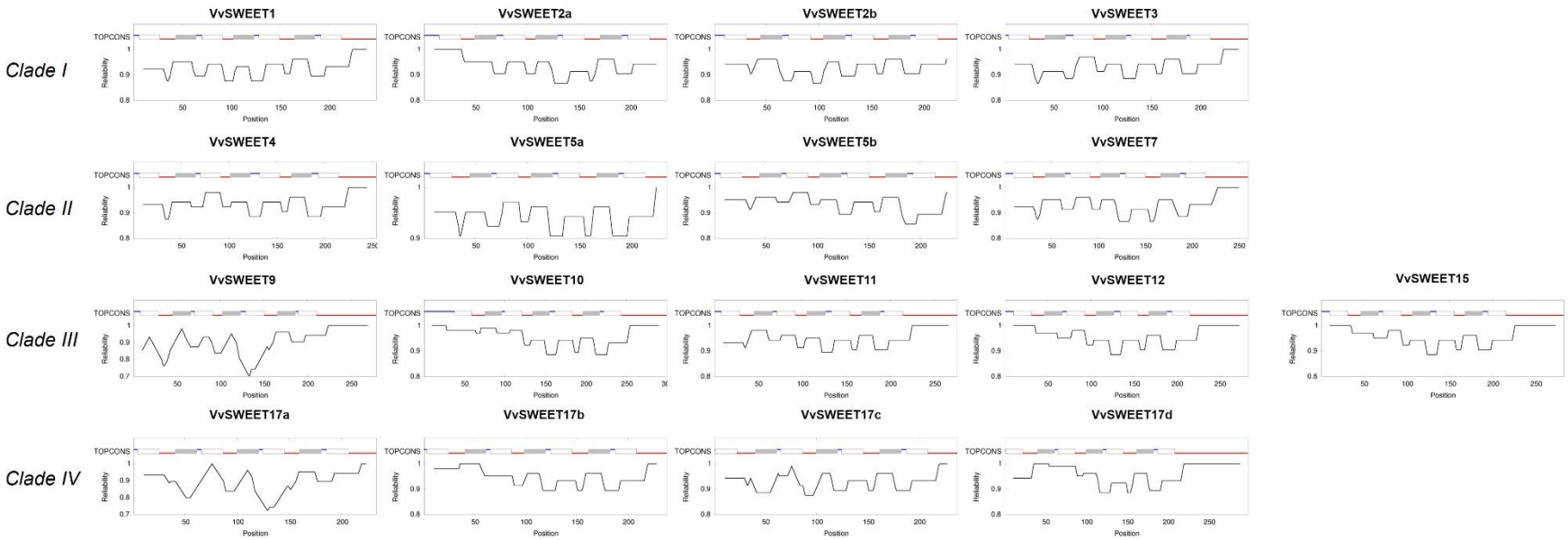
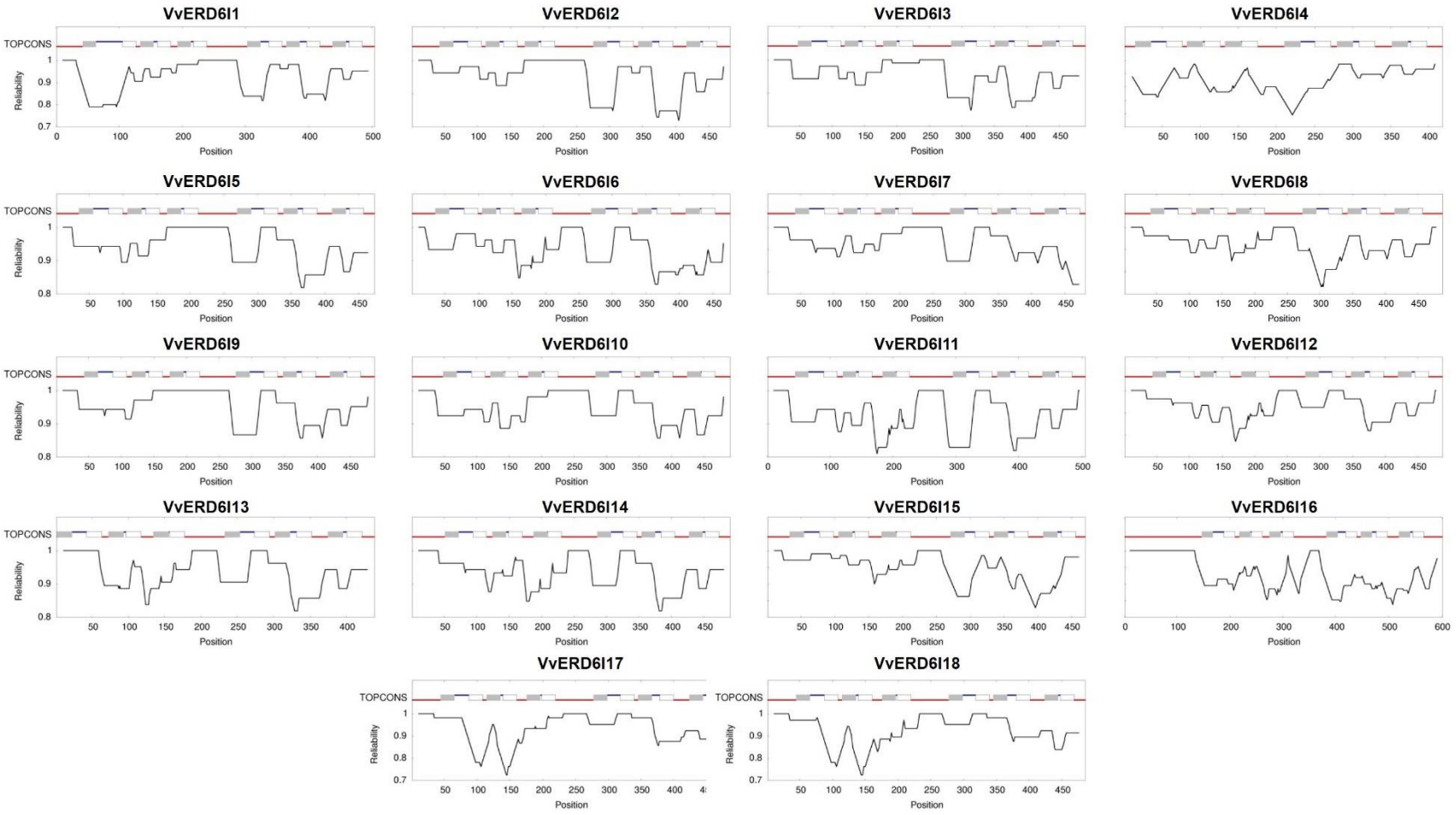
Annexes



Annex Figure 1 – Topology of all grapevine SWEET proteins predicted by TOPCONS software (Consensus prediction of membrane protein topology and signal peptides).



Annex Figure 2 –Topology of all grapevine ERD6l proteins predicted by TOPCONS software (Consensus prediction of membrane protein topology and signal peptides).

Annex table 1 - Cis-acting elements identified in the VvSWEETs promoter sequence via PLACE (Higo et al., 1999). Cis-element name, signaling pathway and number of copies on the promoter are shown.

Cis-acting elements	Response	SW1	SW2a	SW2b	SW3	SW4	SW5a	SW5b	SW7	SW9	SW10	SW11	SW12	SW15	SW17a	SW17b	SW17c	SW17d
Sugar responsive																		
ACGTABOX	Sugar-repression	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2
MYBGAHV	Gibberellin; sugar repression	1	3	0	1	3	0	4	2	1	0	2	2	2	0	1	2	4
PYRIMIDINEBOXOSRAMY1A	Gibberellin; sugar repression	1	2	11	2	2	3	2	1	2	3	2	4	3	4	2	2	4
SBOXATRBCS	Sugar responsive; ABA	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1
SREATMSD	Sugar repression	1	1	3	2	6	0	0	2	1	3	0	0	0	0	0	0	4
TATCCAOSAMY	Sugar; Hormone regulation	0	0	1	2	5	3	0	2	1	1	1	0	0	2	0	1	1
TATCCAYMOTIFOSRAMY3D	Sugar repression	0	0	1	0	5	1	0	0	0	1	1	0	0	1	0	0	0
WBOXHVISO1	Sugar responsive	5	3	4	4	2	3	2	5	3	1	5	4	0	3	1	1	6
SURE1STPAT21	Sucrose responsive	2	1	0	1	0	0	0	0	0	0	0	0	0	0	0	3	0
SURE2STPAT21	Sucrose responsive	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
CGACGOSAMY3	Sucrose starvation	0	0	0	0	0	0	0	1	0	0	2	0	0	0	0	0	0
TOTAL		**10**	**11**	**20**	**12**	**23**	**10**	**8**	**14**	**8**	**9**	**15**	**10**	**6**	**10**	**4**	**9**	**22**
Pathogen responsive																		
GT1GMSCAM4	Pathogen defense: salt stress	15	10	17	9	6	3	12	5	7	6	3	4	4	7	1	3	4
SEBFCONSSTPR10A	Pathogenesis-related	1	1	0	2	0	2	1	0	0	0	3	1	2	2	0	0	2
WBOXATNPR1	Pathogen defense	2	3	1	4	1	2	2	2	3	1	6	8	2	3	1	2	1
WRKY71OS	Pathogen defense; gibberellin	7	7	11	12	5	8	6	11	7	10	14	14	11	14	2	6	14
HSELIKENTACIDICPR1	Pathogen responsive	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
TOTAL		**25**	**21**	**29**	**29**	**12**	**15**	**21**	**18**	**17**	**17**	**26**	**27**	**19**	**26**	**4**	**11**	**21**
Abiotic stress responsive																		
GT1GMSCAM4	Pathogen defense: salt stress	15	10	17	9	6	3	12	5	7	6	3	4	4	7	1	3	4

MYBCORE	Drought	1	1	4	4	1	1	3	7	3	6	4	2	4	7	1	3	1
MYCCONSENSUSAT	Drought, cold, ABA	2	4	20	22	8	10	16	18	12	26	30	22	20	28	0	12	20
ABREATCONSENSUS	ABA, abiotic stress	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ASF1MOTIFCAMV	Abiotic and biotic stress related	0	0	0	0	0	0	1	0	0	1	2	3	0	1	0	2	1
DRE2COREZMRAB17	Drought; ABA; salt tolerance	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
DRECRTCOREAT	Drought; high light; cold; salt	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1
LTRECOREATCOR15	Drought; ABA	0	1	0	0	0	1	0	1	3	0	1	0	2	3	0	0	2
MYCATERD1	Drought responsive	0	1	3	3	0	0	2	3	0	1	3	2	2	1	0	0	2
MYCATRD22	Drought responsive	0	1	3	3	0	0	2	3	0	1	3	2	2	1	0	0	2
CBFHV	Cold; low temperature	2	2	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1
CRTDREHVCBF2	Cold	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LTRE1HVBLT49	Cold; low temperature	0	0	0	0	0	0	0	0	0	0	0	1	2	1	0	0	0
LTREATLTI78	Low temperature	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
TOTAL		**22**	**22**	**47**	**41**	**15**	**18**	**36**	**37**	**27**	**41**	**46**	**36**	**38**	**49**	**2**	**20**	**36**
Hormone responsive																		
ATHB6COREAT	Hormone responsive	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
GARE2	Gibberellin	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0
CAREOSREP1	Gibberellin	2	0	1	0	0	1	1	0	0	1	1	0	0	0	1	1	1
GARE1OSREP1	Gibberellin	0	0	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0
GARE2OSREP1	Gibberellin; ABA	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0
CATATGGMSAUR	Auxin	0	0	0	0	0	2	2	0	0	4	2	0	2	2	0	0	4
GAREAT	Hormone regulation	2	4	1	2	3	0	4	2	2	0	2	2	0	0	1	2	4
PYRIMIDINEBOXHVEPB1	Gibberellin; ABA	0	0	3	2	1	0	1	1	3	0	1	1	0	0	0	0	0
TATCCACHVAL21	Gibberellin	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0
ABRELATERD1	ABA	1	2	3	0	0	1	0	0	3	3	10	1	0	4	1	2	2
ABREMOTIFAOSOSEM	ABA	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0

ACGTABREMOTIFA2OSEM	ABA	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
ARFAT	ABA; auxin	2	1	0	3	0	2	1	1	0	1	2	1	1	1	0	0	0
DPBFCOREDCDC3	ABA	2	1	4	2	1	0	3	4	2	0	4	3	0	1	0	1	0
MYB1AT	ABA	4	3	0	5	2	1	1	2	4	5	3	2	2	4	0	6	5
MYB2CONSENSUSAT	ABA	1	0	3	1	0	1	0	3	1	3	2	2	3	3	0	2	0
PROXBBNNAPA	ABA	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
RYREPEATBNNAPA	ABA	0	1	0	2	1	2	7	5	1	3	5	3	6	1	0	1	1
RYREPEATVFLEB4	ABA; GA	0	0	0	2	0	2	4	0	0	0	4	0	0	0	0	0	0
MYBGAHV	Gibberellin; sugar repression	1	3	0	1	3	0	4	2	1	0	2	2	2	0	1	2	4
PYRIMIDINEBOXOSRAMY1A	Gibberellin; sugar repression	1	2	11	2	2	3	2	0	2	3	2	4	3	4	2	2	4
SBOXATRBCS	Sugar responsive; ABA	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1
TATCCAOSAMY	Sugar; Hormone regulation	0	0	1	2	5	3	0	2	1	1	1	0	0	2	0	1	1
TOTAL		**16**	**19**	**29**	**25**	**20**	**19**	**31**	**24**	**21**	**29**	**41**	**21**	**20**	**26**	**6**	**21**	**27**

Annex table 2 - Cis-acting elements identified in the VvERD6l promoter sequence via PLACE (Higo et al., 1999). Cis-element name, signaling pathway and number of copies on the promoter are shown. Asterisks indicate shorter promoter regions.

Cis-acting elements	Sequence	Response	1	2	3	4	5*	6	7	8	9*	10*	11	12	13	14	15	16	17	18
Sugar responsive																				
AMYBOX2	TATCCAT	Sugar starvation	0	0	0	0	0	0	0	0	1	0	2	0	0	0	0	1	0	0
ACGTABOX	TACGTA	Sugar-repression	4	0	0	2	0	0	0	0	0	0	2	0	0	0	0	0	0	0
MYBGAHV	TAACAAA	Gibberellin; sugar repression	1	2	0	2	1	1	2	3	0	0	2	0	1	0	1	0	1	2
PYRIMIDINEBOXOSRAMY1A	CCTTTT	Gibberellin; sugar repression	4	2	5	3	1	2	2	5	2	3	2	3	7	1	2	3	6	5
SBOXATRBCS	CACCTCCA	Sugar responsive; ABA	0	0	0	0	0	0	2	0	0	0	0	0	0	0	1	0	0	0
SREATMSD	TTATCC	Sugar repression	1	1	1	0	1	0	0	2	0	0	1	0	3	0	1	2	2	0
TATCCAOSAMY	TATCCA	Sugar; Hormone regulation	1	1	1	2	0	0	2	0	1	0	4	0	1	0	0	1	1	0
TATCCAYMOTIFOSRAMY3D	TATCCAY	Sugar repression	0	0	0	1	0	0	0	0	1	0	2	0	1	0	0	1	1	0
WBOXHVISO1	TGACT	Sugar responsive	8	1	2	2	0	0	5	2	2	2	5	5	3	6	3	7	0	1
SURE1STPAT21	AATAGAAAA	Sucrose responsive	0	0	1	3	0	0	0	0	0	1	0	0	2	0	0	0	0	0
SURE2STPAT21	AATACTAAT	Sucrose responsive	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
CGACGOSAMY3	CGACG	Sucrose starvation	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	0
TOTAL			**19**	**9**	**10**	**15**	**3**	**4**	**13**	**13**	**7**	**6**	**20**	**8**	**18**	**8**	**8**	**15**	**12**	**9**
Pathogen responsive																				
GT1GMSCAM4	GAAAAA	Pathogen defense: salt stress	1	6	13	15	1	7	6	7	2	8	4	5	7	4	7	7	6	7
SEBFCONSSTPR10A	YTGTCWC	Pathogenesis-related	2	1	2	2	0	2	2	0	0	2	0	3	0	1	0	2	0	2
WBOXATNPR1	TTGAC	Pathogen defense	5	3	3	1	0	0	3	1	3	2	5	6	3	4	3	7	4	4

WRKY71OS	TGAC	Pathogen defense; gibberellin	16	7	12	10	1	1	15	7	6	8	18	11	9	13	11	17	7	9
ELRECOREPCRP1	TTGACC	Elicitor response; pathogen	0	3	0	0	0	0	1	0	1	1	2	1	2	1	0	0	0	2
BIHD1OS	TGTCA	Disease resistance	5	2	8	4	1	1	4	4	2	4	8	4	3	5	5	7	6	4
TOTAL			**29**	**22**	**38**	**32**	**3**	**11**	**31**	**19**	**14**	**25**	**37**	**30**	**24**	**28**	**26**	**40**	**23**	**24**
Abiotic stress responsive																				
GT1GMSCAM4	GAAAAA	Pathogen defense: salt stress	1	6	13	15	1	7	6	7	2	8	4	5	7	4	7	7	6	7
MYBCORE	CNGTTR	Drought	2	3	6	3	0	2	8	0	4	0	3	4	1	3	2	6	0	6
MYCCONSENSUSAT	CANNTG	Drought, cold, ABA	12	8	12	12	16	4	20	6	8	14	8	14	14	18	16	16	12	14
ABREATCONSENSUS	YACGTGGC	ABA, abiotic stress	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
ASF1MOTIFCAMV	TGACG	Abiotic and biotic stress related	3	0	1	1	0	0	2	0	1	0	1	1	1	0	0	2	1	1
DRE2COREZMRAB17	ACCGAC	Drought; ABA; salt tolerance	0	1	1	0	0	0	2	0	0	0	0	0	0	0	0	1	0	0
DRECRTCOREAT	RCCGAC	Drought; high light; cold; salt	0	1	1	0	0	0	2	0	0	1	0	0	0	0	0	1	0	0
LTRECOREATCOR15	CCGAC	Drought; ABA	0	2	1	0	0	0	2	0	0	1	0	1	2	0	0	0	0	1
MYCATERD1	CATGTG	Drought responsive	2	1	1	0	1	0	1	0	0	2	1	2	3	2	2	1	0	0
MYCATRD22	CACATG	Drought responsive	2	1	1	0	1	0	1	0	0	2	1	2	3	2	2	1	0	0
CBFHV	RYCGAC	Cold; low temperature	1	2	1	0	0	0	2	0	0	1	0	1	0	1	0	2	0	2
CRTDREHVCBF2	GTCGAC	Cold	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
LTRE1HVBLT49	CCGAAA	Cold; low temperature	0	0	3	1	0	0	0	1	0	0	0	1	0	0	1	0	0	0
LTREATLTI78	ACCGACA	Low temperature	0	1	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
ABRELATERD1	ACGTG	ABA; early response dehydration	1	0	6	0	0	0	0	0	0	0	0	3	3	2	1	0	4	3

ACGTABREMOTIFA2OSEM	ACGTGKC	ABA; drougth and salt stress	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
ACGTATERD1	ACGT	Early response dehydration	10	2	8	4	0	0	6	0	2	0	6	8	0	8	2	0	14	6
MYB2AT	TAACTG	Drought stress	0	1	0	1	0	0	0	0	2	0	1	1	0	2	0	2	0	1
MYBATRD22	CTAACCA	Water stress	1	1	0	0	0	0	0	0	1	1	1	0	0	0	0	1	0	0
TOTAL			**35**	**30**	**57**	**37**	**19**	**13**	**54**	**14**	**20**	**30**	**26**	**43**	**36**	**42**	**33**	**40**	**37**	**43**
Hormone responsive																				
ATHB6COREAT	CAATTATTA	Hormone responsive	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
CAREOSREP1	CAACTC	Gibberellin	0	1	2	0	1	1	0	0	0	1	1	2	1	3	1	1	0	0
GARE1OSREP1	RTAACARANTCYGG	Gibberellin	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
GARE2OSREP1	TAACGTA	Gibberellin; ABA	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
CATATGGMSAUR	CATATG	Auxin	1	6	13	15	1	7	6	7	2	8	4	5	7	4	7	7	6	7
GAREAT	TAACAAR	Hormone regulation	3	2	0	2	1	2	2	4	0	0	2	1	1	1	1	0	3	2
PYRIMIDINEBOXHVEPB1	TTTTTTCC	Gibberellin; ABA	0	0	1	2	0	0	1	1	0	1	0	1	1	1	2	2	2	0
TATCCACHVAL21	TATCCAC	Gibberellin	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1
ABRELATERD1	ACGTG	ABA; early response dehydration	1	0	6	0	0	0	0	0	0	0	0	3	3	2	1	0	4	3
ACGTABREMOTIFA2OSEM	ACGTGKC	ABA; drougth and salt stress	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
ARFAT	TGTCTC	ABA; auxin	3	0	1	0	0	2	1	0	0	1	0	2	0	1	0	2	1	1
DPBFCOREDCDC3	ACACNNG	ABA	1	0	1	3	0	0	3	1	0	0	0	4	0	1	0	1	0	2
MYB1AT	WAACCA	ABA	2	5	2	3	1	1	5	3	2	5	4	2	4	5	3	2	1	2
MYB2CONSENSUSAT	YAACKG	ABA	0	2	2	2	0	1	4	0	4	1	1	2	1	2	0	3	0	4
PROXBBNNAPA	CAAACACC	ABA	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
RYREPEATBNNAPA	CATGCA	ABA	2	0	4	0	1	0	2	0	0	0	1	0	0	0	2	3	2	0
RYREPEATVFLEB4	CATGCATG	ABA; GA	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

MYBGAHV	TAACAAA	Gibberellin; sugar repression	1	2	0	2	1	1	2	3	0	0	2	0	1	0	1	0	1	2
PYRIMIDINEBOXOSRAMY1A	CCTTTT	Gibberellin; sugar repression	4	2	5	3	1	2	2	5	2	3	2	3	7	1	2	3	6	5
SBOXATRBCS	CACCTCCA	Sugar responsive; ABA	0	0	0	0	0	0	2	0	0	0	0	0	0	0	1	0	0	0
TATCCAOSAMY	TATCCA	Sugar; Hormone regulation	1	1	1	2	0	0	2	0	1	0	4	0	1	0	0	1	1	0
TOTAL			**19**	**22**	**41**	**36**	**7**	**18**	**32**	**25**	**11**	**20**	**22**	**26**	**29**	**21**	**21**	**25**	**27**	**30**

Annex table 3 – RNAseq experiments available in the NCBI database used in this study for correct structure assess of grapevine ERD6l.

NCBI SSR ID	Description	Reference
SRX012280	RNA-Seq analysis of *Vitis vinifera* post fruit set berry samples	Zenoni et al., (2010)
SRX012281	RNA-Seq analysis of *Vitis vinifera* veraison berry samples	Zenoni et al., (2010)
SRX012282	RNA-Seq analysis of *Vitis vinifera* ripening berry samples	Zenoni et al., (2010)
SRX019208	RNA-Seq analysis of *Vitis vinifera* stem and leaves	Pantaleo et al., (2010)
SRX019209	RNA-Seq analysis of *Vitis vinifera* tendrils	Pantaleo et al., (2010)
SRX019210	RNA-Seq analysis of *Vitis vinifera* inflorescences	Pantaleo et al., (2010)
SRX019211	RNA-Seq analysis of *Vitis vinifera* grape berries	Pantaleo et al., (2010)

Annex table 4 - Sequences of the primers used in the molecular biology approaches of this study.

Primer name		Sequence	Reference
attb-VvSWEET7	*FW*	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGCCATGTCTTCTACAGAA	Designed in this study
	RV	GGGGACCACTTTGTACAAGAAAGCTGGGTTCATGTTCTACGGACCTCAT	Designed in this study
attb-VvSWEET15	*FW*	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAGATGGCTATGGCCATGG	Designed in this study
	RV	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAAACTGTACTTCCATTTG	Designed in this study
qVvSWEET1	*FW*	GGATGCTCATCATGCTCTTCA	Chong et al., 2014
	RV	AAGCAGAGAGAAGGCAGTTGAG	
qVvSWEET2a	*FW*	CGTTCTCTGTTGTTGCCAGTC	Chong et al., 2014
	RV	ACCAAGCAGTTTAGGAGAGCA	
qVvSWEET2b	*FW*	AATGTCGGGATTATTGACAGCA	Chong et al., 2014
	RV	AGGGATGCAACACTCAAATATCC	
qVvSWEET4	*FW*	GGCTCGGACTGTGATTGGTA	Chong et al., 2014
	RV	ACATGCAGTTCATCACTGTGG	
qVvSWEET7	*FW*	ACCGCAGTTGGCATCCTA	Chong et al., 2014
	RV	GCAAGGTAGGGAACTGGTGA	
qVvSWEET10	*FW*	CCATTCACCATCCTTTGGTTT	Chong et al., 2014
	RV	CCACGTAGGGAACAGACTGAA	
qVvSWEET11	*FW*	GGGACGTGCATAGAAGCTACA	Chong et al., 2014
	RV	GCAGACCCAACCGACTATCTT	
qVvSWEET15	*FW*	GGCCAAGAAACAAACTCTCAAA	Chong et al., 2014
	RV	GCCACTGAGAATGAAGCACAG	
qVvSWEET17a	*FW*	GGTTTTGGTGTGGTTGTTGAA	Chong et al., 2014
	RV	AGCTAGAAACCCCACATCCAA	
qVvSWEET17d	*FW*	CTGGCGGCTTACTTGTCCT	Chong et al., 2014
	RV	AAAGCCAACATCCAATACGG	
qVvACT1	*FW*	GTGCCTGCCATGTATGTTGCCATTCAG	Conde et al., 2015
	RV	GCAAGGTCAAGACGAAGGATAGCATGG	
qVvGAPDH	*FW*	CACGGTCAGTGGAAGCATCA	Conde et al., 2015
	RV	CCTTGTCAGTGAACACACCAG	

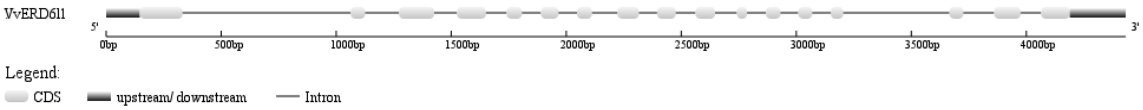
qVvSUC11	*FW*	TGTGCCAATCTCAAGTCTGCC	Pastenes et al., 2014
	RV	CCTGGGCTGCTGTTATGCTT	
qVvSUC12	*FW*	ACCAGCCTCACCATTTATCAGAC	Pastenes et al., 2014
	RV	ATTTCATAACTGCTCTCAGGGTTG	
qVvSUC27	*FW*	TGCTTGGCACTGACGGTACT	Pastenes et al., 2014
	RV	GCTGTAGGTGATCGCAAGAGG	
qVvHT3	*FW*	TAATCGAACGGGGATCAAG	Hayes et al., 2007
	RV	CCCCCAGAAATCAATAAAACTC	
qVvTMT1	*FW*	GTTGCCGTCAACTTCGCAAC	Hayes et al., 2007
	RV	GAAGGAATTTAGCTATGGCAGAG	
qVvCwINV	*FW*	ATGAATCATCTAGYGTGGAGCAC	Hayes et al., 2007
	RV	CTTAAACGATATCTCCACATCTGC	
attb-VvERD6l13	*FW*	GGGGACAAGTTTGTACAAAAAAGCAGGCTTATGGATGGTTTTGCTAAGC	Designed in this study
	RV	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAAGAATCAATGCATGCT	Designed in this study
qVvERD6l13	*FW*	CCTTTGAGTTCGGATCATGTGTGG	Designed in this study
	RV	TCTTCCCGGATGGCAGATTGAG	Designed in this study

Annex Table 5 –Grapevine *ERD6-like* genes structures identified in this study.

Name	**VvERD6-like 1**
GenBank Gene ID	LOC104881446
Genoscope 12x ID	GSVIVG01022029001
GenBank ID	XM_010661753.1
VCost_v3 ID	Vitvi14g00310

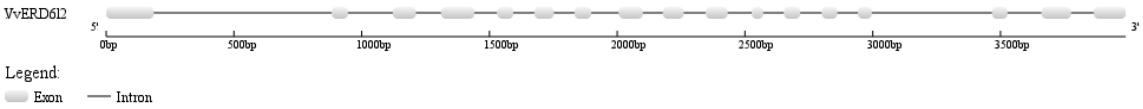
***Genbank correct annotation**

Gene Structure



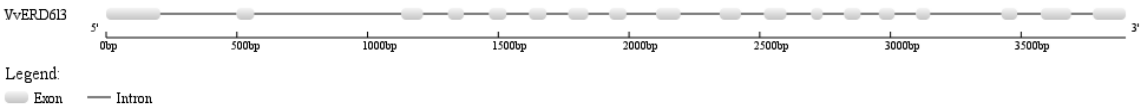
Name	**VvERD6-like 2**
GenBank Gene ID	LOC100247058
Genoscope 12x ID	GSVIVG01022026001
GenBank ID	XM_010661756.2
VCost_v3 ID	Vitvi14g00311

Gene structure



Name	**VvERD6-like 3**
GenBank Gene ID	LOC100267582
Genoscope 12x ID	GSVIVG01022030001
GenBank ID	XM_010661752.2
VCost_v3 ID	Vitvi14g02582

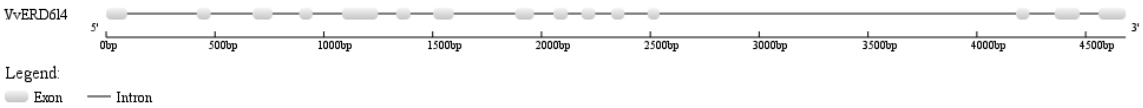
Gene Structure



Name	**VvERD6-like 4**
GenBank Gene ID	LOC100241924
Genoscope 12x ID	GSVIVG01022025001
GenBank ID	XM_019224810.1
VCost_v3 ID	Vitvi14g00312

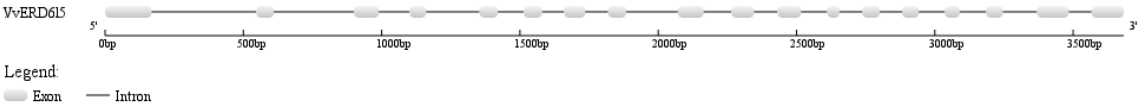
b-Vcost_v3 correct annotation

Gene structure



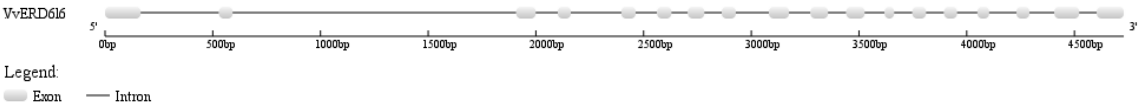
Name	**VvERD6-like 5**
GenBank Gene ID	LOC100245278
Genoscope 12x ID	GSVIVG01022032001
GenBank ID	XM_010661747.2
VCost_v3 ID	Vitvi14g02580

Gene structure



Name	**VvERD6-like 6**
GenBank Gene ID	LOC104881445
Genoscope12x ID	GSVIVG01022031001
GenBank ID	XM_010661751.2
VCost_v3 ID	Vitvi14g02581

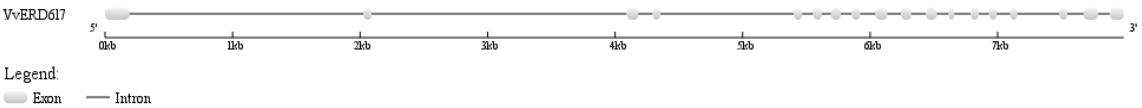
Gene structure



Name	**VvERD6-like 7**
GenBank Gene ID	LOC100854088
Genoscope12x ID	GSVIVG01022033001
GenBank ID	XM_010661765.2
VCost_v3 ID	Vitvi14g00308

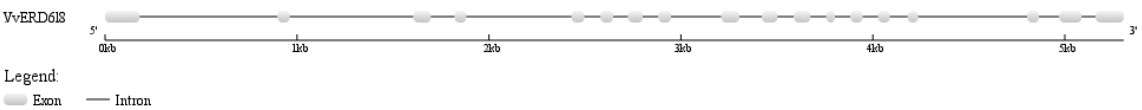
a-GenBank correct annotation

Gene structure



Name	**VvERD6-like 8**
GenBank Gene ID	LOC100265873
Genoscope 12x ID	GSVIVG01022034001
GenBank ID	XM_002263382.2
VCost_v3 ID	Vitvi14g00305

Gene structure

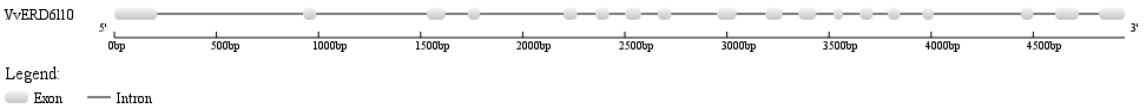


Name	**VvERD6-like 9**
GenBank Gene ID	LOC100253784
Genoscope 12x ID	GSVIVG01022035001
GenBank ID	XM_010661769.1
VCost_v3 ID	Vitvi14g00304

Gene structure

Name	**VvERD6-like 10**
GenBank Gene ID	LOC104881447
Genoscope 12x ID	GSVIVG01022024001
GenBank ID	XM_010661760.2
VCost_v3 ID	Vitvi14g00314

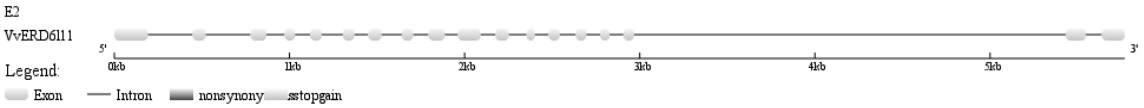
Gene structure



Name	**VvERD6-like 11**
GenBank Gene ID	LOC100263082
Genoscope 12x ID	GSVIVG01017845001
GenBank ID	XM_002278618.4
VCost_v3 ID	Vitvi05g01870

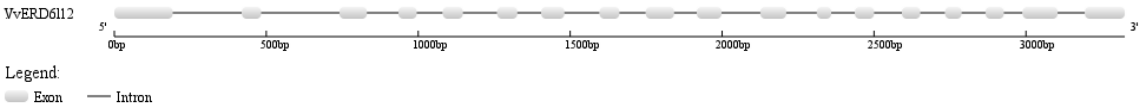
b-Vcost_v3 correct annotation

Gene structure



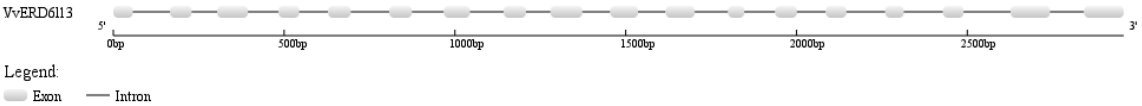
Name	**VvERD6-like 12**
GenBank Gene ID	LOC100240820
Genoscope 12x ID	GSVIVG01017844001
GenBank ID	XM_010651521.2
VCost_v3 ID	Vitvi05g01869

Gene Structure



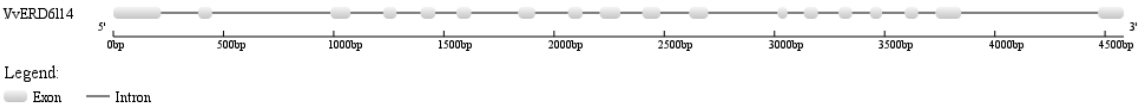
Name	**VvERD6-like 13**
GenBank Gene ID	LOC100261307
Genoscope 12x ID	GSVIVG01017836001
GenBank ID	XM_002278161.3
VCost_v3 ID	Vitvi05g00377

Gene structure



Name	**VvERD6-like 14**
GenBank Gene ID	LOC100263109
Genoscope 12x ID	GSVIVG01011047001
GenBank ID	XM_002270891.4
VCost_v3 ID	Vitvi07g00207

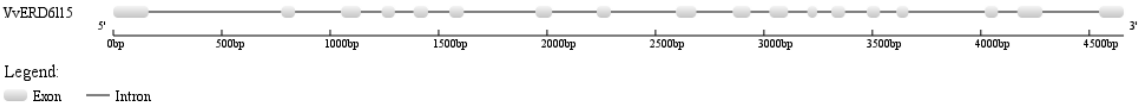
Gene structure



Name	**VvERD6-like 15**
GenBank Gene ID	LOC100264207
Genoscope 12x ID	GSVIVG01022022001
GenBank ID	XM_010661745.2
VCost_v3 ID	Vitvi14g02586

c-Manually curated sequence

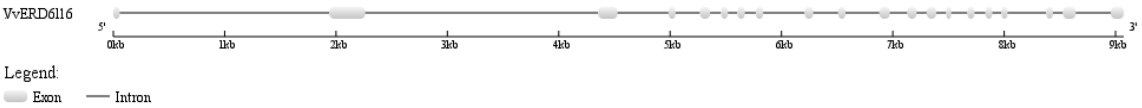
Gene structure



Name	**VvERD6-like 16**
GenBank Gene ID	LOC104881444
Genoscope 12x ID	GSVIVT01022023001
GenBank ID	XM_010661740.1
VCost_v3 ID	Vitvi14g02585

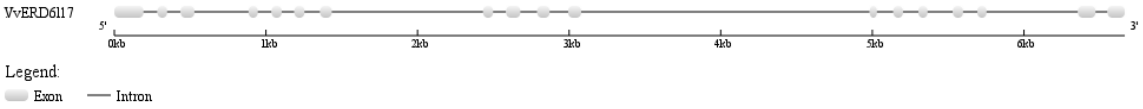
d-Genoscope correct annotation

Gene structure



Name	**VvERD6-like 17**
GenBank Gene ID	LOC100263259
Genoscope 12x ID	GSVIVG01018949001
GenBank ID	XM_002266668.3
VCost_v3 ID	Vitvi04g01302

Gene structure



Name	**VvERD6-like 18**
GenBank Gene ID	LOC100266019
Genoscope 12x ID	GSVIVG01009719001
GenBank ID	XM_019216809.1
VCost_v3 ID	Vitvi18g00970

Gene structure