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Mestrado em Fisiologia Molecular de Plantas

Trabalho efectuado sob a orientação do
Prof. Doutor Hernâni Gerós
e da
Prof. Doutora Sandra Paiva

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DECLARAÇÃO

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Abstract

Vitis vinifera is a major crop worldwide and in Portugal. Berry content in sugars, organic acids, phenolics and aroma compounds are fundamental for fruit and wine quality. These compounds are accumulated/metabolized during the development of the berry. In particular, berry sugar content is directly related to the final alcoholic content of wine, and regulates the development of its aromatic and organoleptic properties. Massive sugar accumulation in berry mesocarp occurs after *véraison* due to a combined action of monosaccharide (MSTs) and disaccharide transporters (DSTs).

High-temperatures affect berry set and development and alter the normal sugar content of the fruit. Also, peaks of high temperature, nowadays more and more frequent, may stop the ripening progress. We have been exploring the mechanisms involved in sugar import and compartmentation into the berry. VvHT1 (*Vitis vinifera* Hexose Transporter 1) is a high affinity plasma membrane H^+-dependent symporter with broad specificity for monosaccharides abundant at early stages of berry development. The expression of this transporter is tightly regulated by sugars at transcriptional and post-translational levels. In the present study we aimed at the elucidation of the effect of high temperature and temperature fluctuations on sugar transport in grape cells. Results showed that a temperature treatment of 38ºC for 12 h decreased by 40% the V_{max} of ^{14}C-glucose transport in CSB (Cabernet Sauvignon Berry) cells. Contrarily, abscisic (ABA) and salicylic acid (SA) stimulated sugar uptake by 28.7% and 62.5%, respectively. ABA and SA also stimulated ^{14}C-glucose accumulation in intact grape berries by 88.7% and 67.8%, respectively. The down-regulation of glucose uptake mediated by high temperature corroborated the observed decrease of the VvHT1 levels in the plasma membrane. Moreover, after high-temperature treatment the intracellular ROS levels and lipid peroxidation increased by 97% and 29%, respectively. Proteomic analysis of the plasma membrane of CSB cells, allowed the identification of several proteins up-regulated in response to high temperature. It is hypothesised that intracellular ROS levels can mediate this cellular response to high-temperature. To study the recycling and turnover of VvHT1 in response to high-temperature a VvHT1-GFP expression clone was produced and a protocol for transient protoplast transfection is currently being optimized.

Resumo

A videira (*Vitis vinifera*) é uma espécie agrícola de elevada importância ao nível mundial e em Portugal. O conteúdo do bago em açúcares, ácidos orgânicos, compostos fenólicos e aromáticos determina a qualidade final do fruto e do vinho. Estes compostos são acumulados/metabolizados durante o desenvolvimento do fruto. O conteúdo do bago em açúcares condiciona o teor alcoólico do vinho, além de regular o desenvolvimento das suas propriedades aromáticas e organolépticas. A acumulação massiva de açúcares que ocorre no mesocarpo após a fase de pintor (*véraison*) resulta da acção combinada de transportadores membranares de mono (*MST*) e de dissacarídeos (*DST*).

Temperaturas elevadas podem afectar a frutificação e o desenvolvimento do bago, bem como alterar o seu conteúdo normal em açúcares. Adicionalmente, picos de temperatura, muito frequentes no contexto das modificações climáticas em curso, podem comprometer o processo de amadurecimento. O nosso grupo de investigação tem dedicado atenção particular ao estudo dos mecanismos envolvidos no transporte e compartimentação de açúcares no bago. O transportador da membrana plasmática VvHT1 (*Vitis vinifera* Hexose Transporter 1), expresso nas fases iniciais do desenvolvimento do bago, medeia a incorporação da glucose e de outros monossacarídeos por um mecanismo de simporte com H^+. A expressão deste transportador é finamente regulada pelo teor em açúcares ao nível transcricional e pós-transcricional. No presente trabalho foi estudado o efeito de temperaturas elevadas, e de flutuações de temperatura, no transporte de açúcares em células de videira. Os resultados demonstram que um pico de temperatura de 38ºC aplicado durante 12 h reduz em 40% a V_{max} de transporte da ^{14}C-glucose em culturas celulares (CSB, *Cabernet Sauvignon Berry*). Contrariamente, o ácido abcísico (ABA) e o ácido salicílico (SA) estimularam o transporte de açúcar em 28,7% e 62,5%, respectivamente. O ABA e o SA também estimularam a incorporação de ^{14}C-glucose em bagos intactos em 88,7% e 67,8%, respectivamente. A repressão do transporte da glucose causada por temperaturas elevadas correlacionou-se com a detecção de níveis diminuídos do VvHT1 na membrana plasmática. Adicionalmente, a exposição a elevadas temperaturas aumentou os níveis intracelulares de ROS e de peroxidação lipídica em 97% e 29%, respectivamente. Uma análise de proteómica efectuada em membranas plasmáticas de células CSB, permitiu a identificação de diversas proteínas especificamente expressas em resposta a temperaturas elevadas. É discutido que o aumento observado dos níveis intracelulares de ROS podem mediar esta resposta celular a elevadas temperaturas. No sentido de estudar a reciclagem e *turnover* do transportador VvHT1 em resposta a temperaturas elevadas, foi construído um clone de expressão VvHT1-GFP e optimizado um protocolo de transformação de protoplastos.

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Abbreviations and acronyms

2-NBDG	2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose
ABA	Abcisic acid
ASR	ABA abscisic acid-, stress-, and ripening-induced
BOR1	Boron transporter 1
BSA	Bovine serum albumin
cDNA	Complementary DNA
CHIP	Carboxyl terminus of Hsc70-interacting protein
CSB	Cabernet Sauvignon berry
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
dpm	Disintegrations per minute
DST	Disaccharide transporter
DW	Dry weight
EDTA	Ethylenediamine tetraacetic acid
FAO	*Food and agriculture organization*
FDA	Fluorescein diacetate
FW	Fresh weight
GFP	Green fluorescent protein
GUS	β-Glucuronidase
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HSP	Heat shock protein
kDa	Kilodalton
LB	Luria broth
LC MS/MS	Liquid chromatography-tandem mass spectrometry
MDA	Malondialdehyde
MES	2-(*N*-morpholino)ethanesulfonic acid
MFS	Major facilitator superfamily
MS	Murashige and Skoog
MST	Monosaccharide transporter
NADPH	Nicotinamide adenine dinucleotide phosphate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethylsulfonyl fluoride

PVPP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-PCR
SA	Salicylic acid
SDS	Sodium dodecyl sulphate
SE/CC	Sieve elements/companion cell
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane
VvHT	*Vitis vinifera* hexose transporter
VvMSA	*Vitis vinifera* maturation-, stress-, ABA-induced protein
VvSK1	*Vitis vinifera* sugar-inducible protein kinase 1
VvSUC	*Vitis vinifera* sucrose transporter

Introduction

Grapes (*Vitis* spp.) are economically the most important fruit species in the world. More than 50 species are recognized in the grape genus *Vitis*, but almost all world wine is made from *Vitis vinifera*, native to the area south of the Caucasus Mountains and the Caspian Sea (Kunkee and Goswell 1996). In 2009 the vineyard area cultivated in the world and total fruit production was of 7,437,141 ha and 66,935,199 t, respectively. In the same year in Portugal the vineyards occupied the largest cultivated area for a fruit crop (222,700 ha) and had the greatest production (487,800 t) (FAO 2009). Approximately 71% of grape production is used for wine, 27% as fresh fruit, and 2% as dried fruit (Conde et al. 2007). Throughout antiquity the conversion of grapes into wine was considered a gift from the gods and the best wines were reserved for the elite of the society. Nowadays, wine is an integral component of the culture of many countries, a form of entertainment in others, and a libation of choice for advocates of its health benefits. Unlike many modern foods, wine's attraction relies not on strong consistent flavors, but upon a subtle array of shifting sensations that make its charm difficult to define (Bisson et al. 2002).

Wine is composed of various constituents that include water, sugars, alcohol, phenolics, acids and mineral salts. The main constituent of wine is water accounting for 75 to 90% (v/v), and this variation is explained by the amount of the other constituents that form the wine extract that differ from wine to wine. The second largest constituent is ethyl alcohol, which, according to the type of wine, varies from 8% to 15% (v/v). Another important constituent is sugar, which is directly responsible for the final alcoholic content of the wine. A normal dry wine generally has less than 2 g sugar/L, while in a botrytized sweet wine it can reach almost 200 g sugar/L (Dominé et al. 2004).

Vineyards can be found in Europe, Northern and Southern America, Africa and Asia. Although this worldwide distribution, the most important factor for viticulture is climate and, above all, temperature. Grapes clearly prefer moderate conditions, and rarely thrive where temperatures rise above 25ºC in the summer months. In a large part of Western Europe, the location of the majority of Europe's classic viticultural regions, average July temperatures vary between 15 and 25℃. Rainfall and drought also play an important role, and it is almost impossible to grow vines with less than 200 mm of rain a year. On the other hand, too much rain also makes it difficult to cultivate grapes. A moderate climate, with adequate to relatively high rainfall, provides ideal conditions for producing both fragrant white wines with a good structure and acidity, and well-balanced red wines with good potential for maturing (Dominé 2004). Wine quality largely depends on the vineyard and on the vine grower. Most of the wine compounds are produced by the plant itself, in the leaves (sugars and acids), and in

berry (acids and phenolics). Furthermore, some molecules related to aroma and taste are produced during the fruit development and ripening, being their spectrum specific to a given variety. Theses aromas, called “varietal” or primary aromas, are the grape’s signature, recognizable by the consumer during degustation. Thus, the growth and the fructification of grapevines in the vineyard are of utmost importance to wine quality (Blouin and Guimberteau 2000).

1.1 Grape berry structure and development

Grape berry, a non-climateric fruit, is essentially an independent biochemical factory (Gholami et al. 1995), and is mainly composed of three distinct tissues, skin, flesh and seeds (Figure 1), with the sheer bulk of wine being derived from the flesh. These tissues vary considerably in composition and contribute differently to the overall wine composition. The flesh accounts for 75-85% of total berry weight, while the skin and the seeds account for about 7% and 4%, respectively (reviewed by Conde et al. 2007; Jackson 2008). Also, the grape berry is richly supplied with vascular tissue, which after it enters the fruit via the pedicel, branches out to supply the developing seeds, the flesh and the skin (Hardie et al. 1996).

The grape berry skin contributes to the integrity of the whole berry by protecting inner tissues against mechanical damage or pathogen attack, promotes seed dispersion by providing a high contrast between background foliage and fruits as well as providing protection from UV light exposure (Grimplet et al. 2007). The vacuole of skin cells is where most of the aromas that arise from volatile compounds, such as terpenes, norisoprenoids, and thiols, are stored as sugar or amino acid conjugates. These compounds are fundamental for wine making, and the variability of skin composition plays an important role in determining the color, aroma, and other organoleptic properties of wine (Lund and Bohlmann 2006).

The primary role of the flesh is to provide a high value nutritional content for dispersal agents, including high concentrations of free amino acids and hexose sugars (mainly sucrose and fructose). During wine production, the flesh contributes with the majority of sugars, which are transformed into alcohol during the fermentation process (Grimplet et al. 2007). Also, the berry flesh accumulates organic acids (mainly tartaric and malic), mineral cations (especially K^+), nitrogenous compounds (soluble proteins, ammonia and amino acids), pectic substances (cell wall structural material composed of galacturonic acid polymers) and non-flavonoid phenolics (primarily benzoic and cinnamic acid derivatives).

The seeds are the main source of flavan-3-ol monomers and procyanidins that have an important contribute to the organoleptic properties of wine including bitterness and astringency (Robichaud and Noble 1990).

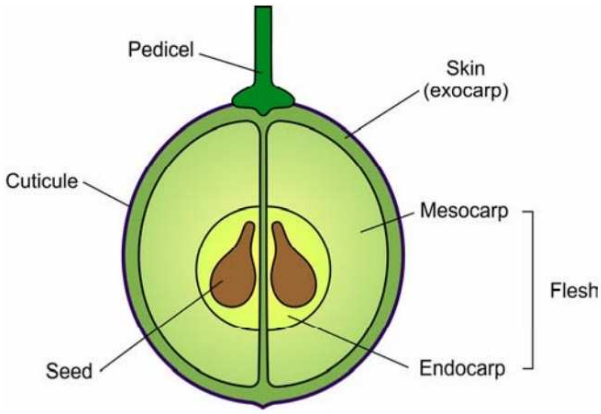

Figure 1. Structure of a ripe grape berry, depicting the three primary tissues: skin, flesh and seeds (Conde et al 2007)

Grape berry development, from fruit set to full maturation, is a complex series of processes requiring the coordination of a large number of events involving substantial and rapid changes in a number of tissues (Conde 2007). In grapes fruit set is defined as the stage when the berry diameter is between 1.6 and 3.2 mm. Climatic factors have a significant effect on fruit set, and, particularly, temperature can inhibit pollen tube growth and ovule fertilization. Also, fruit set is greatly reduced when temperatures fall below 18.3℃ or exceed 37.8℃ (Dookozlian 2000).

The development of the grape berry has been divided into three phases. Increases in berry weight, volume, or diameter during development are typically characterized by a double-sigmoid curve resulting from two consecutive stages of growth separated by a phase of slow or no growth (Figure 2; Coombe 1992; Dookozlian 2000). The first phase (green stage) is a period of rapid berry growth that occurs immediately after bloom. During this time, berries grow both through rapid cell division and cell enlargement. In this phase berry texture is firm, while its color is green due to the presence of chlorophyll. The sugar content of the berry remains low, while organic acids accumulate, mainly tartaric and malic acids (Figure 2). Berry growth during stage I is very sensitive to temperature. Temperatures exceeding 35℃ reduce growth rate and size at harvest and light is also important for optimum berry growth. Berries subject to heavy shade immediately after berry set are significantly smaller at harvest than berries that have been well exposed to light. The second phase (lag

phase) is marked by decrease in the growth rate while its organic acid concentration reaches its highest level and the berries remain firm and begin to lose the chlorophyll content. This phase ends with the *véraison*, a French term that defines the onset of ripening, and the last phase of berry development begins (Figure 2). The third phase (ripening phase) of berry development is characterized by striking changes in fruit characteristics. The berry dramatically increases in size mainly due to cell expansion, which is caused by massive sugar accumulation, water import and also to the deposition of phenolics, among others (Reviewed by Conde et al. 2007). Also, the berries soften and lose chlorophyll, the concentration of organic acids greatly declines, and is observed a colour development and the synthesis and accumulation of aroma and flavour compounds (Figure 2). This phase of berry development is of remarkable importance to wine industry, considering that the accumulation of these compounds results in an increase of the organoleptic properties of the fruit and wine. When conditions are warm and degree-days accumulate rapidly, ripening is accelerated. Prolonged periods of excessively high temperatures following berry softening, for instance 3 to 4 consecutive days above 40.6℃, may retard berry ripening. The effects of elevated temperatures on fruit ripening are temporary and, depending upon the degree of heat stress, sugar accumulation can proceed normally once temperatures return to a normal range (Dookozlian 2000).

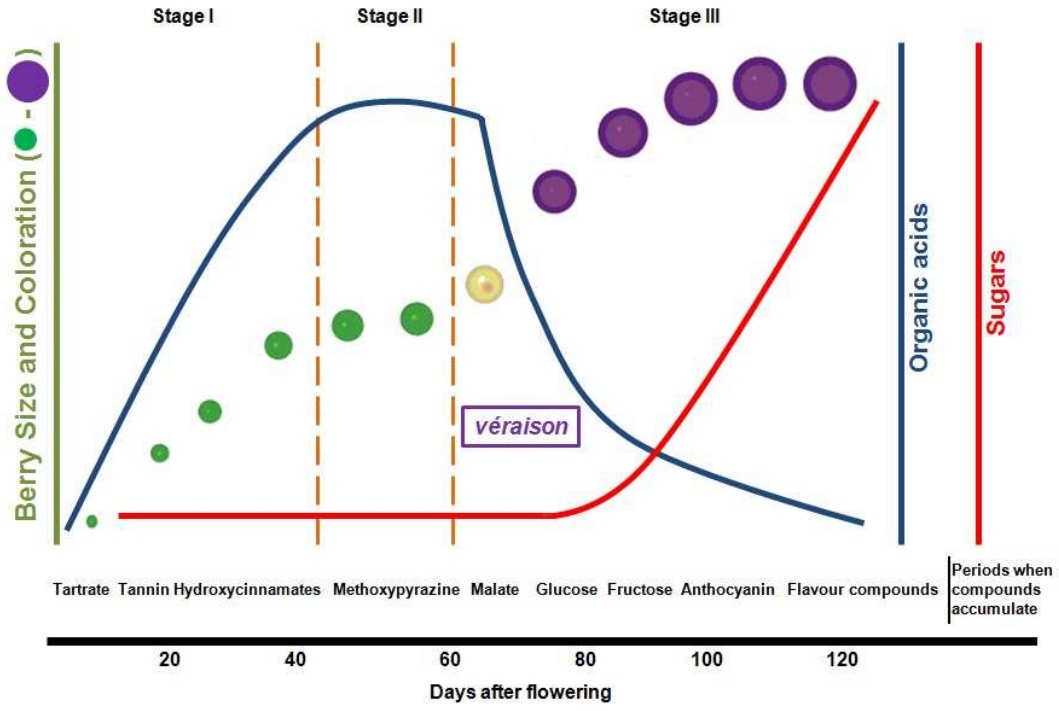

Figure 2. Grape berry development and ripening.

1.2 Hormonal control of berry development

Grape berry development is under tight hormonal regulation, and several hormones have been identified and characterized as regulators of fruit ripening. The pattern of ABA accumulation during berry development is well defined, with high free ABA levels in the flesh of young berries, followed by a rapid decreased to low levels that remains during most of the pre-*véraison* phase (Wheeler et al. 2009). After *véraison*, a peak of ABA level occurs simultaneously with an increase in sugar accumulation and color development. Following this peak in ABA levels after *véraison*, the levels declined as the fruit reaches full ripeness (Kondo et al. 1998; Owen et al. 2009). This pattern of accumulation provides correlative evidence for a role of ABA in ripening and perhaps in its initiation. Also, ABA is mainly found in the phloem of the berry which is consistent with a role in the unloading and uptake of photoassimilates (Kataoka et al. 1982; Shiozaki et al. 1999).

Salicylic acid is involved in signalling in plants, particularly in the induction of defense and stress responses (Bari and Jones 2009). The levels of salicylic acid during grape berry development have not yet been reported, and so it is difficult to propose a developmental role for this hormone.

Like other plant hormones cytokinins are involved in a diverse range of processes (Werner and Schmülling 2009). Cytokinins levels are high in one week old berry flesh but decreased rapidly to low levels by the time of véraison (Zhang et al. 2003). This pattern of accumulation is in agreement with the proposed roles for cytokinins in flower development and fruit set, and is also consistent with the ability of cytokinins to delay berry development (Werner and Schmülling 2009).

Brassinosteroids are generally associated with plant growth and stress response (Haubrick and Assmann 2006). Recently a role for brassinosteroids in fruit development, in particular during ripening, was described (Symons et al. 2006). High levels of brassinosteroids where found in flowers and young leaves, declining prior to *véraison*. Furthermore it has been described a peak in brassinosteroids after *véraison* followed by a rapid decline (Wang et al. 2001). This pattern of accumulation suggest that brassinosteroids may play a role in ripening-associated processes, for example, in the post-*véraison* phase of berry growth or perhaps it is part of a response to the stress resulting from the massive sugar influx that occurs after véraison.

Auxins levels are high in the early phases of berry development, after which they decline steadily to very low levels at *véraison* (Inaba et al. 1976). The high levels early in berry development are in agreement with the proposed role for auxin in cell division and expansion.

Gibberellins are regulators of many processes during plant development involving cell division and expansion (Olszewski et al. 2002). The consensus of a number of studies is that gibberellins levels in the flesh of seeded berries were high at around flowering and early in berry development after which they decreased steadily (Pérez et al. 2009). The observed pattern of gibberellins accumulation is consistent with the proposed role in cell division and expansion in the initial stages of berry development.

1.3 Effect of temperature on grape berry

High temperatures are a major threat to crop productivity. As it was already mentioned, temperature has a dramatic effect in the berry development altering normal berry set, inhibiting pollen tube growth and ovule fertilization. Also, high temperatures during the initial stages of berry development dramatically reduce the berry size and weight at harvest, and it is known that high temperature peaks may stop the ripening progress (Dookozlian 2000).

High temperature affects berry composition, especially titratable acidity, total soluble solids, and anthocyanin content (Poudel et al. 2009). It has been reported that temperature has a dramatic effect on anthocyanin accumulation in grape berry skin, a major contributor to wine organoleptic properties and quality (Mori et al. 2005; Yamane et al. 2006). It was found that a 30℃ night temperature greatly reduces the coloration of Cabernet Sauvignon grapes as compared to fruits ripened at night time temperatures of 15 and 20℃ (Kliewer 1972). The limiting factor behind this decrease in anthocyanin synthesis and accumulation under high temperature appears to be a reduced accumulation of soluble sugars in fruit (Mazza and Miniati 1993). In fact high temperatures appear to limit glucose and fructose accumulation in the berry. Poudel et al (2009) reported that vines exposed to 30℃ had a lower glucose and fructose content than vines subjected to 20 and 25 ℃. The enhanced expression of various genes of the anthocyanins biosynthetic pathway in the berry skin may be correlated with the concomitant accumulation of sugars in the flesh and, concordantly, sucrose treatment promotes anthocyanin synthesis in *V. vinifera* cell cultures (Agasse et al 2007; Agasse et al. 2008). Although some studies have been developed to evaluate the effect of temperature on the grape berry composition, little information is available at the molecular level.

1.4 Long distance sugar transport

Efficient assimilation and use of nutrients by plants is of prime importance for the optimization of crop productivity. The grape berry is considered to be mainly a sink for primary metabolites essential for plant survival, and rely on the use of available carbohydrate resources produced by photosynthesis to support their growth and development. Sugars transport and allocation between the photosynthetic “source tissues” and the heterotrophic “sink tissues” is known as assimilate partitioning and is a major determinant of plant growth and productivity (Kingston-Smith 2001).

In the majority of plants sucrose is the main sugar transported via the floem, although several other solutes have been identified, including raffinose, stachiose and the sugar-alcohols mannitol and sorbitol. The main advantage for long distance transport of sucrose is its non-reducing nature and relative insensitivity to metabolism (Salisbury and Ross 1994), allowing long distance transport without the problem of metabolism easily encountered with glucose. Also, according to Munch’s mass flow hypothesis, sucrose, as the major osmotically active constituent in the phloem, also provides the driving force for translocating all other compounds in the phloem sap (Conde et al. 2006). Sucrose derived from leaf photosynthesis is exported via the phloem to the berries. From *véraison* and throughout ripening the berries accumulate roughly equal amounts of glucose and fructose, reaching over 1 M of each hexose, suggesting that phloem transported sucrose is hydrolyzed at some step during its transport from the leaves to the vacuole of the mesocarp cell (Figure 3; Coombe 1987; Conde et al. 2006; Conde et al. 2007; Agasse et al. 2009).

From its point of synthesis in the mesophyll sucrose may be loaded into the SE/CC complex either through plasmodesmata or via the apoplast. Apoplastic loading requires sucrose export from the mesophyll or the vascular parenchyma by a sucrose exporter that remains unidentified, and reuptake into the SE/CC complex by a sucrose/H^+ symporter. When in the phloem, hydrostatic pressure drives phloem sap movement toward sink tissue. Passive leakage can take place along the path and reuptake of leaked sucrose occurs along the phloem. Phloem unloading also occurs through a symplastic (via the plasmodesmata) and apoplastic mechanism. Apoplastic phloem unloading implies the existence of a sucrose exporter that remains unidentified, at the sink tissue. In the berry tissues symplastic connections via plasmodesmata between sieve tubes and mesocarp cells remain for quite a long period during fruit development. However, several lines of evidence indicate that the apoplastic pathway play a major role at late stages of grape berry development. Indeed, there is a shift from symplastic to apoplastic phloem unloading at the onset of ripening (Zang et al.,

2006). The data available on invertase activity also support an apoplastic pathway of sugar unloading during grape berry development (Agasse et al. 2006; Agasse et al 2009). It appears that sugar accumulation in sink organs would rather result from the coordinated action of several mechanisms, involving various transporters and hydrolytic enzymes. Therefore, the presence of an apoplastic step requires the involvement of membrane-located sugar transporter proteins mediating the exit of sucrose from the phloem, and the uptake and compartmentation of sugars across the plasma membrane and the tonoplast of flesh cells. Concordantly, both DST and MST families have been characterized in plants (reviewed by Williams et al. 2000)

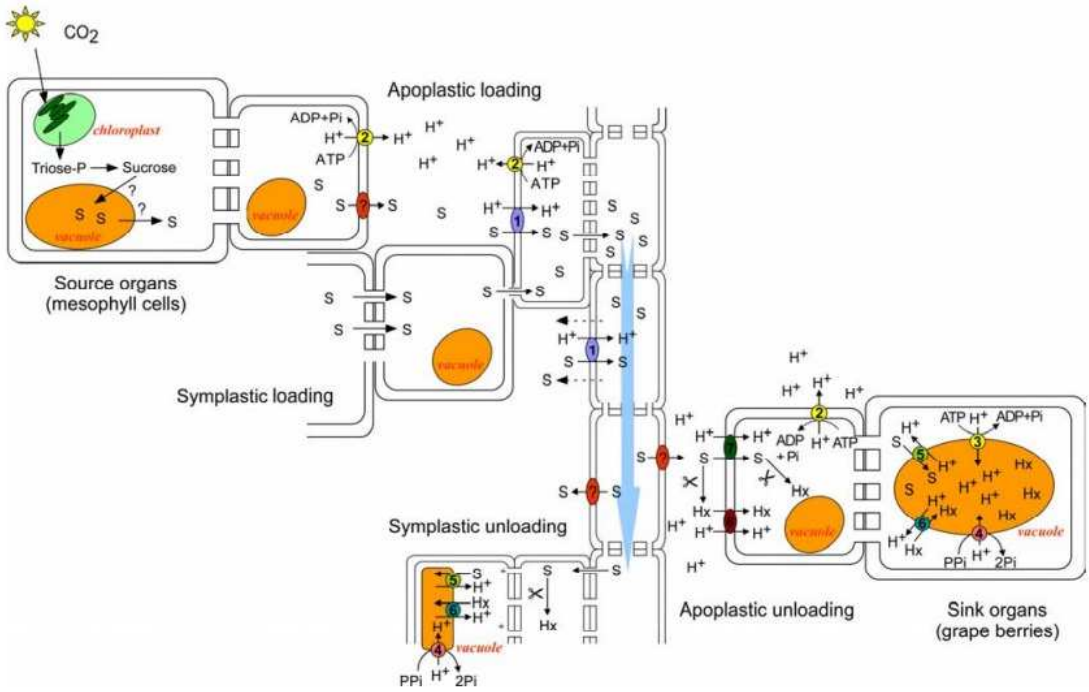

Figure 3. Long distance sugar transport through the floem. From the place of sinthesys in the leaves mesophyll, sucrose may be loaded into the sieve elements/companion cell complex by apoplastic and symplastic mechanisms. Hydrostatic pressure drives phloem sap movement toward sink tissues. The unloading of the floem may occur via the apoplast, in coordination with cell wall invertases and monosaccharide transporters, or through plasmodesmata (Adapted from Lalonde et al. 1999).

1.5 Disaccharide transporters

It has already been mentioned that sucrose is the main carbohydrate transported through the phloem. The first plant sucrose transporter was identified in spinach and named SoSUT1 (*Spinacea oleracea* Sucrose Transporter 1), followed by the cloning of the potato sucrose transporter, StSUT1 (*Solanum tuberosum* Sucrose Transporter 1)

(Reismeiner et al 1992; Reismeiner et al 1993). In situ studies showed that the sucrose carrier from potato, StSUT1, is highly expressed in the phloem of the leaf minor veins, the major site of phloem loading (Reismeiner et al 1993). The cloning of several DST led to the establishment of a topological protein model. The protein structure is composed of 12 α-helices domains highly conserved and a cytoplasmatic loop with a high degree of variability.

The importance of sucrose transporters in phloem loading was further supported by studies on transgenic potato and tobacco plants. In these studies, antisense RNAs were used to reduce the level of the sucrose carrier SUT1 in the phloem (Reismer et al 1994; Kuhn et al. 1996; Lemoine et al. 1996; Burkle et al. 1998). Results correlated with the expected function of SUT1 in phloem loading, considering that the antisense plants showed a retarded growth phenotype on soil, and their source leaves were found to export fewer sugars causing an accumulation of carbohydrates in the leaves and a concomitant decrease in the sink tissues. These observations clearly showed that sucrose transporters in the phloem are essential for carbohydrate partitioning, at least in tobacco and potato, both members of the Solanaceae family (Tuernit 2001).

In *Vitis vinifera* three DST cDNAs were cloned (*VvSUC11*, also identified as *VvSUT1, VvSUC12* and *VvSUC27*) and characterised as proton-dependent sucrose transporters, whereas 9 DSTs sequences are present in *Arabidopsis* genome (Sauer et al. 2004). VvSUC11 and VvSUC12 are intermediate affinity sucrose transporters with 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 with a K_m of about 10 mM (Zhang et al. 2008). *VvSUC11* is expressed in flowers and fruits whereas *VvSUC12* expression is restricted to berries and young leaves. *VvSUC27* expression is closely related to sink activity since its transcripts are strongly accumulated in flowers and unripe berries, roots and tendrils but poorly present in mature leaves being associated with the early stages of berry development (Davies et al. 1999). Furthermore, *VvSUC11* and *VvSUC12* transcription increases with post-*véraison* sugar accumulation, which suggests a direct pathway for sucrose acquisition by berry cells (Davies et al. 1999). In spite of the available information regarding grapevine sucrose transporters, the knowledge about the localization of sucrose transporters in berry flesh and the mechanisms of sucrose uptake along ripening is scarce and further investigation is needed.

1.6 Monosaccharide transporters (MST)

Monosaccharides represent the most important carbon and energy source for the majority of heterotrophic organisms. In the Calvin cycle and gluconeogenesis the carbon (CO_2) fixated through photosynthesis is converted to monosaccharides, like glucose and fructose (Buttner and Sauer 2000). The first MST cloned was the *HUP1* (*Hexose Uptake Protein 1*) from the unicellular algae *Chlorella kessleri*. These cells can switch from an autotrophic to a heterotrophic metabolism inducing hexose transporters when sugars are available. This interesting feature of the algae metabolism led to the identification of a 49 kDa protein by differential screening of cDNA libraries of induced and non-induced cells (Sauer and Tanner 1989). The functional characterization of the protein by complementation of hexose transport null-mutant yeasts demonstrated that the transport was a hexose-proton symport (Sauer et al. 1990). Since then several MST have been identified. *A. thaliana* genome has 53 homologous sequences encoding putative MSTs (Büttner 2007). A remarkable feature is that all MST share the same 12 transmembrane domains with N- and C- cytoplasmic termini structure, a characteristic of all sugar transporters belonging to the Major Facilitators Superfamily (Figure 4; Williams et al. 2000, Delrot et al. 2001).

In *V. vinifera* 59 putative hexose transporters encoding genes have been identified based on protein motif recognition (Samson et al. 2004, Jaillon et al. 2007). Six full length cDNAs encoding for MST and named VvHT1-6 (*V. vinifera* Hexose Transporter 1-6) were previously cloned from various grape cultivars such as Pinot noir, Ugni blanc, Chardonnay, Cabernet Sauvignon and Syrah (Fillion et al. 1999; Vignault et al. 2005; Hayes et al. 2007). The predicted peptides share about 60% identity to each other (Büttner and Sauer 2000, Büttner 2007). Interestingly, VvHT6 is related to AtTMT2 (*Arabidopsis thaliana* Tonoplast Monosaccharide Transporter 2), a member of the Tonoplast Monosaccharide Transporter subfamily of the Major Facilitators Superfamily. AtTMTs are tonoplastic hexose-proton antiporters induced by abiotic stresses such as cold or drought and were suggested to play a role as sensors (Wormit et al. 2006).

Uptake activities of VvHT1, VvHT4 and VvHT5 have been demonstrated by heterologous expression in the *hxt*-null mutant yeast EBY VW 4000 (Wieczorke et al. 1999). All three VvHTs are high affinity, H^+-dependent transporters mediating the uptake of radiolabelled D-[U-^{14}C]glucose according to saturable Michaelis-Menten kinetics. VvHT1 exhibits the highest affinity for glucose (K_m of 70 µM) compared to VvHT4 and VvHT5 (K_m about 150 µM and 100 µM, respectively) and is the only one able to restore the growth of the complemented yeast on glucose. VvHT3 was not able

to transport any of the tested radiolabelled sugars in the deficient yeast model (Vignault et al. 2005; Hayes et al. 2007). Up to date, attempts to confirm the transport activity of both VvHT2 and VvHT6 in yeast has had little success.

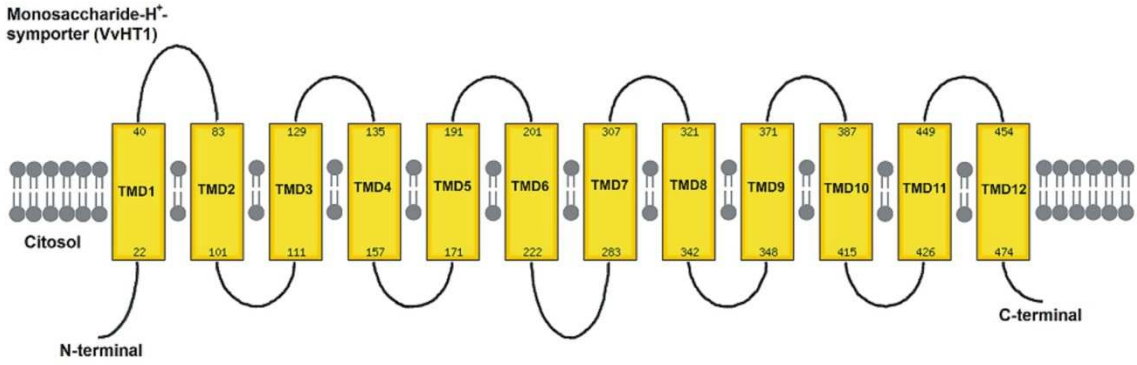

Figure 5. Topological model of VvHT1, a typical MST, with 12 trans-membrane domains with N- and C- cytoplasmic termini structure characteristic of the Major Facilitators Superfamily.

1.7 Identification, localization and characterization of the grape berry monosaccharide transporter VvHT1

VvHT1 was firstly identified and cloned by Fillion et al. (1999) from two different grape cultivars, Pinot Noir and Ugni-Blanc. Preliminary studies by reverse transcription-PCR suggested that VvHT1 was mainly expressed in the berries, with a first peak of expression at anthesis, and a second peak about 5 weeks after véraison. Although it has been reported that VvHT1 expression slightly increases after véraison in Ugni-Blanc berries (Fillion et al. 1999), detailed microarray analysis suggested that the second peak of expression does not occur in berries from Chardonnay, Shiraz, and Cabernet Sauvignon varieties (Terrier et al. 2005). VvHT1 was characterized as an MST by heterologous expression in both *Nicotiana tabacum* and yeast (Leterrier et al. 2003; Vignault et al. 2005). Studies performed on tobacco Bright-Yellow cells transformed with different lengths of the VvHT1 promoter transcriptionally fused to β-glucuronidase reporter gene (VvHT1-GUS) suggested that this MST is regulated by sucrose, the non-transported sucrose isomer palatinose and glucose, whereas fructose did not affect it. Furthermore, the authors demonstrated that in grape cell suspension sucrose and palatinose increase the expression of *VvHT1* (Figure 5; Atanassova et al

2003). These results provided the first example of a putative sugar transporter, which is induced by both glucose and sucrose in higher plants (Atanassova et al 2003).

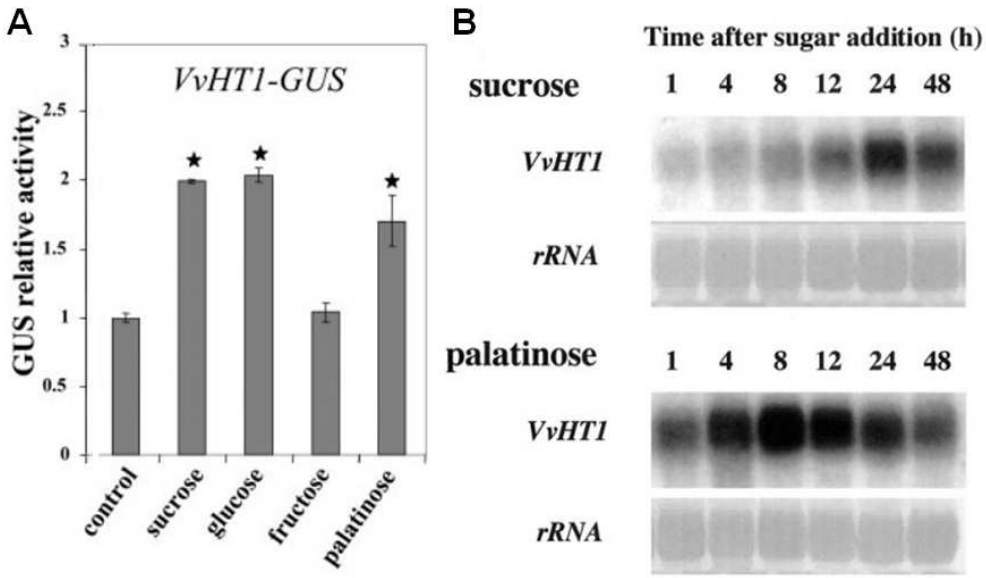

Figure 5. Studies of the regulation of *VvHT1* expression. A) Regulation by different sugars studied with a fusion of the *VvHT1* promoter with the reporter gene GUS. B) Analysis of the VvHT1 transcripts enhanced by sucrose and sucrose analog palatinose (Adapted from Atanassova et al. 2003).

Following this line of work, Cakir et al. (2003) identified an ASR protein in grape, VvMSA (*Vitis vinifera* Maturation-, Stress-, ABA-induced protein), by means of a yeast one-hybrid approach using as target the proximal promoter of the *VvHT1*, that contains two sugar boxes and is induced by sucrose and glucose, as it was previously mentioned. VvHT1 and VvMSA are both inducible by sucrose in grape berry cell culture, and sugar induction of VvMSA is enhanced strongly by ABA. Gel-shift assays demonstrated a specific binding of VvMSA to the *VvHT1*, suggesting that this ASR protein may be part of the transcriptional complex mediating the sugar-inducible expression of *VvHT1*. Also, the positive regulation of *VvHT1* promoter activity by VvMSA in planta was confirmed by coexpression experiments (Cakir et al. 2003). VvHT1 localization was elucidated by Vignault et al. (2005) using *in situ* hybridization, immunofluorescence and immunogold labelling experiments. *In situ* hybridization showed that *VvHT1* transcripts are primarily found in the phloem region of the conducting bundles, and immunofluorescence and immunogold labelling experiments localized VvHT1 in the plasma membrane of the sieve element/companion cell interface and of the flesh cells. These studies suggested that VvHT1 is involved in the retrieving of the monosaccharides needed to provide the energy necessary for cell division and cell growth at an early stage of berry development (Vignault et al. 2005).

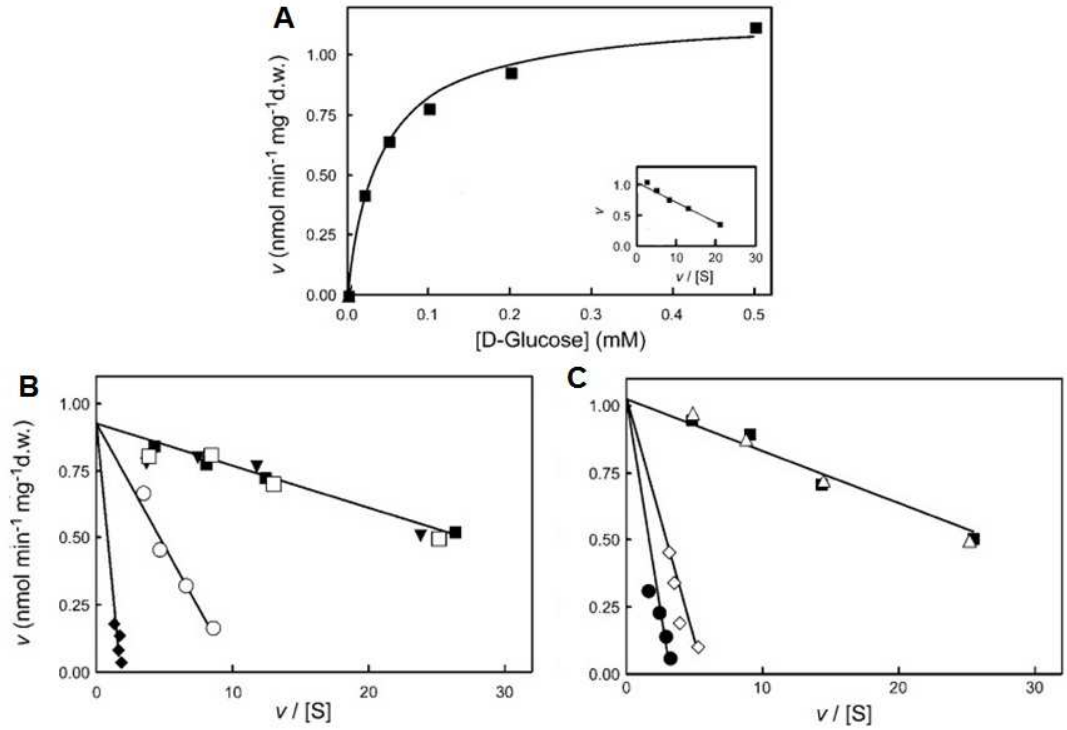

Figure 6. Kinetics and specificity of the monosaccharide transport system of *V. vinifera* cultured cells (CSB, Cabernet Sauvignon Berry). A) Initial uptake rates of 0.02 - 0.5 mM D-[^{14}C]glucose. B) Eadie-Hofstee plots of the initial uptake rates of D-[^{14}C]glucose in the absence of other sugars (closed squares) and in the presence of 5 mM xylitol (open circles), 5 mM galactose (lozenge), 5 mM mannitol (open squares), and 5 mM arabinose (inverted triangles). C) Eadie-Hofstee plots of the initial uptake rates of D-[^{14}C]glucose in the absence of other sugars (closed squares) and in the presence of the glucose analogs L-glucose (8 mM; open triangle), 2-deoxy-D-glucose (0.5 mM; open lozenge) and 3-ortho-metil-glucose (0.5 mM; closed circles). Adapted from Conde et al. (2006).

In grape cell suspensions Conde et al. (2006) characterized VvHT1 as a high-affinity (K_m= 0.05 mM glucose), broad-specificity (Figure 6) monosaccharide-proton co-transport system. The high affinity measured for the H^+-dependent monosaccharide transport may be important for cell growth in media with limiting sugar supply.

As shown in Figure 7, *VvHT1* transcription, VvHT1 protein amount and glucose transport are tightly regulated by sugar availability in the culture medium. Additional evidences supported the involvement of hexokinase as a sugar sensor. When high levels of glucose are present, energy-independent, diffusional uptake is the preferred mode of sugar absorption. Under these conditions, *VvHT1* expression is maintained at basal levels due to the balance between a positive induction signal generated by the presence of glucose and a repression signal due to high glucose levels sensed by

hexokinase. Additionally, high glucose levels seem to repress glucose transport activity at the protein level, triggering inactivation, mistargeting, and/or proteolysis of VvHT1. The absence of the repression signal generated by hexokinase allows the increase of *VvHT1* transcripts and, in accordance, the number of high-affinity monosaccharide/H^+ symporters in the plasma membrane (increase of V_{max}), ensuring a high-transport capacity at limiting glucose conditions (Figure 8). These findings correlate well with the expression of the *VvHT1* in the early stages of berry development, when the sugar levels in the berry are low.

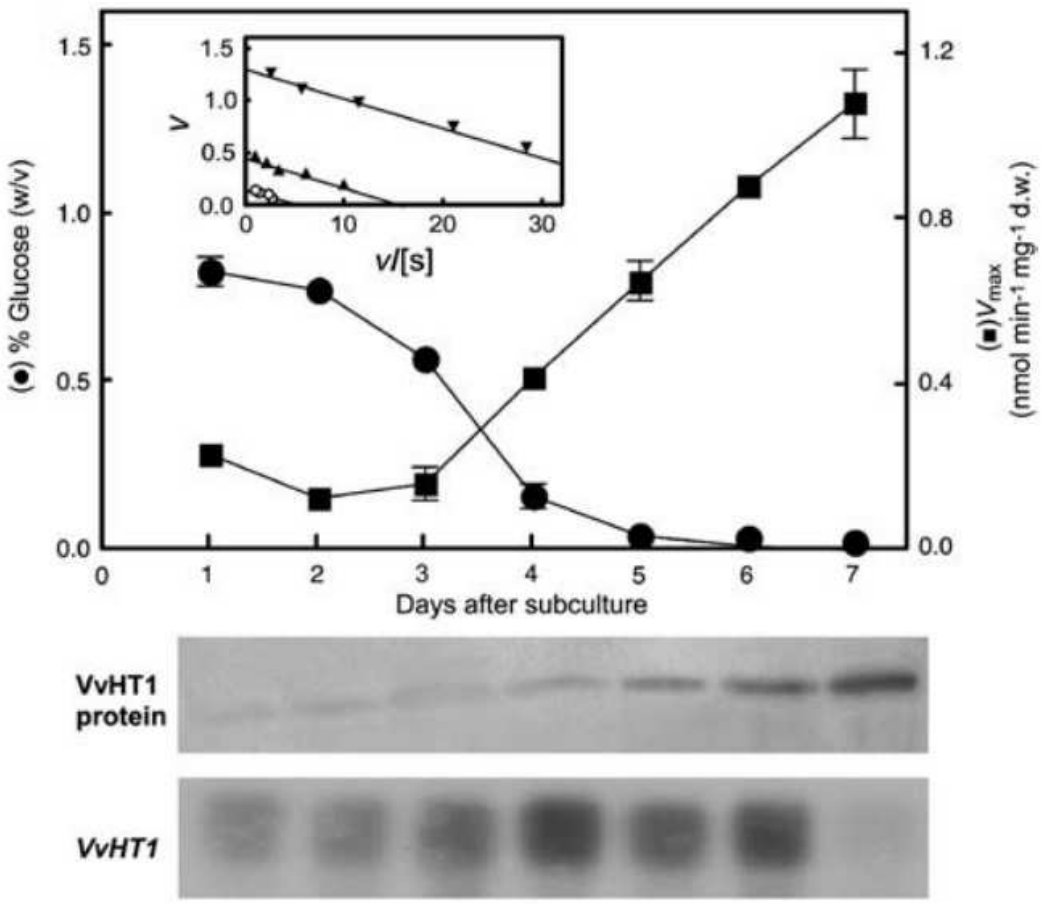

Figure 7. Regulation of *VvHT1* transcripts and protein levels in CSB cells by glucose concentration in the growth medium (Conde et al. 2006).

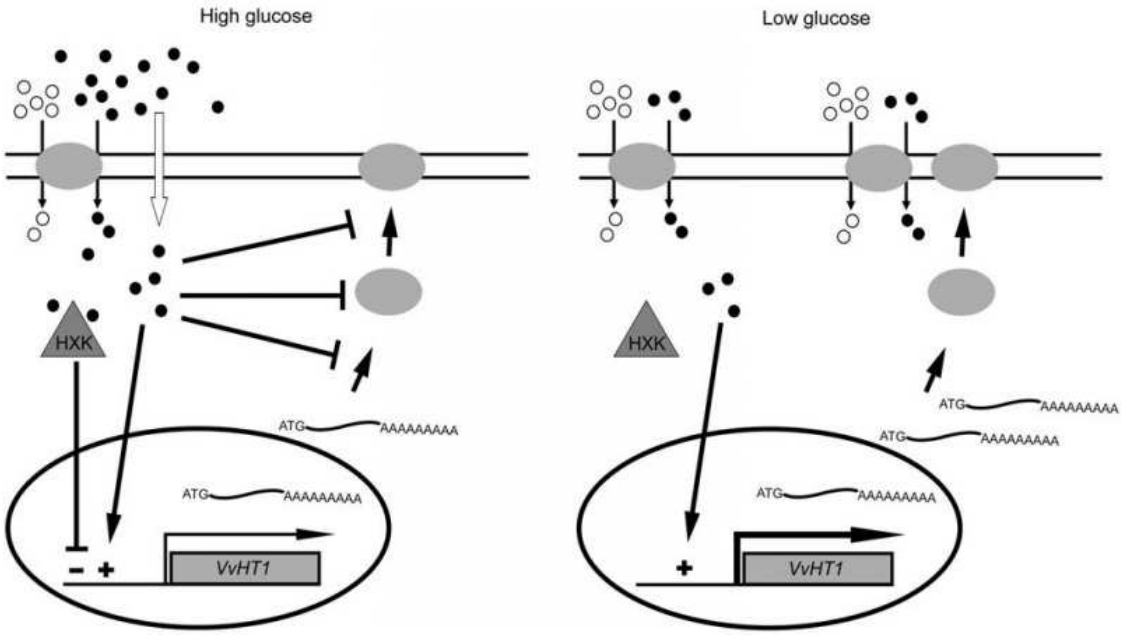

Figure 8. Model of the glucose regulation of *VvHT1* expression and glucose transport activity. Glucose (closed circles), proton (opened circles), hexokinase (HXK). (Conde et al. 2006)

Material and Methods

2.1 Biological Material

2.1.1 CSB cultured cells

CSB (Cabernet Sauvignon Berry) cells were maintained in 250 mL Erlenmyers with MS medium (Murashige and Skoog 1962) supplemented with 2% (w/v) sucrose, in an orbital shaker at 100 rpm and 23℃ in the dark. The cells were sub-cultured after 7 days, at the end of the exponential growth phase.

2.1.2 Intact grape berries

Grape berries from the Alvarinho cultivar were used to study sugar incorporation. Berries were randomly collected from the bunches 8 weeks after fruit set (10 to 12 mm; 0.75 to 0.85 g) and maintained at 4ºC before use.

2.1.3 Bacterial cells

The *E. coli* cells used to amplify the plasmid vectors were from the One Shot MAX Efficiency DH5α-T1R (Invitrogen), and were transformed with a chemical method, accordingly to the manufacturer's instructions. For long term storage, glycerol stocks prepared by adding 400 µL 45% (v/v) sterile glycerol to 200 µL bacterial culture were maintained at -80℃.

2.2 Uptake studies of radioactive sugars

2.2.1 Cell treatments

Cell suspensions were collected at day 6 after subculture by centrifugation at 3000 *g* for 3 min, washed twice in MS medium without carbon source and resuspended in the same medium. The cells were placed for 12 h in the orbital shaker, in the same conditions to overexpress *VvHT1* (Conde et al. 2006). In the following day cells were centrifuged at 3000 *g* for 3 min and resuspended at approximately 5 mg FW/mL in MS medium without carbon source, pH 5.0. This pH provides the necessary proton gradient to energize the monosaccharide/H^+ transport system (Conde et al. 2006). To study the influence of high temperature on sugar transport, the cells were incubated for 12 h at 38ºC prior uptake studies. To study the influence of SA and ABA on sugar transport the cells were incubated with 150 µM of both hormones for 24 h at 23ºC prior uptake studies.

2.2.2 Determination of initial velocities of D-[^{14}C]glucose transport

One mL of cell suspension was added to 10 mL flasks under constant agitation (100 rpm). After 5 min incubation at room temperature, the reaction was started with 40 µL of a radioactive solution with a specific activity of 500 dpm/nmol glucose. Different solutions were used according to the desired final concentrations (0.02-0.5 mM). After 3 min incubation the reaction was stopped by adding 5 mL of cold MS medium without carbon source. This step stops the transport reaction by both decreasing the temperature and diluting the reaction mixture. The cells were then separated from the culture medium by filtration under vacuum through a GF/C membrane (Whatman), and washed in 10 mL of cold MS medium. The membranes were placed in 20 mL scintillation vials containing 5 mL of scintillation liquid (OptiPhase HiSafe II, LKB) and the radioactivity incorporated in the cells was measured in a Packard Tri-Carb 2200 CA (Packard Instruments Co., Inc., Rockville, Md) scintillation counter. D-[^{14}C]glucose was obtained from the Radiochemical Center (Amersham).

2.3 Western Blot analysis

2.3.1 Plasma membrane isolation and purification

Plasma membrane enriched vesicles from both control cells and temperature treated cells were isolated and purified as previously described (Conde et al. 2006). Approximately 200 mL of suspension cells were centrifuged at 3000 *g* for 3 min at 4℃, washed twice in ice-cold MS medium without carbon source, and resuspended in 250 mL of ice-cold extraction buffer [250 mM sucrose, 2 mM EDTA (pH 8.0) 2 mM DTT, 1 mM PMSF, 70 mM Tris-HCl (ph 8.0), 3 mM $MgCl_2$, 100 mM KCl, 0.1% (w/v) BSA and 0.2% (w/v) PVPP], and all subsequent procedures were carried out at 4℃. The cells were homogenized with an ultra-turrax T25 (IKA WERKE, Janke and Kumkel IKA, Germany) for 5 min with approximately 20 s pulses, and centrifuged at 10000 *g* for 10 min. The pellet was discarded and the supernatant was filtered through 3 layers of cheese cloth and centrifuged at 100000 *g* for 60 min to obtain the microsomal fraction. After discarding the supernatant, the pellet was aspirated and gently homogenized in 8 mL of resuspension buffer [20 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 7.5), 1 mM DTT, 1 mM PMSF and 15% (v/v) glycerol]. The microsomal fraction was layered over a 32% and 46 % (w/v) discontinuous sucrose gradient [32/46% sucrose (w/v), 20 mM Tris-HCl (pH 7.5), 1mM EDTA (pH 7.5), 1mM DTT and 1 mM PMSF] and centrifuged at 80000 *g* for 3 h in a Beckman SW 28 rotor. The plasma membrane-enriched fraction was

collected from the 32/46% sucrose interface with the help of a pipette, diluted 4 times in resuspension buffer and centrifuged at 100000 *g* for 30 minutes. The supernatant was discarded and the pellet resuspended in 600 µL of resuspension buffer, aliquoted and quickly stored at -80℃.

2.3.2 SDS-PAGE

SDS-PAGE of membrane proteins was performed according to Laemmli (1970). A stacking gel [4% (w/v) polyacrylamide, 250 mM Tris-HCl (pH 6.8) and 0.1% (w/v) sodium dodecyl sulphate (SDS)] was placed on the top of the resolving gel [10% polyacrylamide (w/v), 375 mM Tris-HCl (pH 8.8) and 0.1% (w/v) SDS] and submerged in TRIS-Glycine running buffer [25 mM Tris-HCl, 250 mM glycine and 0.05% (w/v) SDS]. The samples were heated at 70℃ for 10 min prior to separation in Laemmli buffer [50 mM TRIS-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 10% (v/v) glycerol] and 15 µg of protein from each sample was loaded into the wells.

2.3.3 Membrane transfer and immunoblotting

The proteins separated by the SDS-PAGE were electro-transferred to a 0.45 µm thick Immobilion-P^{SQ} nitrocellulose membrane (Millipore) in transfer buffer [50 mM Tris-HCl, 380 mM glycine, 0.02% (w/v) SDS and 20% (v/v) methanol] in a TE Series transfer electrophoresis unit (Hoeffer Scientific Instruments). The nitrocellulose membrane was incubated 1 h in the blocking solution [5% (w/v) fat-free milk powder, 1% (w/v) BSA in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4), containing 0.1% (v/v) Tween-20] at room temperature in the dark with small agitation. All the following steps were performed at room temperature in the dark. The membrane was then placed 15 min in the washing solution [1% (w/v) fat-free milk powder, 1% (w/v) BSA in PBS, containing 0.1% (v/v) Tween-20] for 15 min, followed by 2 washes in the same solution for 5 min. The membrane was then incubated with the primary antibodies against the C-terminus of VvHT1 diluted 1:1000 in PBS containing 01% (v/v) Tween-20 and 0.3% (w/v) BSA for 1 h, followed by 3 washes, of 5 min each, with the washing solution. A 1 h incubation of the secondary antibodies, anti-rabbit immunoglobulin G (IgG) conjugated to goat peroxidase, diluted 1:3000 in PBS containing 0.1% (v/v) Tween 20 and 0.3% (w/v) BSA was performed and followed by 3 washes, 5 min each, with the washing solution. The immunodetection was accomplished using the chemiluminescent ECL detection substrate (Amersham). Relative levels of antigens on the nitrocellulose membranes were analyzed by the KEMIDOK and quantified in the Quantity One Software (Bio-Rad).

2.4 Study of solute incorporation in intact grape berries

The method used to study substrate compartmentation into intact grape berries was based in an approach reported several years ago (Kriedmann 1968). The berries were placed in 126-wells ELISA plates in contact with the desired radioactive solution via the pedicel. After incubation with the radioactive substrates (30 mM of D-[^{14}C]glucose or [^{14}C]sucrose at 250 dpm/nmole) at 23ºC during 12 h, the pedicel was cut and each berry was placed in 6 mL scintillation liquid and homogenized with a pestle. The radioactivity entrapped in the mesocarp tissue was measured in a Packard Tri-Carb 2200 CA (Packard Instruments Co., Inc., Rockville, Md) scintillation counter, and the results expressed in disintegrations per minute (dpm). SA or ABA (150 µM) were added to the radioactive solution to study their effect on glucose internalization by grape berries. To study the effect of high temperature on the incorporation of D-[^{14}C]glucose the berries were placed in a green-house at 38ºC 12 h. The incorporation of the fluorescent glucose analog 2-NBDG was also tested. The berries were incubated overnight with 1.35 mM of 2-NBDG and the results visualized under a epifluorescent microscope.

2.5 Intracellular ROS quantification

ROS production was measured using the H_2DCFDA probe (Molecular Probes, Invitrogen). Cultured cells (1 mL) were incubated with 2 µM H_2DCFDA for 15 min in the dark. After a quick centrifugation, 0.5 mL of the supernatant was collected and diluted in 2.5 mL of sterile water. The fluorescence was measured in a Perkin Elmer LS50 spectrofluorimeter at 488 nm excitation and 525 nm emission with 1 s integration (Franklin et al. 2008).

2.6 Malondialdehyde quantification

MDA was determined according to Heath and Packer (1968). Briefly, 0.5 g of filtered cells were grounded in liquid nitrogen and homogenized in 0.5 mL of 10% (w/v) TCA and centrifuged at 18000 *g* for 10 min at 25ºC. 250 µL of the supernatant was mixed with 250 µL of reaction buffer [10% (w/v) TCA and 0.6% (w/v) TBA], incubated for 30 min at 95℃ and then quickly cooled on ice. The absorbance of the supernatant was measured at 532 nm, and the value for non specific absorption at 600 nm was

subtracted. The amount of MDA complex was calculated from the extinction coefficient 155 $mM^{-1}cm^{-1}$.

2.7 Production of a VvHT1-GFP expression clone

2.7.1 Isolation and purification of total RNA from CSB cells

For the isolation of total RNA approximately 2 mL from CSB cultured cells were filtered and grounded in liquid nitrogen with a mortar and pestle. The TRI Reagent (Ambion) was used to isolate the total RNA, according to the manufacturer's instructions. Total RNA was purified with the RNeasy purification kit (QIAGEN), according to the manufacturer's instructions. RNA concentration was measured in a Nanodrop ND-1000, and the 260/280 nm and 230/260 nm ratios were used to estimate the quality of the isolated RNA. Also, the integrity of the isolated RNA was checked by a 1% agarose gel electrophoresis.

2.7.2 First strand cDNA synthesis

First strand cDNA synthesis was performed with the LongRange 2Step RT-PCR (QIAGEN) following the manufacturer's instructions.

2.7.3 Gateway technology

2.7.3.1 Production of *att*B-PCR products

To insert *VvHT1* into the donor vector by site specific recombination the *att*B regions were added to both ends of the *VvHT1* cDNA. Primers were designed, accordingly to the manufacturer's instructions, to incorporate the *att*B regions by PCR. The primers included a gene specific template sequence and the 25 bp *attB*1 and *attb*2 sequences (VvHT1 forward: ggg acaa gttt gta caa aaa agc agg ctt caa tat gcc ggc tgt cgg agg ctt tga taa g; VvHT1 reverse: ggg gac cac ttt gta caa gaa agc tgg gtt tac att ctt aac agg gta gtt ttc ctt gac cag ttc gac). The PCR was performed with the Phusion kit (FINNZYMES) according to the manufacturer's instructions. The DNA polymerase of the Phusion kit is a PFU (isolated from the hyperthermophilic *Pyrococcus furiosus*) and as a superior thermostability and proofreading properties compared to other thermostable polymerases.

2.5.3.2 BP recombination reaction

The *VvHT1* entry clone, pDONR-VvHT1, was generated by a BP recombination reaction between the *att*B-PCR product and the donor vector pDONR221, witch has the gene that confers resistance to kanamycin. The reaction mixture was composed of 75 ng *VvHT1 att*B-PCR product, 75 ng pDONR221, 1 µL BP clonase enzyme and TE buffer [10 mM TRIS, pH 8.0 and 1 mM EDTA, pH 8.0] to a final volume of 5 µL. After overnight incubation bacterial cells were transformed and plated on solid LB medium supplemented with 50 µg/mL kanamycin to allow the selection of transformed bacteria. Positive clones were identified by colony PCR.

2.7.3.3 Colony PCR

To identify positive clones a colony PCR was performed with the Hot Star Taq DNA Polymerase kit (QIAGEN), accordingly to the manufacturer's instructions. The primers are those used for the *VvHT1* and a sample of the selected colonies was used as template. The results were checked by 1% agarose gel electrophoresis. The positive clones were inoculated in liquid LB with the appropriate antibiotic and growth overnight at 37℃ with vigorous shacking. In the following day an aliquot of the cells was used to make glycerol stocks, and rest to isolate plasmid DNA.

2.7.3.4 Isolation of plasmid DNA (miniprep)

The Easy Spin kit (Citomed) was used to isolate plasmid DNA according to the manufacturer's instructions.

2.7.3.5 LR recombination reaction

The *VvHT1* expression clone, VvHT1-pH7FWG2, was generated by a LR recombination reaction between pDONR-VvHT1 and the entry vector pH7FWG2 which confers resistance to spectinomycin. The reaction mixture was composed of 75 ng pDONR-VvHT1, 75 ng pH7FWG2, 1 µL of LR clonase and TE buffer to a final volume of 5 µL, and incubated overnight. In the following day, bacteria was transformed and plated on solid LB medium supplemented with 100 µg/mL spectinomycin to allow the selection of transformed cells. Positive clones were identified by colony PCR.

2.8 Protoplast isolation

The suspension cells were collected at day 7 after subculture, centrifuged 4 min at 3000 *g*, and resuspended in digestion buffer [Gamborg B5 medium supplemented

with 0.4 mM sucrose, 1.15% (v/v) Y-C cellulase and 0.15 % Y-23 pectoliase], and incubated overnight at 25 rpm and 22℃ in the dark. In the following day, the digestion mixture was centrifuged 8 min at 750 *g* and the protoplasts remain in the upper fraction that is collected, diluted 1:1 with digestion buffer without enzymes, to remove all the remaining enzymes from the protoplasts, and centrifuged again in the same conditions. A discontinuous gradient was performed by adding W5 medium [5 mM glucose, 154 mM NaCl, 125 mM $CaCl_2$ and 5 mM KCl (pH 5.8)] to the protoplasts suspension and centrifuged 8 minutes at 750 *g*. The protoplasts were collected from the interface of the discontinuous gradient, diluted 1:1 in W5 medium and centrifuged 8 minutes at 750 *g*. The supernatant was discarded and the pellet washed and resuspended in MMM medium [400 mM mannitol, 15 mM $MgCl_2$ and 5 mM MES (pH 8.0)] (Papadakis et al. 2009; Fontes et al. 2010).

2.9 Protoplast transient transformation

Transient protoplast transfection protocol was adapted from Yoo et al. (2007). 50,000 protoplasts were centrifuged for 3 min at 90 g, the supernatant was discarded and 100 µL of transfection buffer [600 mM mannitol, 15 mM $CaCl_2$ and 5 mM MES, pH 5.7] was added. Following, 15 µg of plasmid DNA was added, incubated 15 min at room temperature, 110 µL of PEG [40% (w/v) PEG 4000, 300 mM mannitol and 100 mM $Ca(NO_3)_2$] was added and incubated 2 min at room temperature. After this, 440 µL of W5 medium [154 mM NaCl, 162 mM $CaCl_2$, 2.5 mM KCl and 2 mM MES, pH 5.7] was added, centrifuged 1 min at 110 g and the supernatant was discarded, except ~ 50 µL. Finally, 100 µL of culture buffer [600 mM mannitol, 4 mM MES and 4 mM KCl, pH 5.7] was added and incubated overnigh at 20ºC. The transformation was confirmed under an epifluorescence microscope.

2.10 Protein quantification

Protein concentration was determined by the method of Lowry (1951), using BSA as the standard.

2.11 LC MS/MS analysis

Plasma membrane proteins were separated by SDS-PAGE (Laemmli 1970), in 10% acrylamide gel, and the proteins stained with colloidal Blue G-250. A specific section of the gel was sliced (~ 31-65 kDa) and immediately subjected to in-gel tryptic digestion (Shevchenko et al. 1996). Tryptic peptides were further fractionated by reverse-phase chromatography coupled online to an LCQ Deca XP ion trap mass spectrometer (Thermo Finnigan). The peptides were analyzed by MS full scan and MS/MS scans of the three most intense parent ions. MS/MS data sets were searched by SEQUEST through Bioworks 3.2 interface (Thermo Finnigan) against a subset of the NCBI protein database downloaded on 08/12/2008 consisting of *Vitis vinifera* sequences. *Vitis vinifera* homologs were identified using the BLAST search of the NCBI (http://ncbi.nlm.nih.gov/)

Results

3.1 Effect of high-temperature on glucose transport

A detailed kinetic characterization of glucose transport by CSB cells was performed in a previous paper (Conde et al., 2006). In the present study the effect of high temperature was evaluated. For this purpose, D-[^{14}C]glucose uptake in derepressed cells ([glucose]$_{medium}$ << 0.02%) incubated for 12 h at 38ºC and 25ºC (control cells) was measured (Figure 9).

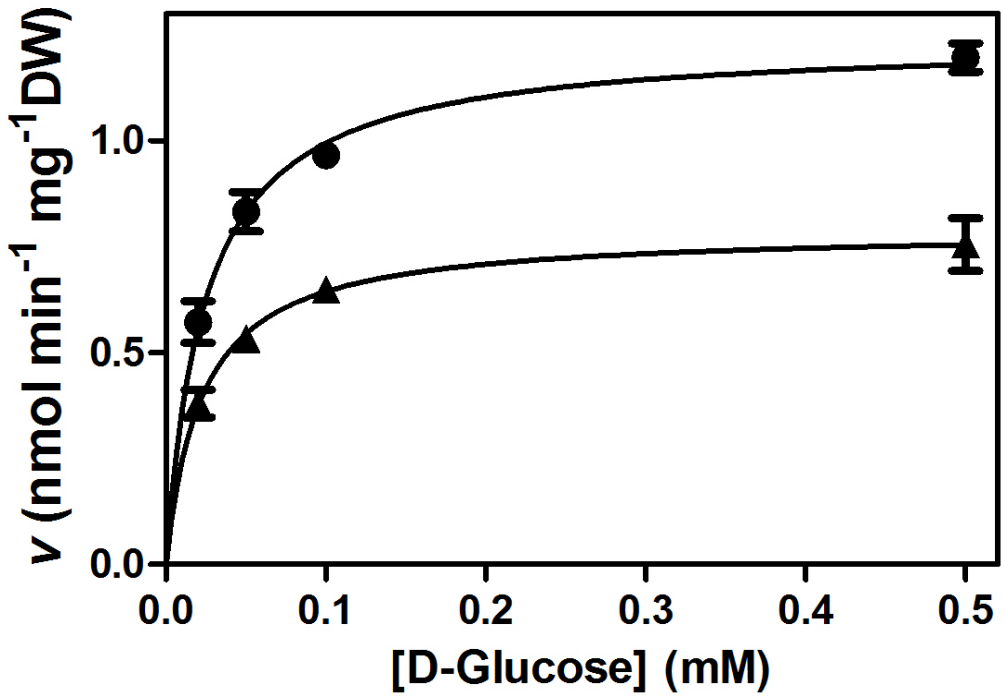

Figure 9. Glucose transport by CSB cells cultivated with 2% sucrose up to the mid-exponential growth phase and transferred to a sugar-depleted medium before treatment at different temperatures (derepressed conditions). Initial uptake rates at pH 5.0 by control cells cultivated at 25ºC (circles) and by cells exposed for 12 h to high temperature (38ºC) (triangles). Inset, Eadie-Hofstee plot of the initial glucose uptake rates. Uptake rates are mean values ± SE; n = 3.

The kinetic parameters exhibited by control cells were as follows: K_m, 0.024 ± 0.003 mM glucose and V_{max}, 1.24 ± 0.04 mmol glucose min^{-1} mg^{-1} DW, similar to those reported before (Conde et al., 2006). In high-temperature treated cells, the K_m was similar (0.024 ± 0.003 mM) but the V_{max} decreased by 36%, to 0.79 ± 0.04 mmol glucose min^{-1} mg^{-1} DW. To study if high temperature affects sugar transport at protein activity level or by altering protein amount a Western-Blot analysis with a polyclonal

antibody targeting to the C-terminal end of the VvHT1 protein was performed (Figure 10). Plasma membranes from control and temperature-treated cells were purified, as described in Material and Methods.

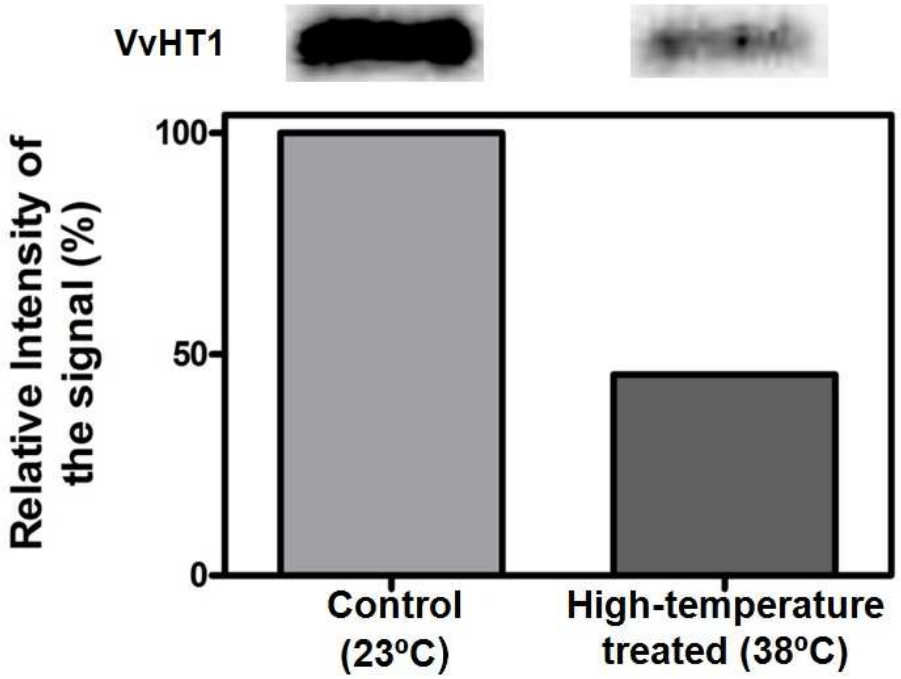

Figure 10. Effect of high-temperature on VvHT1 levels at the plasma membrane of CSB cells. The western-blot analysis was performed on purified plasma membrane fractions from control cells (incubated at 23ºC for 12 h) and high temperature treated cells (incubated at 38ºC for 12 h).

The analysis of the results by the Quantity One software (Biorad) showed that the overnight incubation at 38℃ reduces the amount of the VvHT1 protein in the plasma membrane by 55%, which correlated to the decrease of the V_{max} of glucose uptake.

Changes induced by high-temperature in the plasma membrane polypeptide pattern were assessed by SDS-PAGE analysis. As shown in Figure 11, a group of polypeptides with a molecular mass of ~ 31-65 kDa was up-regulated by high-temperature. LC MS/MS analysis (Pole Proteomic; Bordeaux, France) allowed the identification and characterization of these proteins (Table I).

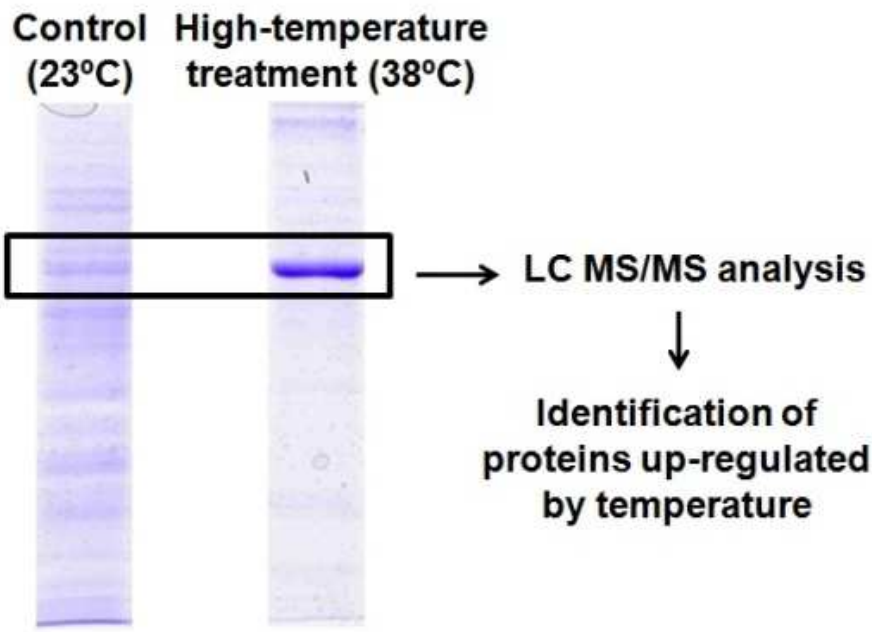

Figure 11. Polypeptide pattern of plasma membrane proteins from CSB cultured cells after SDS-PAGE. Purified plasma membranes were isolated from control cells (cultivated at 23℃) and high-temperature treated cells (cultivate d at 38℃).

3.2 Effect of high temperature on ROS homeostasis

High-temperature is commonly associated with ROS production (Dat et al. 2000). In the present study the influence of a 12 h incubation of CSB cells at 38ºC in the redox state was evaluated. The estimation of the intracellular ROS levels with the H_2DCFDA fluorescent probe showed that CSB cells exposed to 38ºC increased the levels of fluorescence by 97%, when compared to the control (Figure 12). Following this result, the effect of the increased intracellular ROS levels on membrane damage was evaluated by measuring lipid peroxidation with the MDA method (Figure 13). Results showed a 28.7% increase in MDA amount, when the cells were exposed to high-temperature.

Table I. Characterization of the proteins up-regulated by temperature identified by LC MS/MS analysis of the excised band depicted in Figure 14.

Acc. No.[1]	Protein Function	Taxonomy	MW (kDa)[2]
gi|147828051	Plasminogen activator inhibitor 1 RNA-binding protein, putative	*Ricinus communis*	39.17
gi|147834848	Hsp70-interacting protein 1	*Vitis labrusca*	41.02
gi|147845028	Chaperone protein dnaJ, putative	*Ricinus communis*	45.49
gi|147769068	Chaperone protein dnaJ 15	*Arabidopsis thaliana*	45.88
gi|147818771	Methylthioribose kinase, putative	*Ricinus communis*	47.93
gi|147783001	Eukaryotic peptide chain release factor subunit, putative	*Ricinus communis*	49.10
gi|147780810	Eukaryotic peptide chain release factor subunit, putative	*Ricinus communis*	49.10
gi|147780179	Glycosyl hydrolase family-like protein	*Salvia miltiorrhiza*	49.59
gi|147856780	Serine/threonine-protein kinase PBS1, putative	*Ricinus communis*	50.06
gi|147790061	Leucine rich repeat-containing protein, putative	*Ricinus communis*	58.47
gi|147784740	Chaperonin containing t-complex protein 1, gamma subunit, tcpg, putative	*Ricinus communis*	60.44
gi|147790061	Leucine rich repeat-containing protein	*Ricinus communis*	58.47
gi|147784740	Chaperonin containing t-complex protein 1, gamma subunit, tcpg, putative	*Ricinus communis*	60.44
gi|147805226	UBQ10 (Polyubiquitin 10)	*Arabidopsis thaliana*	51.16
gi|147834511	Polyubiquitin	*Zea mays*	60.25

[1] Accession number in the NCBI database

[2] Theoretical molecular weight

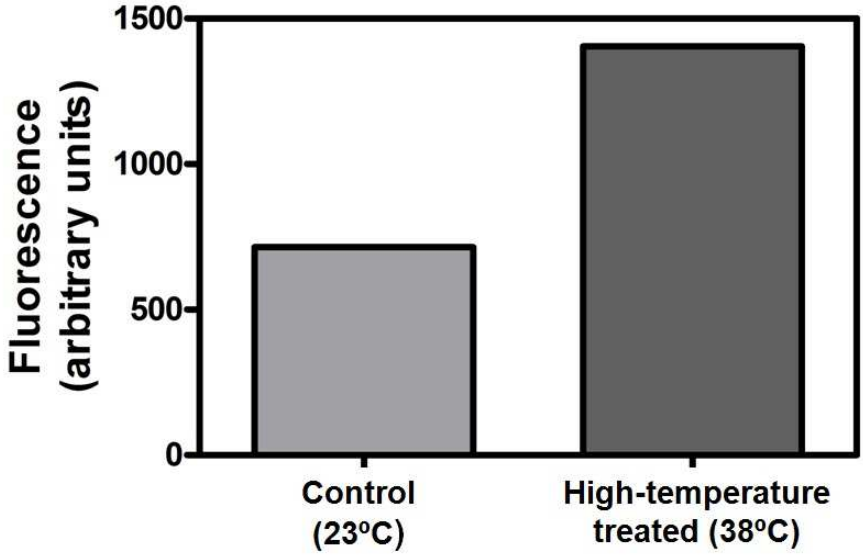

Figure 12. Intracelular ROS quantification with the fluorescent probe H_2DCFDA in CSB cells incubated for 12 h at 38ºC (high temperature treated cells) and in control cells (cultivated at 23ºC). Mean of two independent experiments are shown.

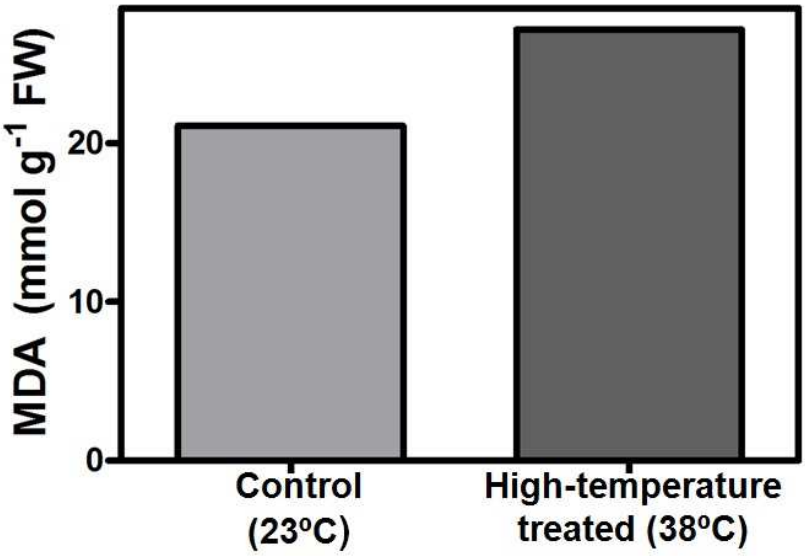

Figure 13. Quantification of MDA in CSB cells incubated for 12 h at 38ºC (high temperature treated cells) and in control cells (cultivated at 23ºC). Mean of two independent experiments are shown.

3.3 Effect of ABA and SA on glucose transport

As previously reported (Cakir et al. 2003) VvHT1 is regulated by an ASR protein, VvMSA, which specifically binds to its promoter region. Also, VvMSA expression is strongly enhanced by ABA and glucose, suggesting that ABA can regulate VvHT1. Furthermore, ABA levels increase in parallel with sugar accumulation in the grape berry and color development and it is mainly found in the phloem of the berry suggesting a role on the unloading and uptake of sugars (Kataoka et al. 1982; Shiozaki et al. 1999). In the present study the incubation of CSB cells with 150 µM ABA for 12 h promoted a consistent increase of the V_{max} of the glucose transport system from 1.15 ± 0.02 nmol glucose min^{-1} mg^{-1} DW (control cells) to 1.48 ± 0.01 nmol glucose min^{-1} mg^{-1} DW (Figure 14).

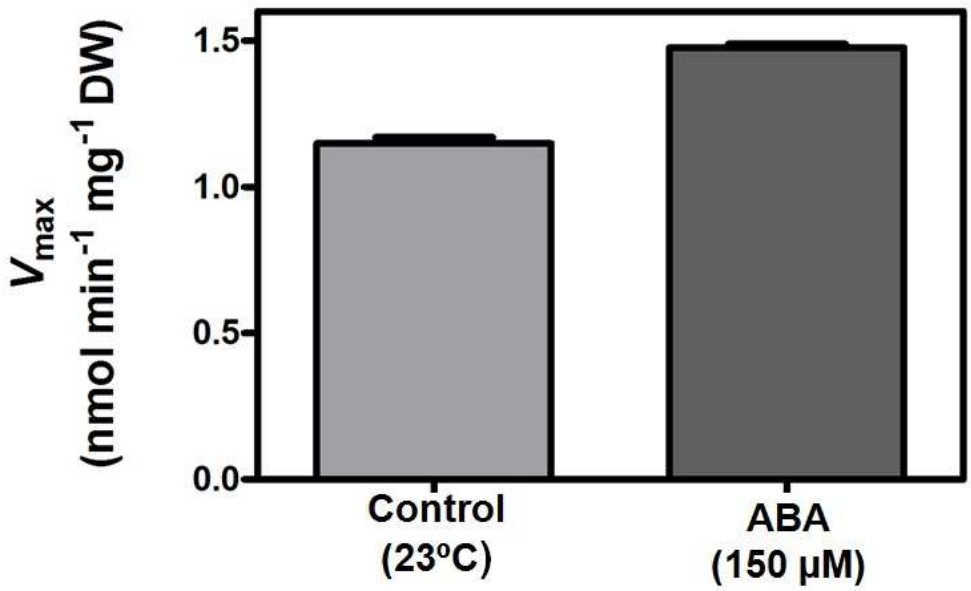

Figure 14. Effect ABA (150 µM; 12 h incubation) on the activity of glucose transport in CSB cells. Mean of two independent experiments are shown.

SA has been involved in signaling in plants, particularly in the induction of defense and stress responses (Bari and Jones 2009), but its role during grape berry development remains unclear. In the present study the incubation of CSB cells with 150 µM SA for 12 h increased the V_{max} of the glucose transport system from 0.80 ± 0.06 nmol glucose min^{-1} mg^{-1} DW (control cells) to 1.30 ± 0.04 nmol glucose min^{-1} mg^{-1} DW (Figure 15).

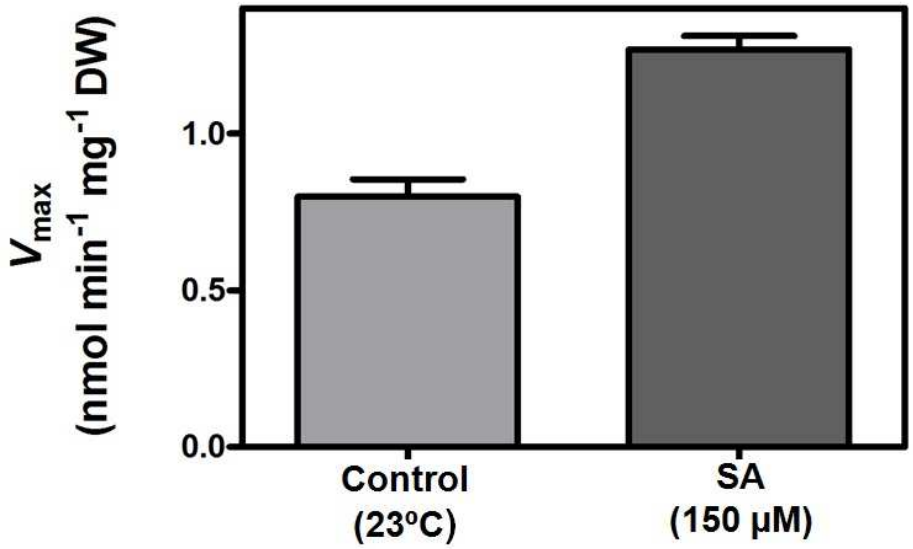

Figure 15. Effect SA (150 µM; 12 h incubation) on the activity of glucose transport in CSB cells. Mean of two independent experiments are shown.

3.4 Compartmentation studies in intact grape berries

In a paper published several years ago (Kriedmann 1978) the pattern of glucose incorporation into intact grape berries incubated via the pedicel with radioactive substrates was evaluated by autoradiography. That work was the inspiring basis of this study where intact grape berries were incubated in ELISA plates with radioactive sugars and sugar analogs to evaluate the effect of high temperature, ABA and SA on sugar incorporation (Figure 16). As shown in figure 17, after the incubation of intact grape berries with 1.35 mM 2-NBDG for 12 h the fluorescent sugar distributed throughout the mesocarp tissue. Figure 18 shows that high temperature (38ºC), 150 µM ABA and 150 µM SA increased D-[^{14}C]glucose uptake by grape berries, after 12 h incubation as follows: control, 2.55 ± 0.47 nmol glucose h^{-1} g^{-1} FW; 38ºC treatment, 4.86 ± 1.30 nmol glucose h^{-1} g^{-1} FW; ABA treatment, 4.81 ± 0.72 nmol glucose h^{-1} g^{-1} FW and SA treatment, 4.28 ± 0.85 glucose h^{-1} g^{-1} FW.

Figure 16. Experimental approach to study the influence of high temperature, hormones and inhibitors on sugar incorporation into intact grape berries via the pedicel.

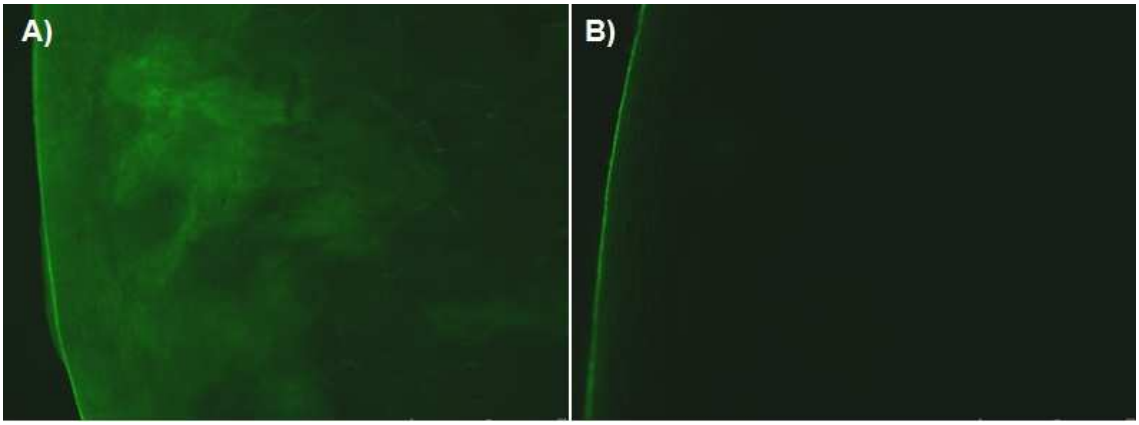

Figure 17. Study of sugar compartmentation in the mesocarp of intact grape berries by the incorporation of the fluorescent glucose analog 2-NBDG via the pedicel. Transversal sections of berries incubated with 1.35 mM 2-NBDG for 12 h (A) and of non-incubated berries (B) observed under the epifluorescence microscope.

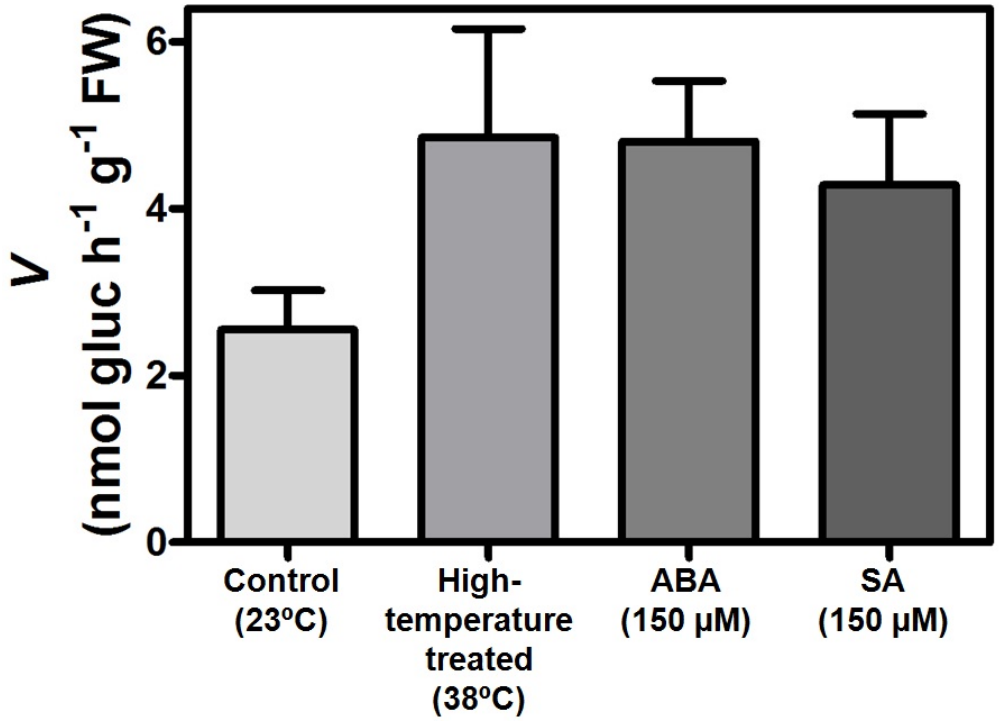

Figure 18. Study of different treatments on glucose compartmentation into intact grape berries incubated with D[^{14}C]glucose for 12 h. Results are mean values ± SE; n = 5.

3.5 Study of VvHT1 recycling and turnover induced by glucose repression and high-temperatures

The information regarding transmembrane transporters recycling induced by glucose repression and high temperatures in plants is still scarce. Contrarily, in yeast the problematic of catabolic repression is a well studied mechanism. Paiva et al. (2002), using *Saccharomyces cerevisiae*, studied the recycling of the lactate permease Jen1p after glucose catabolic repression. In this study the authors demonstrated that after a glucose pulse the protein was targeted for degradation in the vacuole, and that this pathway was dependent on an ubiquitination step. To study the VvHT1 recycling and turnover in response to sugar addition and high temperature, a transient protoplast transformation protocol was set up, as previously reported (Yoo et al. 2007). The green fluorescente protein was used to study the subcellular localization and expression of VvHT1. Up until now a clone harboring VvHT1-GFP under the control of 35S promoter was produced (Figure 19). Moreover, the transfection protocol for grape protoplasts was already optimized. Protoplasts from grape cells (Figure 20) were prepared following the protocol previously optimized by our group. (Fontes et al. 2010)

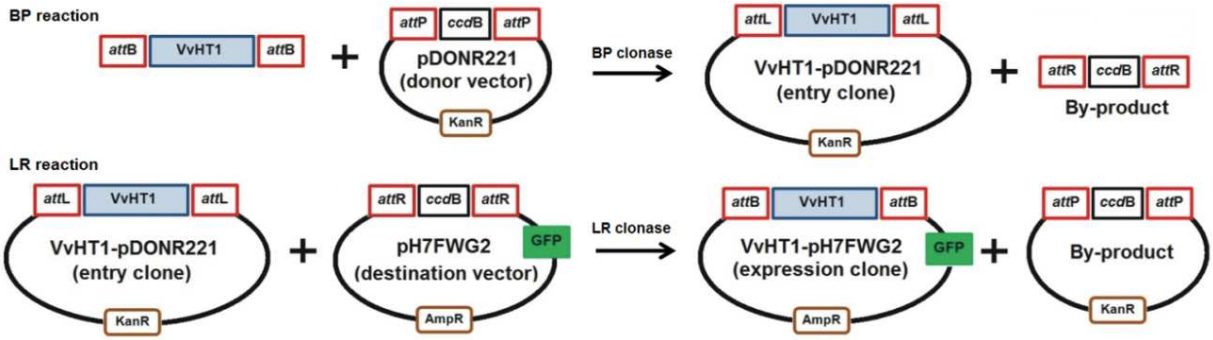

Figure 19. Production of a VvHT1-GFP expression clone using Gateway cloning for transient protoplast transformation.

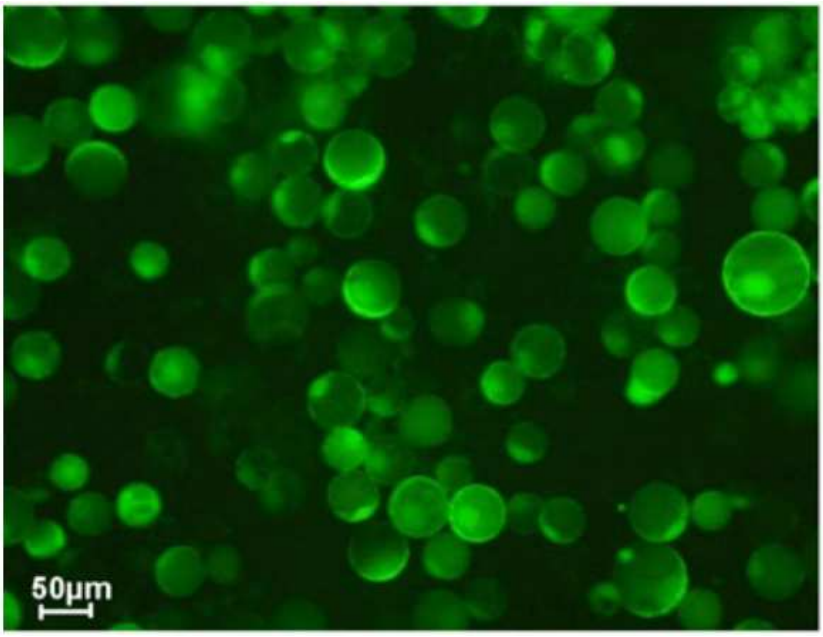

Figure 20. Protoplasts of CSB cultured cells stained with FDA and observed under the epifluorescence microscope.

Discussion

4.1 Temperature affects sugar transport/compartmentation in grape cells

Climate, and above all, temperature are fundamental factors influencing vineyard crop productivity. High-temperature has a profound effect in the grape berry altering its normal development and ripening. Besides reducing berry size and weight at harvest (Dookozlian 2000) high temperature alters its normal sugar content (Poudel et al. 2009). The hypothesis that grape sugar accumulation is negatively regulated by high temperature has been proposed for several years. Radler (1965) reported reduced sugar accumulation when clusters of Sultana vines were exposed to day and night high-temperatures. More recently, Poudel et al. (2009) demonstrated that high-temperatures reduce the accumulation of glucose and fructose in the interspecific hybrid wine grape Kadainou R-1, although the molecular mechanisms involved are far from being elucidated. Also, the synthesis of anthocyanines is negatively influenced by high temperature (Mori et al. 2005; Yamane et al. 2006).

In the present study, the influence of high-temperature on glucose transport by CSB cultured cells was evaluated. This transport system was previously studied in detail at the biochemical and molecular level (Conde et al. 2006). Results showed that the application of a temperature shock of 38ºC over 12 h reduced the transport capacity, which correlated with the observed decrease in the VvHT1 protein amount at the plasma membrane level measured by immunodetection with an anti-VvHT1 antibody. These findings suggest that high-temperature regulates VvHT1 activity by reducing the amount of protein in the plasma membrane, probably decreasing gene expression and reducing protein amount by stimulating the degradation of the sugar transporter. Further studies are needed to depict the molecular basis of sugar transport regulation by high temperatures. The effect of high temperature on the expression of monosaccharide transporters, including *VvHT1*, is currently being studied by Northern-blot analysis by our group.

Recent studies in plants regarding the recycling of transmembrane transporters may help us to understand the dynamics of these regulatory mechanisms. Takano et al. (2005) described the recycling of the root boron transporter from *A. thaliana* (BOR1, Boron Transporter 1). This transporter is expressed in the root xylem when the leaves contain low levels of boron, and is endocytosed, sorted and degraded in the vacuole when boron levels are normalized. This protein recycling pathway could be responsible for the observed decrease of the VvHT1 levels at the plasma membrane after a high-temperature peak (this work) or after sugar repression, as previously observed (Conde et al. 2006). As referred in the Results section, we have already produced a VvHT1-GFP fusion, and preliminary studies on the recycling/turnover of the VvHT1 after a

temperature/sugar pulse are underway in transiently transfected grape protoplasts (homologous expression).

In contrast with the observed reduction of sugar transport by CSB cells, a peak of 38ºC for 12 h promoted an apparent increase in sugar incorporation in intact grape berries. However, these data must be interpreted with caution and further studies are needed since glucose could be metabolized during the experiment, ^{14}C subsequently being incorporated in other organic substrates/CO_2. Then, the apparent increase on sugar compartmentation could result from a positive effect of temperature on sugar metabolism. The utilization of radiolabelled non-metabolizable sugar analogs will clarify this hypothesis.

4.2 High-temperature affects ROS homeostasis in grape cells

ROS have been considered as central components of plant adaptation to both biotic and abiotic stresses, whereas high-temperature is generally associated to a ROS production in several plant species (Dat et al. 2000a). The present data suggested that high-temperature substantially increased (96.7%) ROS levels in CSB cells and lipid peroxidation (29%), as suggested before in other plant models (Davidson et al. 2001; Larkindale and Knight 2002; Vacca et al. 2004).

The increase in the intracellular ROS could be explained by their dual role during plant stress responses, exacerbating the damage caused by high temperature and activating defense responses (Dat et al. 2000a). It was shown that within 15 min after a heat shock potato leaf tissues produced an oxidative burst (Doke 1997) and more recently the exposure of tobacco seedlings to 40℃ for 1 h in the light was shown to induce a significant increase in H_2O_2 (Dat et al. 2000b; Foyer et al. 1997). Also, a similar accumulation of H_2O_2 after a heat treatment was measured in mustard seedlings (Dat et al. 1998). Furthermore, this effect could be caused or potentiated by the inhibition of catalase, which is inhibited by high temperature, and is one of the most important anti-oxidant mechanisms of plant cells (Lopez-Delgado et al. 1998; Dat et al. 1998; Feierabend et al. 1992; Hertwig et al. 1992). In plants, the generation of H_2O_2 appears to be mediated by a plasma membrane complex, NADPH oxidase, and the inhibition of this pathway abolishes stress-induced H_2O_2 accumulation (Yu et al. 2004; Wang and Ma 2008). Furthermore, high-temperature can have a devastating effect on plant metabolism, acting on the quaternary structure of protein complexes. When different pathways are uncoupled, electrons that have a high-energy state are transferred to molecular oxygen to form ROS (Asada and Takahashi 1987; Mittler

2002), such as H_2O_2, O_2^-, and HO capable of causing oxidative damage to cell macromolecules.

We cannot conclude, at the moment, what mechanism or mechanisms are behind the observed increase in intracellular ROS levels. This effect can be caused by an inhibition of catalase, an increase in the activity of the NADPH oxidase, or even an increase in the levels of NADPH caused by an overexpression of the Glucose-6-phosphate dehydrogenase, an enzyme responsible for the production of NADPH in the pentose phosphate pathway. Moreover, it is possible that the increase in the intracellular ROS levels may be due to a cellular response, independent of catalase and NADPH, to cope with high-temperature, or even a synergistic effect of these mechanisms.

High-temperature may cause oxidative damage to proteins, DNA, and lipids generated by the uncoupling of metabolic pathways (Asada and Takahashi 1987; Mittler 2002). For instance, the impairing of mitochondrial functions at high temperature results in the induction of oxidative damage and causes lipid peroxidation (Davidson and Schiestl 2001, Larkindale and Knight 2002; Vacca et al. 2004). MDA is formed as an end product of the degradation of polyunsaturated lipids caused by ROS, and its quantification is commonly used to extrapolate lipid peroxidation. Our results confirmed that in high-temperature treated cells higher lipid peroxidation was increased.

4.3 A preliminary proteomic analysis of the plasma membrane from grape cells reveals the induction of key proteins in response to high temperature

Several proteomic studies have been performed in grape berries, including the exocarp proteome (Deytieux et al.2007), mesocarp proteome (Sarry et al., 2004), berry proteome (Giribaldi et al., 2007), and the plasma membrane proteome from CSB suspension cultured cells (Zhang et al., 2008). Furthermore, proteomic approaches have been used to study the berry development (Sarry et al. 2004; Vincent et al. 2006; Giribaldi et al. 2007), the berry response to water and salinity stresses (Vincent et al. 2007; Jellouli et al. 2008) and ABA treatment (Giribaldi 2010). The berry proteome in response to high-temperature stress was not yet explored. In the present study a group of proteins differentially expressed in response to high-temperature, including HSP's related proteins and polyubiquitins, were identified and characterized (Table I).

When plants are exposed to high temperatures they synthesize both high molecular mass HSP's (from 60 to 110 kDa) and small HSP's (from 15 to 45 kDa) (Miernyk 1997; Renaut et al. 2006), and, concordantly, a HSP70-interacting protein 1,

two Chaperone protein dnaJ and two Chaperonin containing t-complex protein 1 were identified in purified plasma membranes from high-temperature cells. HSP's (Reviewed by Wang et al. 2004) interact with a wide range of co-chaperone proteins that regulate their activity or aid in the folding of specific substrate proteins (Bukau and Horwich 1998; Hartl 1996; Frydman 2001; Buchner 1999). HSP70 chaperones, together with their co-chaperones (DnaJ/HSP40 and GrpE) have essential functions in preventing aggregation and in assisting refolding of non-native proteins under both normal and stress conditions (Hartl 1996; Frydman 2001). They are also involved in protein import and translocation processes, and in facilitating the proteolytic degradation of unstable proteins by targeting the polypeptides to lysosomes or proteasomes (Hartl 1996). In plants, many HSP70 proteins have been identified in different species (Boston et al. 1996; Vierling 1991), and the Arabidopsis genome contains at least 18 genes encoding members of the HSP70 family (Lin et al. 2001; Sung et al. 2001), and 12 HSP70 members have been identified in the spinach genome (Guy et al. 1998). The importance of these proteins in stress responses was further emphasized when overexpression of HSP70 genes promoted thermotolerance (Lee and Schoffl 1996) and resulted in enhanced tolerance to salt and water (Alvim et al. 2001; Sung and Guy 2003; Sugino et al. 1999; Ono et al. 2001; Leborgne-Castel et al. 1999). Chaperonins or HSP60 are a class of molecular chaperones found in prokaryotes and in the mitochondria and plastids of eukaryotes (Boston et al. 1996; Hartl 1996). This chaperons play a crucial role by assisting a wide range of newly synthesized and newly translocated proteins to achieve their native forms (Bukau and Horwich 1998; Frydman 2001). Also, chaperonins are classified into two subfamilies: the GroE chaperonins that are found in bacteria, mitochondria and chloroplasts and chaperonins containing t-complex polypeptide 1 (TCP1), also named CCT chaperonins, and are found in Archaea and in the cytosol of eukaryotes (Ranson et al. 1998). Functional characterization of plant chaperonins is limited, but CCTα, a chaperonin from the mangrove plant *Bruguiera sexangula*, enhances salt- and osmotic-stress tolerance of *E. coli* transformants suggesting an important role for these chaperones in stress responses (Yamada et al. 2002).

In grape, some studies have reported the expression of HSP's in response to heat stress. Morrel et al. (1997) described the overexpression of two HSP70 isoforms in dormant grape buds after 30 min at 44ºC by two-dimensional western immunoblot. Zhang et al. (2008) using immunogold electron microscopic techniques localized HSP70 predominantly in the chloroplast, starch granules, mitochondria and nucleus. More recently, Kobayashi et al. (2010) identified four heat shock-induced genes in grapevine, HSG1, HSG4, HSG14, and HSG19, using a cDNA subtraction method. In

spite of the advances that are being made, the cellular mechanisms of HSP70 function in plants under stress conditions remains unclear.

It has been suggested that plant cells sense ROS via redox-sensitive transcription factors, such as nitrogen permease reactivator (NPR1) or heat shock transcription factors (HSF's) (Mittler et al. 2004), which in turn activate HSP's expression, pointing out the fact that most HSP's are intimately associated with ROS (Suzuki and Mittler 2006). Our results suggest a similar correlation. We observed an increase in the intracellular ROS levels in grape cultured cells after an overnight incubation at 38ºC, and an increase in the expression of proteins related to stress-responses such a HSP70-interacting protein 1, two Chaperone protein dnaJ and two Chaperonin containing t-complex protein 1 (Table I). Also, these results strengthened the role of ROS as a signal molecule in stress responses in CSB cultured cells, by possibly mediating the high-temperature stress response.

Furthermore, we identified the expression of UBQ10 (Polyubiquitin 10) and a Polyubiquitin (Table I). These polypeptides comprise 10 tandemly repeated ubiquitins that are intimately related to protein degradation. Virtually all aspects of a plant's life cycle are controlled by the regulated synthesis of new polypeptides and the precise degradation of preexisting proteins. Via this "protein cycle," up to 50% of the total protein is replaced by plants every week (Vierstra 1993). In this pathway, the highly conserved 76-amino-acid protein ubiquitin serves as a reusable tag for selective protein breakdown in a cascade of reactions involving several enzymes E1-E2-E3 (E1 or ubiquitin-activating enzyme, E2 or ubiquitin-conjugating enzyme and E3 or ubiquitin-protein ligase). The resulting ubiquitin–protein conjugates are then recognized and degraded either by the multisubunit 26S proteasome with the concomitant release of ubiquitin for reuse (Vierstra 2003; Smalle and Vierstra 2004) or by the lysosome/vacuole in the case of plasma membrane proteins. Furthermore, a role for HSP's in the protein degradation via ubiquitin-proteasome 26S has been proposed. In this model, the chaperones play an important role in protein degradation, mediated by the ubiquitin ligase CHIP (Hohfeld et al. 2001). This E3 can interact with HSP70 and HSP90, through three tandem TPR's located in the N-terminus, and impair the ability of the chaperones to assist cellular protein folding. Also, in the C-terminus of CHIP a U-box required for ubiquitin ligase activity is present. This suggests an elegant solution to the problem of how the degradation machinery recognizes aberrant proteins, and, according to this model, a balance of protein folding and degradation would be achieved through regulation of chaperone activity (Hohfeld et al. 2001). Our results showing an increase in ubiquitin expression may indicate that protein degradation is being used in response to high-temperature stress, by possibly reusing the resulting

amino acids to synthesize new proteins. Also, the identification of proteins that are only present in high-temperature treated CSB cultured cells is consistent with the proposed role for proteolysis under high-temperature stress.

4.4 ABA and SA stimulate sugar incorporation in grape cells

As referred previously, ABA levels are high in the flesh of young berries, and a peak is also observed after véraison. In fact, this peak in ABA occurs simultaneously with an increase in sugar accumulation and color development, and is mainly found in the phloem of the berry which is consistent with a role in the unloading and uptake of photoassimilates (Wheeler et al. 2009). In CSB cultured cells a grape ASR protein appears to regulate the monosaccharide transporter VvHT1, by integrating the transcriptional complex mediating the sugar-inducible expression of *VvHT1* (Cakir et al. 2003). The observation that 150 µM of ABA increased the glucose transport by CSB cells is in agreement with these data. Although Cakir et al. (2003) demonstrated that *VvHT1* could be regulated by an ASR and overexpressed by ABA, an increase in the transport activity was not described. Furthermore, several transporters and channels can be post-translationally regulated by phosphorylation/dephosphorylation mechanisms. In *O. europaea* the stimulation of glucose uptake by the protein kinase inhibitor staurosporine, in glucose-sufficient cells, suggests the involvement of a phosphorylation-mediated regulation that may affect protein levels and activity (Conde et al. 2010). Also, the first sugar-inducible protein kinase, VvSK1 (*Vitis vinifera* Sugar-Inducible Protein Kinase 1), has been described (Lecourieux et al. 2010). *VvSK1* is mainly expressed in flowers, roots and in the berries, mainly after véraison, when the berries accumulate glucose, fructose, and ABA. In grapevine cell suspensions, *VvSK1* transcript abundance is increased by sugars and ABA. This data and the presence of phosphorylation consensus sequences in the VvHT1 polypeptide suggest that this transporter could be regulated by phosphorylation after ABA incubation. Further studies will clarify whether ABA regulates VvHT1 at transcriptional or post-transcriptional levels.

The role of SA in the berry development and sugar accumulation is still obscure. It is known that SA is a key signal, involved in the activation of plant defense responses to fungal, bacterial and viral attacks. Also, SA is important in the transduction of stress signals, in particular during pathogenesis, and many effects of cell infection are mediated by the increased level of SA (Hayat and Hamad 2007). Recently, it was reported that *VvHT5*, a Stress-Inducible Hexose Transporter, is up-regulated in

response to biotrophic fungal infection (Hayes et al. 2010). Furthermore, Burmistrova et al. (2009) described that low concentrations of SA (10-100 µM) regulate phloem unloading in the root tip of *Zea mays*. These results may suggest that SA regulates sugar accumulation into sink tissues, which is supported by the data of the present study obtained both in CSB cells and intact grape berry. In both models SA stimulated sugar uptake.

4.5 Future prospects

Studies of sugar incorporation in cultured cell suspensions have several advantages over plant tissues where bulk diffusion, tissue penetration barriers, and cell heterogeneity impair kinetic studies. Although studies in cell suspensions allowed the characterization of several sugar transporters, the extrapolation of the results obtained in this model to a plant level is sometimes difficult. To overcome this limitation we studied sugar import both in grape cell suspensions and intact grape berries. This model of intact grape berry will allow a deeper research on sugar incorporation and compartmentation into flesh cells at different stages of fruit development, and how temperature, SA and ABA affect sugar status at both biochemical and molecular levels. In particular, the expression of hexose transporters, including *VvHT1*, and disaccharide transporters, will be studied in response to high-temperature.

The indications that ABA may regulate VvHT1 by a post-translational mechanism will enable us to identify VvHT1 protein interactors and their functional significance for transport activity. This may be studied by coimunnoprecipitation experiments with specific antibodies, aiming at the identification of molecular partners of VvHT1 by LC MS/MS.

The increase of glucose transport upon treatment with SA is puzzling. SA has been mainly associated with plant responses to pathogen attack. The expression of VvHT1 at transcript and protein levels, after eliciting CSB cultured cells with known grape pathogens, such as *Botrytis cinerea*, will elucidate if the increased sugar transport is associated with a plant pathogen defence response, as suggested by Azevedo et al. (2006). In this work, D-[14C]glucose uptake in *Pinus pinaster* cell suspensions was enhanced by elicitation with *Botrytis cinerea* spores. This increase in glucose accumulation was dependent on calcium influx and on a burst in ROS, mediated by NADPH oxidase.

Following the observation of increased intracellular ROS caused by high-temperature, the elucidation if the observed ROS peak contributes to the decrease of

the VvHT1 in the plasma membrane is of utmost interest. This may be accomplished in the presence of antioxidants, for example ascorbic acid or glutathione, during high-temperature stress, followed by the evaluation of VvHT1 transport activity and level of expression. Additionally, the study of the activity and expression of the primary enzymes involved in ROS protection, such as glutathione S-transferase, catalases, superoxide dismutase and ascorbate peroxidises, as well as ROS production, namely plasma membrane NADPH oxidase and glucose-6-phosphate dehydrogenase will be a good challenge. This will allow us to evaluate whether ROS function as a signal molecule or as a by-product of the deleterious effects of high-temperature.

The proteomic analysis of high-temperature treated cells showed that HSP's and ubiquitins were overexpressed in response to high-temperature. To evaluate the contribution of these proteins in the cell homeostasis at high temperatures, the application of a temperature shock in the presence of the protein synthesis inhibitor cycloheximide, followed by the quantification of sugar transport, ROS production and lipid peroxidation, may provide valuable evidences.

Protein recycling is fundamental, but the mechanisms that regulate this process in plant cells are far from being understood. Our studies aiming at the elucidation of the mechanisms behind VvHT1 recycling at high temperature and under sugar repression were not concluded. Currently, we are optimizing transient protoplast transfection with the VvHT1-GFP fusion, and, in a near future, using this tool we will hopefully be able to respond to these fundamental questions: how is VvHT1 recruited from the plasma membrane under high temperature or after a sugar pulse? What are the mechanisms underlying its degradation?

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