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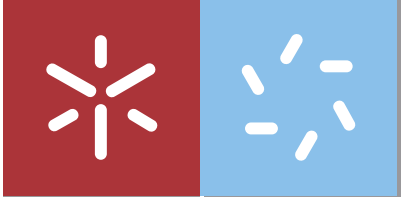

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Grapevine response to cold - study of starch metabolism in woody tissues

Tese de Mestrado
Biologia Molecular, Biotecnologia
e Bioempreendedorismo em Plantas

Trabalho efetuado sob a orientação do
Doutor Henrique Noronha
e do
Professor Doutor Hernâni Varanda Gerós

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“Every living thing is a masterpiece, written by nature and edited by evolution.”

Neil deGrasse Tyson,2014

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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.

Abstract

Grapevine (*Vitis vinifera* L.) is one of the most important agronomical species in the world, but in the context of the ongoing climate changes, extreme temperatures, high light intensities and water deficit are becoming important threats in many important viticultural areas worldwide. Among these, stress caused by cold has been shown to influence its productivity, metabolism and gene expression, which prompted us to study some molecular mechanisms involved in grapevine response to low temperature. In this regard, cv. Vinhão canes were harvested and incubated at 4 ºC for 7, 14 and 30 days to unravel physiological changes, identify and characterize key genes involved in response to cold. Results showed that starch levels steadily decreased during the incubation period at 4 ºC, and that the expression of the amylolytic gene *VvAMY2* increased along time, while the expression of *VvBAM5* decreased. Furthermore, HPLC analysis showed an increase in sucrose, fructose and glucose during the incubation at 4 ºC. Additional expression studies of key-genes involved in sugar metabolism showed that the steady-state transcript levels of *VvpGT2* and *VvSWEET2b* transporters as well as *VvSusy* and *VvSPS* enzymes increased along time, while the expression of the transporters *VvTMT1* and *VvSUC27* was downregulated. The *VvpGT2* gene was selected for further studies regarding organ specific expression and function. *VvpGT2* steady-state transcript levels were high in mature berries and flowers. Following heterologous expression in a mutant yeast (EBY.VW4000) lacking monosaccharide transporters, no *VvpGT2* transport activity of fructose, glucose and sucrose could be detected. Raffinose and stachyose levels in the grapevine canes were quantified by HPLC because the metabolism of raffinose has been linked to cold response. Results showed a steadily increase of raffinose levels along incubation at 4 ºC and transcriptional studies also revealed a noticeable increase of the steady-state levels of the galactinol synthase genes *VvGolS1* and *VvGOLS3* raffinose synthase *VvRafS5*.

Key-words: Abiotic stress, qPCR, Starch metabolism, Sugar transport, *Vitis vinifera*.

Resumo

A videira (*Vitis vinifera* L.) é uma das espécies agrícolas mais importantes ao nível global, contudo temperaturas extremas, altas intensidades de luminosas e a secura constituem ameaças importantes em muitas regiões vitivinícolas ao nível mundial, no contexto das alterações climáticas em curso. Em particular, o stresse causado pelo frio afeta a sua produtividade, metabolismo e expressão genética, o que nos motivou a estudar alguns mecanismos moleculares de resposta da planta a temperaturas reduzidas. Varas atempadas de videira da variedade Vinhão foram colhidas e incubadas a 4 ºC durante 7, 14 e 30 dias para se estudar as modificações fisiológicas e identificar e caracterizar genes-chave envolvidos na resposta ao frio. Os níveis de amido quantificados nas varas de videira diminuíram durante o tempo de incubação e, em paralelo, observou-se um aumento da expressão do gene *VvAMY2* que codifica uma enzima amilolítica. Contudo, os níveis de transcritos do gene *VvBAM5* diminuíram. Estudos de HPLC mostraram que ocorreu um aumento de sacarose, frutose e glucose em resposta ao frio. Estudo da expressão de genes-chave envolvidos no metabolismo dos açúcares mostraram um aumento dos níveis de transcritos dos transportadores *VvpGT2* e *VvSWEET2b* e das enzimas VvSusy e VvSPS, no entanto os níveis de transcritos dos transportadores *VvTMT1* e *VvSUC27* diminuíram. Quando se estudou o perfil de expressão do gene *VvpGT2* em diversos órgãos observou-se que os níveis de transcritos são mais elevados em bagos maduros e flores. Estudos de expressão heteróloga do gene *VvpGT2* numa levedura mutante. Estudos de expressão heteróloga do gene *VvpGT2* numa levedura mutante (EBY.VW4000) incapaz de transportar açúcares, mostraram que a proteína VvpGT2 é incapaz de transportar glucose, frutose e sacarose.

No presente trabalho foram ainda avaliados por HPLC os níveis de rafinose e de estaquiose nas varas de videira uma vez que tem sido observado que o metabolismo dos oligossacarídeos da família da rafinose está envolvido na resposta das plantas ao frio.Com efeito, durante a incubação a 4 ºC observou-se um aumento de rafinose durante o período de incubação a 4 ºC bem como dos níveis de transcritos das enzimas galactinol sintetase, *VvGolS1* e *VvGolS3* ,e da rafinose sintetase, *VvRafS5*

Palavras-chave: qPCR, Metabolismo do amido, Stress abiótico, Transporte de açúcares, *Vitis vinifera*.

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List of abbreviations and acronyms

ADP	Adenosine diphosphate
ADP-Gluc	Adenosine diphosphate glucose
AGPase	Adenosine diphosphate glucopyrophosphorylase
AMY	α-amylase
ATP	Adenosine triphosphate
BAM	β-amylase
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
CO_2	Carbon Dioxide
CTAB	Cetyltrimethyl ammonium bromide
cv.	Cultivar
DNS	3,5-dinitrosalicylic acid
DTT	Dithiothreitol
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FW	Fresh weight
G1-Pi	Glucose-1-phosphate isomerase
G6-Pi	Glucose-6-phosphate isomerase
Gal	Galactose
GBSS	Granular-bound starch synthases
GPT	Glucose-6-phosphate/ phosphate translocator
H^+/ATPase	Proton-pumping Adenosine triphosphatase
H_2O	Water
HCl	Hydrochloric acid
HPLC-RI	High performance liquid chromatography with refractive index
LDA	Limit dextrinase
MES	2-(N-Morpholino)ethanesulfonic acid
MEX	Plastidic maltose transporter

mRNA	Messenger RNA
Na	Sodium
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOAc	Sodium Acetate Anhydrous
NTT	Plastidic nucleotide transporter
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PGM	Plastidic phosphoglucomutase
pGT	Plastidic glucose transporter
PMSF	Phenylmethylsulfonyl Fluoride
PPi	Pyrophosphate
PVPP	Polyvinylpolypyrrolidone
RafS	Raffinose synthase
RFO	Raffinose family oligosacharides
SD	Standard deviation
SE-CC	Sieve element-companion cell
SS	Starch synthases
Suc	Sucrose
SUT/SUC	Sucrose transporter genes
SWEET	Sugars will eventually be exported transporters
TMT1	Tonoplast monosaccharide transporter 1
TPT	Triose-phosphate/phosphate translocator
UDP-Gal	Uridine diphosphate galactose

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1. Introduction

1. Introduction

Grapevine (*Vitis vinifera* L.), a woody perennial domesticated about 6000-8000 years ago, is recognised as one of the most valuable agricultural crops worldwide (McGovern et al., 2017). It has long been cultivated in temperate climate regions with sufficient rain, warm and dry summers and relatively mild winters (Jones et al., 2005; Ali et al., 2010). Due to its socioeconomic importance, grape growing has resulted in various uses of its fruit, from wine making to distillation of liquors, dried raisins and table grapes (Lecourieux et al., 2013). In 2017, it was estimated that 7.5 million hectares throughout the world were occupied by vineyards, corresponding to a total grape production of 73.3 million tons (OIV, 2018).

1.1. Biotic and abiotic stress factors affecting grapevine yield

Viticulture is famously sensitive to climate (White et al., 2006) and, along with other crops, it faces increased biotic and abiotic pressures linked to climate change. As a consequence of this phenomenon, the suitability of different crops like wheat, maize, soybean, rice, olive and grapevine has been affected in many areas, resulting in alterations in the production levels (Van Dingenen et al., 2009; Lobell et al., 2012; Ray et al., 2015; Moriondo et al., 2013a,b).

Grapevine is particularly affected by cold stress (Sawicki et al., 2015), heat stress (Kriedemann and Smart 1971), water shortage (Hardie and Considine, 1976; Lovisolo and Schubert, 1998), water excess (Striegler et al., 1993), and from rises of CO_2 concentration in the atmosphere (Martínez-Lüscher et al., 2015). Gene expression and plant metabolism alterations caused by cold stress will lately affect many biological functions (Chinnusamy et al., 2007; Sawicki et al., 2015). For instance, feedback inhibition of photosynthesis process is a consequence of the cessation of growth, the reduced energy and electron utilization by the Calvin Cycle and photosynthetic chain (Ruelland and Zachowski, 2010; Theocharis et al., 2012). The study of the mechanisms of grapevine response to abiotic and biotic stress has a direct impact in agricultural industry and may assist biotechnology protocols aiming the selection of highly productive and stress-resistant grapevine varieties (Ollat et al., 2017; Nenko et al., 2018).

1.2. Carbon assimilation and sucrose synthesis in autotrophic tissues

The carbohydrate status is responsible for the modulation and coordination of internal regulators and environmental cues that control growth and development (Ruelland et al., 2010; Theocharis et al., 2012). Sucrose, produced through photosynthesis in the mesophyll of mature leaves, is the main

carbohydrate used for long distance transport in grapevine (Swanson and El Shishiny 1958; Keller, 2015). During the day, the Calvin-Benson cycle assimilates carbon from atmospheric CO_2 through the use of products of the light reactions (ATP and NADPH). The intermediates produced in the previous step, trioses-phosphate (Trioses-P), are used not only to synthesize transient starch in the chloroplast but are also translocated to the cytosol by the triose-phosphate/phosphate translocator (TPT, Fischer et al., 1994), serving as precursors of sucrose formation (Figure 1A). During the night, the coordinated action of α- and β-amylases, and the debranching enzymes (isoamylase and limit dextrinase), degrade transient starch to maltose and glucose that are then exported to the cytosol by the plastidic maltose transporter (MEX; Niittylä et al., 2004) and plastidic glucose transporter (pGT; Weber et al., 2000), respectively, allowing a constant sucrose synthesis to provide carbon to sink tissues (Figure 1B).

1.3. Long distant sugar transport

Sugars, synthesized in the mesophyll cells, but also, water, minerals and amino acids, are translocated by plants through the vascular system (Keller, 2015). Sugars transport and allocation between the photosynthetic "source tissues" and the heterotrophic "sink tissues" is known as assimilate partitioning, which has evolved in land plants as a major determinant of plant growth and productivity (Kingston-Smith 2001).

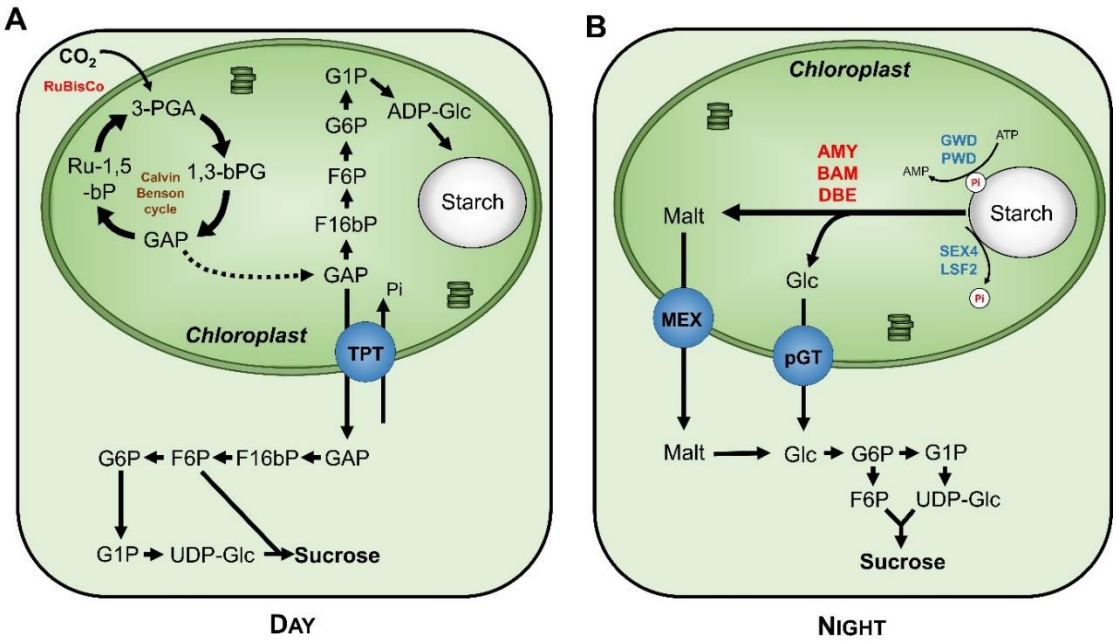

Figure 1. Carbon fixation and starch synthesis and mobilization in autotrophic tissues. A) During the day, carbon from the atmospheric C02 is brought in to the Calvin-Benson cycle through Rubiscos' action leading to the generation of trioses-phosphate (Trioses-P) that will afterward be used to synthesize starch in the chloroplast and sucrose in the cytosol, after

being translocated through TPT. **B)** During the night, starch is degraded to maltose and glucose and then exported to the cytosol through the action of MEX and pGT2 accordingly. After that, sucrose is synthesized to support heterotrophic tissues. 1,3-bPG, 1,3-bisphosphoglyceric acid; 3-PGA, 3-phosphoglyceric acid; ADP-Glc, adenosine diphosphate-glucose; ATP, Adenosine triphosphate; F16bP, fructose 1,6 bisphosphate; F6P, fructose-6-phosphate; GAP, Glyceraldehyde-3-phosphate; Gluc, glucose; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; MEX, maltose transporter; pGT, plastidic glucose transporter; Pi, inorganic phosphate; Ru-1,5-bP, ribulose 1,5-bisphosphate; TPT, triose-phosphate/phosphate translocator; UDP-Glc, uridine diphosphate-glucose. Adapted from Noronha et al., 2018.

1.3.1 Phloem loading and unloading

In higher plants, sucrose is loaded from sites of biosynthesis into the phloem by membrane carriers and transported to the sites of use and storage (Lalonde et al*.,* 2004). For the delivery of sucrose into the SE/CC complex two pathways have been proposed: apoplastic and symplastic. In symplastic loading, sucrose moves from mesophyll cells to the SE-CC complex through small pores between adjacent cells, the plasmodesmata, that allow sucrose to move from the bundle sheath into the companion cell.

Conversely, the aploplastic loading mechanism requires the export of sucrose from the mesophyll cells to the apoplast and its uptake in the SE-CC complex by a H^+/ATPase symporter located in the same cells. Recently it was proposed that sucrose release in the apoplast, more precisely in the vicinity of the SE-CC complex, is mediated by *SWEET* (*sugars will eventually be exported transporters*) facilitators (Chen et al*.,* 2011).

In agreement with Munch's mass flow hypothesis, sucrose moves across the phloem from high concentration sites (autotrophic tissues) to low concentration organs (heterotrophic tissues) (Münch, 1930; Conde et al*.,* 2007). In heterotrophic or sink tissues, unloading takes place either by transmembrane export or through plasmodesmata, although the first steps are often symplastic (Fisher and Oparka, 1996). Also, it has been postulated that sucrose efflux transporters may work as facilitators or as proton antiporters (Walker et al*.,* 1995). Sucrose in the apoplast can be taken up directly to the sink cell through sucrose/H^+ symporters or by hexose transporters after its hydrolysis into glucose and fructose by invertases.

1.4. Carbon storage in grapevine sink tissues

Carbohydrates are stored in perennial structures mainly as starch (Yang and Hori, 1979; Mullins et al*.,* 1992). In all grapevine varieties studied, starch represents the most important part of sugar reserves and is mobilized or accumulated according to the plant needs (Winkler and Williams, 1938; Bouard,

1966; Mullins et al., 1992; Zapata et al., 2001). Starch metabolism and photosynthesis are the two key processes determining sugar availability and uring the annual grapevine cycle, a complex carbohydrate flux is set up between annual and perennial organs.

Sugars accumulated in heterotrophic plastids (amyloplasts, found in roots, woody tissues, fruits, seeds, tubers, and pollen grains) obtained from long-distance sugar transport through the phloem (Lalonde et al., 2004; Lemoine et al., 2013) are the first source of sugars used by emerging shoots in the spring (Scholefield et al., 1978). Tubers, fruit and seeds are also considered to be major sinks during the reproductive stages (Wardlaw, 1990), relying on the use of available carbohydrate resources produced by photosynthesis to support their growth and development.

1.4.1. Starch in grapevine storage organs

Grapevine has an annual growth that can be divided into several distinct developmental stages: dormancy, bud break (budburst), bloom (anthesis or flowering), fruit set (berry set or setting), *veraison* (color change or onset of ripening), harvest (ripeness or maturity), and leaf senescence and subsequent fall (Lebon et al., 2008). During winter dormancy, starch is mainly located in the ray parenchyma of the roots (Zapata et al., 2004), trunks and canes. Plant metabolism is reactivated in early spring, (Huglin, 1986) and starch becomes the only source of carbohydrates in grapevine (Scholefield et al., 1978; Huglin and Schneider, 1998; Zapata et al., 2004), and is progressively mobilized by the growth of annual vegetative and reproductive organs (Bates et al., 2002; Zapata et al., 2004) inducing bud break. Depending on the variety, reserve mobilization in the grapevine occurs approximately until anthesis, (Yang et al. 1980; Candolfi-Vasconcelos et al., 1994; Caspari et al., 1998). After that, starch accumulates again in annual (Bates et al., 2002) and perennial (Mullins et al., 1992; Bates et al., 2002; Zapata et al., 2004) tissues from fertilization until the beginning of berry ripening (*veraison*) (Candolfi-Vasconcelos et al., 1994; Caspari et al., 1998).

1.4.2. Starch structure

Starch is the predominant carbohydrate reserve in many plants found in both photosynthetic and nonphotosynthetic tissues (Slatery et al., 2000). It contributes with 50-70% of the energy in the human diet (Coopeland et al., 2009) and is additionally utilized as commercial feedstock and in numerous industrial applications (Kavakli et al., 2000).

Starch is composed by amylose and amylopectin (Figure 2A), which comprise 98–99% of native granules dry weight, but small amounts of lipids, minerals, and phosphorus in the form of phosphate

esterified to glucose hydroxyls can also be found in starches structure (Copeland et al., 2009). Starch granules range in size (from 1 to 100 mm diameter) and shape (polygonal, spherical, lenticular), and can vary greatly with regard to content, structure and organization of the amylose and amylopectin molecules, the branching architecture of amylopectin, and the degree of crystallinity (Lindeboom et al., 2004) (Figure 2B). These polymers share the same basic structure but differ in their length and degree of branching (Slatery et al., 2000).

Amylopectin, a large branched polymer that typically makes up 75% or more of this polysaccharide, is considered the basic unit of the starch granule (Myers et al., 2000; Denyer et al., 2001; Zeeman et al., 2010). Amylose is an essentially linear polymer of glucosyl residues linked via α-1,4 glucosidic linkages, whereas amylopectin exists as a branched α-1,4:α-1,6 D-glucan polymer (Slattery et al. 2000). Amylose is smaller than amylopectin, 10^6 - 10^8 Da, with longer chains and a limited number of branch linkages (Buléon et al., 1998). Glycogen, a storage polysaccharide of animals, is a glucose polymer composed of α-1,4-linked, α-1,6-branched chains, but a higher degree of branching in comparison with amylopectin (Copeland et al., 2009). The multiplicity in branching is a common feature of both amylopectin and glycogen (Buléon et al., 1998). Plants ability to synthesize amylopectin has evolved from an ancestral capacity to make glycogen (Copeland et al., 2009; Zeeman et al., 2010). The conserved architecture of starch granules, the dense packaging of glucose units in organized clusters, is determined by the packing of amylopectin molecules.

1.4.3. Starch synthesis in heterotrophic tissues

In spite of the fact that most biochemical steps of starch synthesis operating in leaves are conserved in heterotrophic tissues, some are particular to sink organs. For instance, in heterotrophic tissues, starch is synthesized in amyloplasts following the incorporation of glucose-6-phosphate (G6-Pi) from the cytosol by a glucose-6-phosphate/ phosphate translocator (GPT; Kammerer et al., 1998). Initial evidence for the import of G6-Pi into starch synthesizing plastids came from experiments with different plant species (Neuhaus et al., 1993; Kang and Rawsthorne 1994; Wischmann et al., 1999). In *Arabidopsis thaliana* and *Vitis vinifera*, two GPTs have been identified, *AtGPT1*,*2* and *VvGPT1-2* respectively, operating as glucose-6-phosphate transporters *in planta* (Niewiadomski et al., 2005; Noronha et al., 2015).

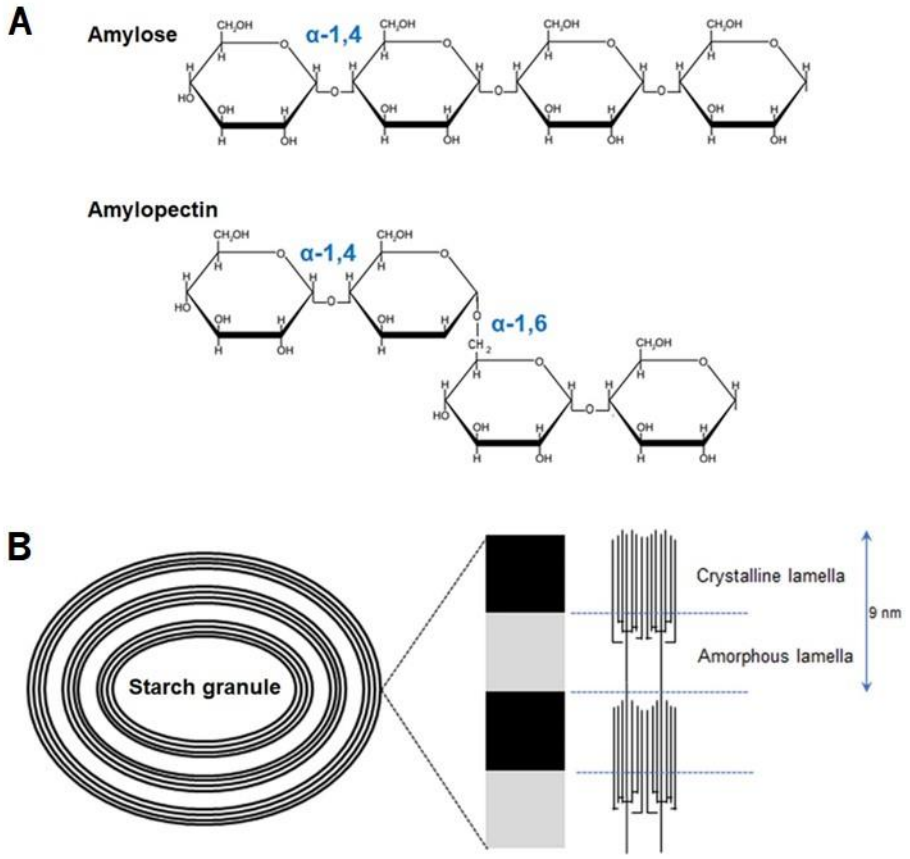

Figure 2. Composition and structure of the starch granule. Starch granules are composed by an array of semicrystalline lamellae, amylopectin and amylose (A), divided by amorphous growth rings. Double helical structures are formed within a cluster as a result of the branch points present in amylopectin. This structure packs together in a consistent manner that allows the formation of a crystalline lamellae, which intersperces with the amorphous lamellae, where the branch points are placed, leading to the formation of a semicrystalline matrix. Within the granule, stacks of semicrystalline lamellae are separated by amorphous growth rings (B). Adapted from Tester et al., 2004 and Huijbrechts 2008.

Additionally, the interconversion of glucose-1-phospate (G1-Pi) and G6-Pi for starch biosynthesis is likely the result of a plastidic phosphoglucomutase (PGM). There are two PGM isoforms in plants, one localized in the plastids and the other in the cytosol (Muhlbach and Schnarrenberger, 1978). Deficiency in the plastidic PGM activity lead to a "*starchless*" phenotype in Arabidopsis and *Nicotiana sylvestris*.

In the case of G6-Pi, ATP required for the synthesis of ADP glucose is imported by heterotrophic plastids from the cytosol. During starch biosynthesis in potato tubers, G1-Pi is converted to ADP-Gluc by AGPase. Plastidic nucleotide transporter (NTT), an ATP/ADP translocator, supplies ATP-dependent reactions in non-photosynthetic plastids, such as starch and fatty acid biosynthesis with cytosolic ATP (Schünemann et al., 1993; Kang and Rawsthorne, 1994).

Higher plants, that produce ADP-Gluc and pyrophosphate (PPi) from G1-Pi and ATP, possess the ADP-Glucopyrophosphorylase (AGPase) catalyzing the first key regulatory step in the starch biosynthetic pathways. Its activity is localized exclusively inside the plastid in all tissues except in the cereal endosperm where most of its activity is cytosolic (Denyer et al., 1996; Burton et al., 2002).

Starch synthases, found within the plastids, consists of two distinct classes designated granular-bound starch synthases (GBSSs) and others that can be found in the soluble phase or granular bound are known as starch synthases (SS). GBSSs are responsible for amylose synthesis (Kuipers et al., 1994) and SS are involved in amylopectin synthesis (Smith et al., 1997). It was reported that *waxy* mutants of cereals, and equivalent mutations also affect the starch of pea embryos, potato tubers, the perisperm of Amaranthus seeds, and Chlamydomonas cells. All of these mutants lack activity of GBSSI and consequently only have amylopectin, supporting the role of GBSS in the amylose synthesis (Slattery et al., 2000).

1.5. Starch metabolism during winter

Chilling markedly reduces grapevines growth, photosynthesis and delays flowering (Hendrickson et al., 2004; Bertamini et al., 2006; Ait Barka et al., 2006; Bertamini et al., 2006). In order to overcome such conditions, woody perennial trees, such as grapevine, not only need to synthesize proline and phenolic compounds, but also need to accumulate starch and soluble sugars (Ait Barka et al., 2006; Fernandez et al., 2012). This will allow a greater degree of freezing tolerance in response to low, non-freezing temperatures and vegetative growth in the following spring (Figure 3).

Carbohydrate metabolism has been reported to have greater instantaneous low temperature sensitivity than other components of photosynthesis (Fernandez et al., 2012). Seasonal dynamics of starch and soluble carbohydrates have been described in the canes of some varieties (Eifert et al., 1960; Bouard, 1966). More precisely, the raffinose family oligosaccharides (RFOs) are responsible for protecting plant cells against damage during abiotic stresses for numerous species, such as poplar (Sauter and van Cleve 1991, 1994), *Pinus strobus* L. (Hinesley et al., 1992), birch (Kasuga et al., 2007) and willow (Ögren 1999).

Carbohydrate accumulation at low temperatures may be explained through the activation of specific enzymes (Bohnert and Sheveleva, 1998; Couee et al., 2006). Galactinol is responsible for donating activated galactose (Gal) moieties to sucrose (Suc). Uridine diphospate (UDP-Gal) and galactinol synthase assure galactinol and myo-inositol synthesis accordingly (Peterbauer and Richter 2001). Raffinose, that is synthesized by raffinose synthase (RafS), will lately receive a Gal residue leading to the

formation of stachyose. The action of specific galactosyltransferases regulates the formation of other oligosaccharides of this pathway (manninotriose and melibiose), using galactinol as a galactosyl moiety donor (Figure 3).

Spinach and Arabidopsis leaves exposed to cold treatment (Schneider and Keller 2009) demonstrated that most of the enzymes involved in raffinose synthesis are localized in the cytosol (Schneider and Keller 2009). In another study, chestnut *CsDSP4*, a homolog of the Arabidopsis *SEX4* phosphatase associated with starch degradation in leaves (Zeeman and Rees 1999; Niittylä et al*.,* 2006), was found to be induced in woody tissues during autumn starch catabolism to increase the accumulation of sugars (Berrocal-Lobo et al., 2011). In Arabidopsis, plants acclimated to low temperature (+5 ºC), up-regulation of *SUTs*, *SUC1* and *SUC2* expression suggests an alternative mechanism for maintaining sucrose transport to sinks, mainly young leaves (Lundmark et al*.,* 2006). The activity of sucrose phosphate synthase, a cytosolic enzyme that synthesizes sucrose-6-phosphate from UDP-glucose and fructose-6-phosphate (Ito et al*.,* 2011), has been reported to increase in various plant species as a response to cold treatments (Guy et al*.,* 1992; Holaday et al*.,* 1992; Hurry et al*.,* 1995).

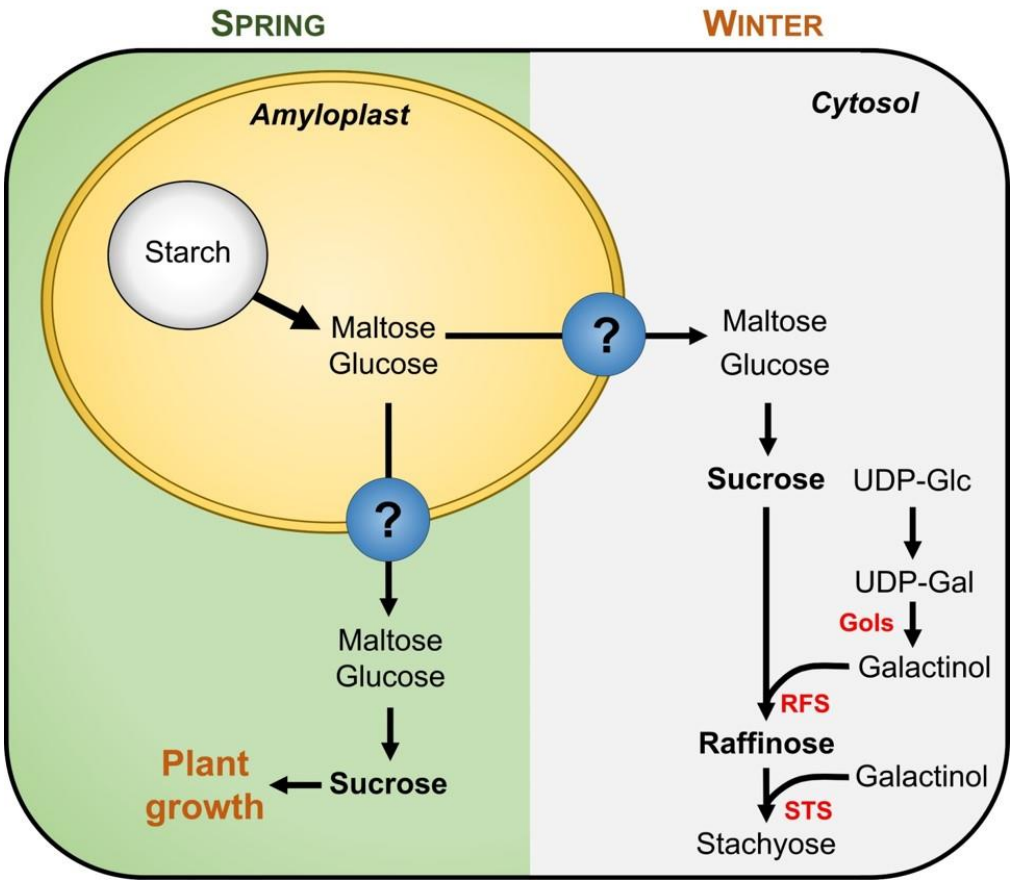

Figure 3. Carbon reserves mobilization during spring and in winter induced by cold in woody tissues. During spring, starch may be converted to maltose and glucose inside the amyloplast followed by the formation of sucrose, to sustain plant growth and metabolism. During winter, maltose and glucose are mobilized to the cytosol allowing the formation of sucrose and its conversion to raffinose and stachyose, in this order. Gols, Galactinol synthase; RFS, raffinose synthase; STS, stachyose synthase; UDP-Gal, uridine diphosphate-galactose; UDP-Glc, uridine diphosphate-glucose. Adapted from Noronha et al., 2018.

1.6. Objectives

The effects of climate change on natural ecosystems and on human activities, including agriculture, have been extensively described (IPCC 2018). In the long term, the sustainability of wine production in several viticultural regions may be threatened (Schultz and Jones, 2010; Hannah et al., 2013). During the past decades, a change in grapevine phenology has been reported for several grape-growing areas (Duchêne and Schneider, 2005, Petrie and Sadras, 2008, Ramos et al., 2008). Among various environmental stresses, low temperature is one of the most critical factors limiting the productivity and distribution of plants (Theocharis et al., 2012). Many physiological and biochemical cell functions have been associated with visible symptoms after cold-stress, such as, cessation of growth, reduced energy

and electron utilization by the Calvin cycle and photosynthetic chain, starch mobilization to fuel the synthesis of compatible solutes (Zheng et al., 2009; Ruelland et al., 2010).

In the present study we wanted to address the hypothesis that the response of grapevine to cold involves modifications of starch metabolism together with the reprograming of the expression of key genes involved in sugar metabolism. To address this hypothesis, *Vitis vinifera* canes from an important regional variety - Vinhão - were incubated for 7, 14 and 30 days at 4 ºC. Starch and soluble sugars content were quantified in tissue extracts and Real Time PCR analysis evaluated expression of key sugar transporters and enzymes involved in starch and RFO metabolism.

2. Materials and Methods

2. Material and Methods

2.1. Plant Material

Cuttings from field-grown grapevines (*Vitis vinifera* L.) of cv. Vinhão were used in the present work. Canes were collected, after fruit harvest, from a commercial vineyard in Ponte de Lima and incubated for 7, 14 and 30 days at 4 ºC. At each incubation period, 4 similar sized canes were selected from each sample (Figure 4) and the bud region and outer layers of the periderm were removed with scissors and scalpel. Then, they were cut in small segments, frozen with liquid nitrogen, and grounded using a mill (IKA) and stored at -80 ºC. In addition, an aliquot of this material was lyophilized in a Christ-Alpha 2-4 LD lyophilizer for subsequent quantification studies (Conde et al., 2015).

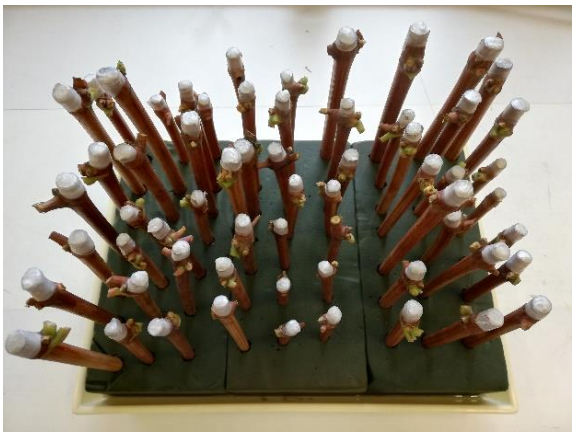

Figure 4. Incubated canes from *Vitis vinifera* cv. Vinhão for 7, 15 and 30 days at 4 °C.

2.2. Starch quantification

Starch was quantified in canes according to the protocol by Smith and Zeeman (2006). In order to remove soluble sugars, lyophilized cane (0.05 g DW) tissues were extracted three times with 5 mL 80% ethanol, and starch grains were gelatinized by autoclaving. Starch was enzymatically degraded due to the inclusion of α-amylase (AMY; 1U, Sigma–Aldrich) and β-glucosidase (10U, Sigma–Aldrich) in a medium containing 200 mM sodium acetate (pH 5.5) and glucose was measured through the DNS method.

2.3. RNA isolation and cDNA synthesis

To isolate total RNA from cane tissues the protocol by Reid et al. (2006) was adapted. For each condition, 500 mg of frozen tissue were used, which were mixed with 1 mL of extraction buffer containing 300 mM Tris HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 2% CTAB, 2% PVPP and 1.0 M DTT. Samples were then incubated at 60 ºC for 15 min and shaken every 5 min. Then, mixtures were extracted twice with 850 µl of chloroform:isoamyl alcohol (24:1) followed by a centrifugation at 13500

rpm for 15 min at 4 ºC for 15 minutes. To the aqueous phase were added 0.1 vol of 3 M NaOAc (pH 5.2), 0.6 vol of isopropanol followed by incubation at -80 ºC for 1 h. The pellet was centrifuged at 13500 rpm at 4 ºC during 30 min and later resuspended in 100 µl of ultrapure H_2O. In order to purify the samples, the GRS Total RNA Kit – Plant (GriSP, Lda.) was used following the manufacturer's instructions from step 5. RNA concentration and purity were quantified by spetrophotometry in the Nanodrop ND-1000 (Thermo Fisher Scientific Inc.) and integrity checked in a 1% agarose gel. The synthesis of the first cDNA strand was performed using Xpert cDNA Synthesis Mastermix protocol (GriSP, Lda.), according to the manufacturer's instructions.

2.4. Real-time PCR studies

Quantitative real-time PCRs were performed with Xpert Fast SYBR Blue (GriSP, Lda.) along with the conditions previously optimized in a CBX96 Real-Time Detection System (Bio-Rad). The amplification protocol included an initial denaturation step at 95 ºC for 3 min, followed by additional 40 cycles which were composed of a denaturation step for 3 seconds at 95 ºC, annealing for 20 seconds at 55 ºC and a final extension step for 20 seconds at 72 ºC. Experiments were done in biological replicates and then interpreted with the software Bio-Rad CFX Manager (Bio-Rad), while *VvGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was used as internal control. The primers presented below (Table 1) were used to study gene expression.

Table 1: Primers forward and reverse used for gene expression analysis by qPCR. Sequences accession numbers were taken from Grape Genome Browser 12x.

Gene	Primer Forward (5'-3')	Primer Reverse (5'-3')	Sequence accession number
VvAMY2	TCAGTCTGCTGCATCTGAAGGG	TGGGTACCATAGTGGGATGCATTG	GSVIVT01031740001
VvAMY5	TGGCCTTCAAGGGCTGTCACATTC	AAGGCCAATGAGCCTGTGTTGAG	GSVIVT01008714001
VvBAM2	CCAGTTTACGTAATGCTCCCTCTG	GCTGTTTCTCAAGCCCGTCTTTG	GSVIVT01030642001
VvBAM3	GTTCAAGGGTGCCAGTGTTT	GCTTTAGTCCATGCCTTTGC	GSVIVT01013272001
VvBAM4	TGCAACCCTTGCATTTGAGGAAAC	CACCAATAGACTGCTGGAACCTTG	GSVIVT01036911001
VvBAM5	CGCCAACCTATCTTTGCAGTCC	TCTTCGACACGGTCCATATCTGC	GSVIVT01014681001
VvMEX	GTCTTGTGGCCGTTGTTATGGC	TGGCATCCACATGAAGAGAAGTG	GSVIVT01024263001
VvpGT2	TCAATGCTCTTGGAGCTGCCATC	CTTCCTCACTGCTTTGCCTGA	GSVIVT01015361001
VvpGT1	ATTGGGAGTTACCTGGGAATGGC	AGTTGTTCATCCACTGGCGAAATG	GSVIVT01030495001
VvpGT4	TAACAAGCTTTGCTGGGATGGC	GGGCACTCCAAGTGAAAGAGAAGG	GSVIVT01031006001
VvSPS1	GGTTTGCCTATTGTTGCCACCAG	CAAGAAGGCCATTGTCAAGTACCC	GSVIVT01012825001
VvSuSy (Hren et al., 2009)	TGTTAAGGCTCCTGGATTTCAATTA	AGCCAAATCTTGGCAAGCA	GSVIVT01015018001

VvSUC27 (Pastene et al., 2014)	TGCTTGGCACTGACGGTACT	GCTGTAGGTGATCGCAAGAGG	GSVIVT01034886001
VvTMT1	TTCTTTGCTGTTGGTGCAAG	CAGAGAGCCCCTGAAAGTTG	GSVIVG01009024001
VvGolS1 (Pillet et al., 2012)	TGATTACAGCAGCGTTTTGCC	CGAGAGTACTGGCCTCTTCTA G	GSVIVG01028174001
VvGolS3	TGCCGAGCAGGACTATTTGA	CAAGGTTGTAGGTGGGTGGA	GSVIVT01031274001
VvRafS1	TCCTGCCAGAGATGGAATAAGCC	GGCAATTGAACACACCCACGAC	GSVIVT01014778001
VvRafS5	CCATCTTGAGGTGCCAGTACTACG	ATGGTGTTGCCGTCGTGAAGAG	GSVIVT01032425001
VvGAPDH (Gainza-Cortés et al., 2012)	TTCCGTGTTCCTACTGTTG	CCTCTGACTCCTCCTTGAT	GSVIVT00009717001

2.5. Sugar extraction and quantification by high performance liquid chromatography (HPLC) analysis

The extraction of sugars was performed as reported by Conde et al. (2015) which was previously adapted from Eyéghé-Bickong et al. (2012). In short, 150 mg of lyophilized cane tissue was mixed with 1 mL of ultrapure H_2O, exhaustively vortexed, and then a proportional volume of chloroform was added to the mixture. The biphasic solvent was promptly vortexed for 5 min to mix and incubated at 50 ºC for 30 min with a continuous shaking (1400 rpm). After this, the samples were centrifuged at 13400 rpm for 10 min at room temperature and the supernatant collected. The extracted sugars were filtered with a 0.45 µm nitrocellulose filter and quantified by HPLC-RI using a Rezex RCM–Monosaccharide Ca^{2+} (8%) column (Phenomenex) column at a flow rate of 0.6 ml min^{-1} at 40 ºC using water as the mobile phase. An injection volume of 20 µl was used. 20 g/L sucrose and 10 g/L of raffinose, glucose, fructose and stachyose were used as pattern curves. Sugars concentration of each sample were identified through the comparison of the peak area and retention time with the standard sample curves.

2.6. Cloning and functional characterization of *VvpGT2*

The *pAG416GPD-VvpGT2* construct was prepared following the Gateway technique (Invitrogen). Recombination sequences were introduced by PCR in the *VvpGT2* cDNA and the fragment recombined into the entry vector pDONR221 with the BP clonase enzyme. *VvpGT2* cDNA was then recombined into the *pAG416GPD* vector by the LR clonase enzyme. The *pAG416GPD-VvpGT2* plasmid was introduced in the hexose transporter-deficient yeast strain EBY.VW4000 using the LiAc/SS-DNA/PEG method (Gietz and Woods 2002) and selected on solid synthetic medium [0.7% YNB (Alfa Aesar), 1.3% dropout (US Biological; without uracil), 0.5% ammonium sulphate, 2% glucose and 2% agar].

Yeast drop-test assays were performed as described previously (Noronha et al., 2016). Briefly, yeast cells harboring *pAG416GPD-VvpGT2* construct and the *pAG416GPD* empty (control) vector were grown in liquid media until the OD640 nm reached 1.0. After this, cells were washed with sterile H_2O,

serial dilutions were made and 5 µL drops were added to YNB media supplemented with 2% sucrose, fructose, glucose or maltose.

2.7. Statistical Analysis

The results were statistically analyzed by Analysis of Variances tests (one-way and two-way ANOVA) using Prism vs. 7 (GraphPad Software, Inc.). Statistical differences between mean values are marked with letters for the different conditions

3. Results

3. Results

3.1. Starch quantification in grapevine woody canes exposed to cold

To study how low winter temperatures can affect starch metabolism in grapevine, *Vitis vinifera* cv. Vinhão canes were incubated for 7, 14 and 30 days at 4 ºC, as described in Material and Methods. As can be seen in Figure 5, a steadily decrease in the total amount of starch in woody canes was observed during the incubation period.

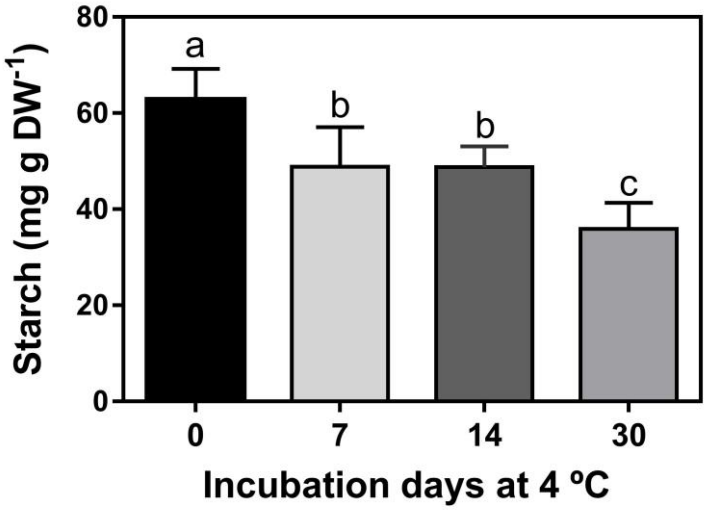

Figure 5. Reserve starch in woody canes of *Vitis vinifera* cv. Vinhão incubated at 4 ºC up to 30 days. Results indicate the mean ± SD of four independent experiments. Different letters stand for significant differences (P≤0.05).

3.2. Transcriptional analysis of grapevine α- and β-amylase genes

The expression of 2 α-amylase and 4 β-amylase genes was studied by qPCR, and results showed that the expression of *VVAMY2* decreased over time, while the expression of *VvAMY5* was only detected after the onset of the cold treatment (Figure 6A). Regarding β-amylases the steady-state transcript levels of *VvBAM2, VvBAM3 and VvBAM4* decreased while the expression of *VvBAM5* was clearly upregulated after 30 days of incubation (Figure 6B).

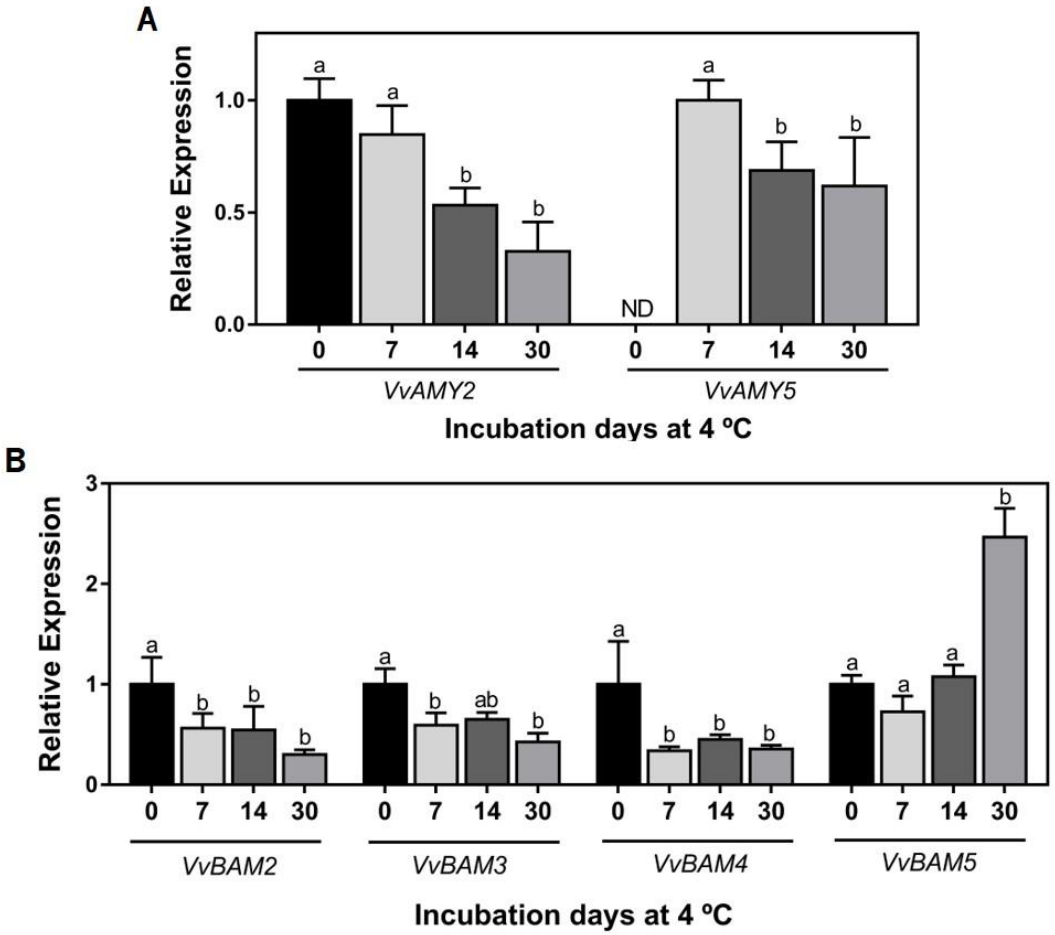

Figure 6. Expression of α-amylases (*VvAMY2* and *VvAMY5*) (A) and β-amylases (*VvBAM2*, *VvBAM3*, *VvBAM4*, *VvBAM5*) (B), from woody canes of *Vitis vinifera* cv. Vinhão incubated at 4 ºC up to 30 days. Results indicate the mean ± SD of four independent experiments. Different letters stand for significant differences (P≤0.05). ND, not detected.

3.3. Low temperatures promote a transcriptional modification of key-genes involved in glucose and maltose metabolism

Following the observed decrease in starch content in grapevine canes incubated at 4 ºC we speculated that the expression of genes involved in the transport of sugars from the plastid to the cytosol could be changed. Results showed that the expression of the plastidic maltose transporter *VvMEX* was downregulated following incubation at 4 ºC, while the expression pattern of three plastidic glucose transporters did not follow a similar trend (Figure 7). In particular, a steadily increase in the expression of *VvpGT2* was observed.

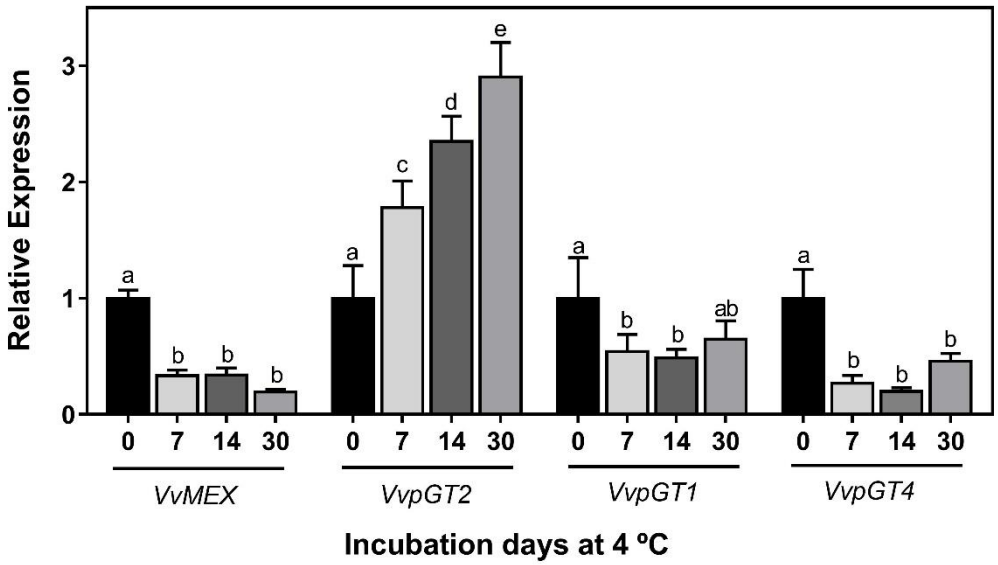

Figure 7. Expression of genes involved in maltose (*VvMEX*) and glucose (*VvpGT1*, *VvpGT2* and *VvpGT4*) plastidial transport from woody canes of *Vitis vinifera* cv. Vinhão incubated at 4 ºC up to 30 days Results indicate the mean ± SD of four independent experiments. Different letters stand for significant differences (P≤0.05).

3.4. Soluble sugar concentration increases in response to cold

Soluble sugars from woody canes were also analyzed by HPLC (see Materials and Methods). Results showed that sucrose, glucose and fructose levels increased throughout the experiment (Figure 8), mainly glucose that changed from 8.31 ± 0.54 mg g FW^{-1} at the time t=0 to 16.12 ± 0.44 mg g FW^{-} after 30 days of incubation at low temperature

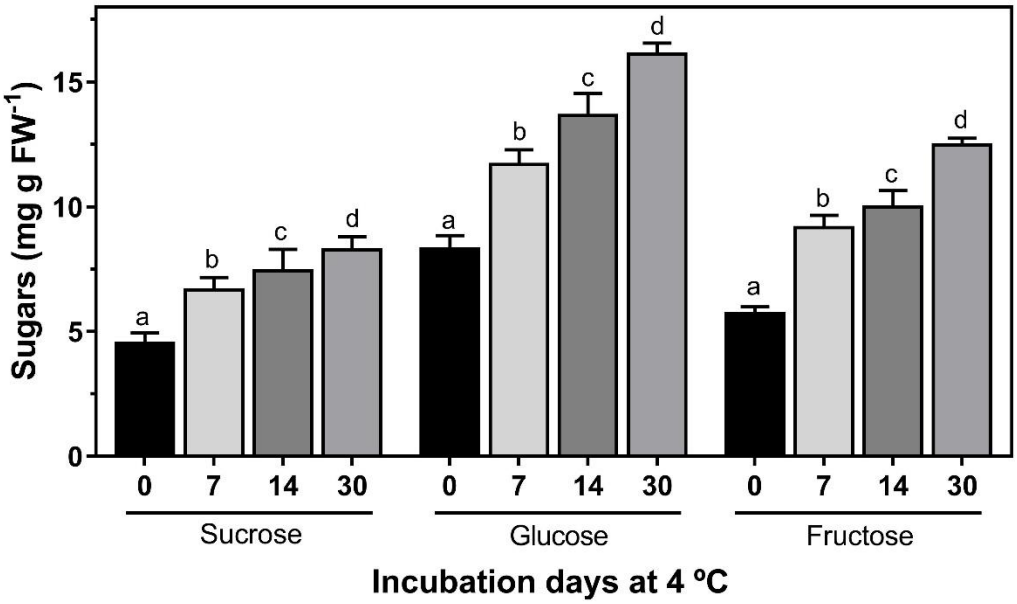

Figure 8. Concentration of major sugars (sucrose, glucose and fructose) from woody canes of *Vitis vinifera* cv. Vinhão incubated at 4 ºC up to 30 days. Results indicate the mean ± SD of four independent experiments. Different letters stand for significant differences (P≤0.05).

3.5. Cold leads to a transcriptional adjustment of key genes involved in the transport and metabolism of soluble sugars

The steady-state transcript levels of *VvSPS* (sucrose synthesis) increased after 7 days of cold treatment and then slightly decreased up to day 30. The expression of *VvSusy* (sucrose degradation) was also upregulated by cold, but 30 days after incubation the steady-state transcript levels decreased abruptly (Figure 9A).

The cold treatment downregulated the expression of the sucrose/H^+ symporter *VvSuc27* and the transcript levels of the plastidic monosaccharide transporter *VvTMT1* also decreased, while a strong upregulation of the expression of *VvSWEET2b* was observed up to day 14. However, thirty days after the experiment the expression of *VvSWEET2b* was reduced to the basal levels observed at the time t=0 (Figure 9B).

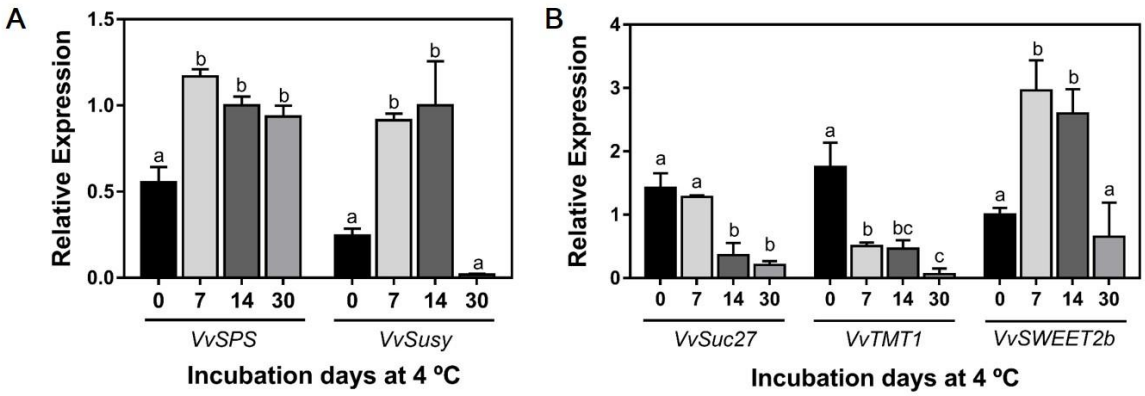

Figure 9. Expression of genes involved in sucrose synthesis and degradation, *VvSPS* and *VvSusy* accordingly (A) and transport, *VvSuc27*, *VvTMT1* and *VvSWEET2b* (B) from woody canes of *Vitis vinifera* cv. Vinhão incubated at 4 ºC up to 30 days. Results indicate the mean ± SD of four independent experiments. Different letters stand for significant differences (P≤0.05).

3.6. Raffinose family oligosaccharides concentration increase with cold treatment

Raffinose and stachyose levels in the grapevine canes were quantified by HPLC because the metabolism of raffinose has been linked to cold response. Results showed that both raffinose and stachyose levels increased during the experiment.

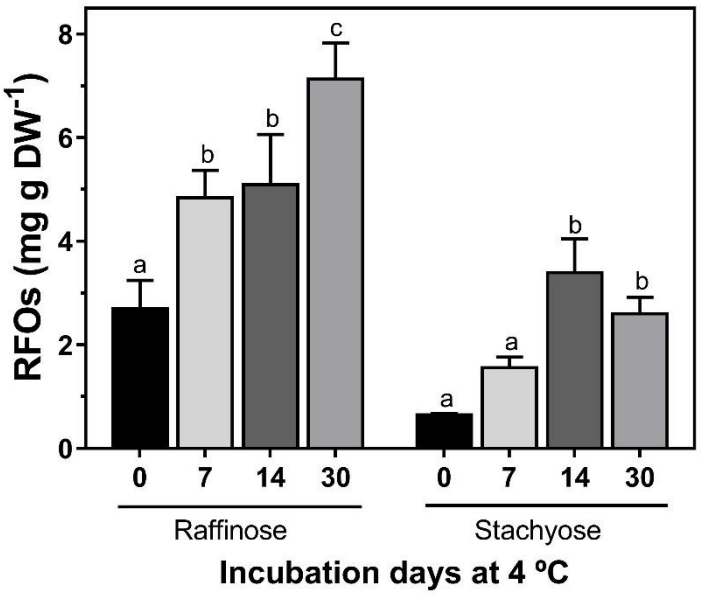

Figure 10. Concentration of oligosaccharides (raffinose and stachyose) from woody canes of *Vitis vinifera* cv. Vinhão incubated at 4 ºC up to 30 days. Results indicate the mean ± SD of four independent experiments. Different letters stand for significant differences (P≤0.05).

3.7. Cold promotes a transcriptional modification of key genes involved in galactinol and raffinose metabolism

Real Time PCR analysis was performed to determine the expression of genes coding for enzymes responsible for the synthesis of RFOs. Results showed that cold treatment strongly upregulated the expression of *VvGolS1* and *VvGolS3* (galactinol synthesis) together with *VvRafS5* (raffinose synthesis) (Figure 11).

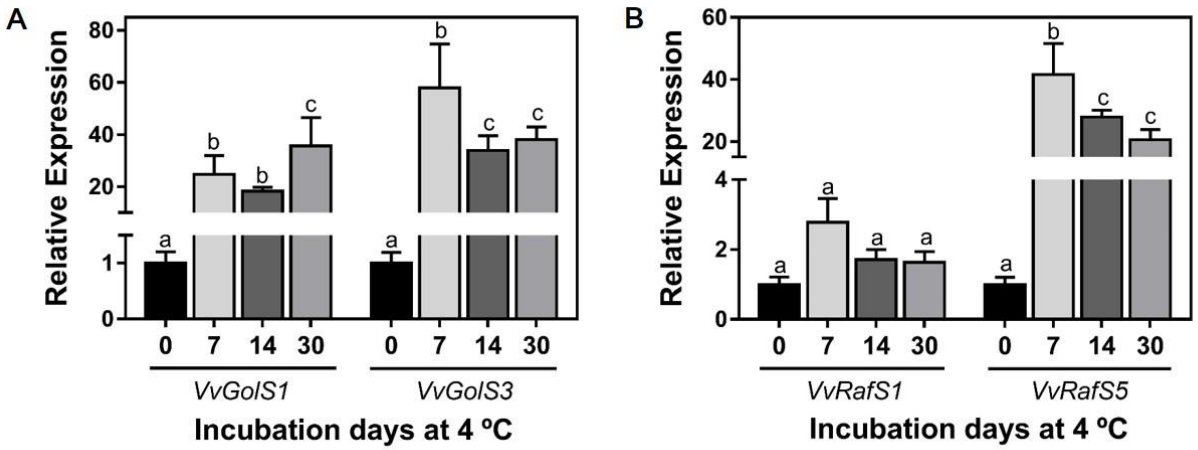

Figure 11. Expression of genes responsible for galactinol synthesis (*VvGolS1*, *VvGolS3*) (A) and raffinose synthesis (*VvRafS1, VvRafS5*) (B) from woody canes of *Vitis vinifera* cv. Vinhão incubated at 4 ºC up to 30 days. Results indicate the mean ± SD of four independent experiments. Different letters stand for significant differences (P≤0.05).

3.8. Tissue specific expression and functional characterization of the plastidic translocator *VvpGT2*

Following the above observation that *VvpGT2* was strongly upregulated during cold treatment we proceeded with the characterization of this gene in more detail. mRNA was extracted from different grapevine organs (canes, flowers and roots) and from berries at different development stages (green, *veraison* and mature), and results showed that showed that *VvpGT2* is mainly expressed in mature berries and in flowers (Figure 12A).

To address the hypothesis that *VvpGT2* codes for a functional sugar transporter, the gene was heterologously expressed in the monosaccharide-defective mutant strain *EBY.VW4000*. Results of the drop-test experiments are shown in figure 12B. As can be seen, the transformed yeast was unable to grow in solid media supplemented with glucose, fructose and sucrose, suggesting that *VvpGT2* is not able to transport none of these sugars. Currently we are checking if the protein is being correctly target to the plasma membrane using GFP fusion proteins. Further transport experiments will be performed with radioactive substrate to improve the accuracy of the analysis.

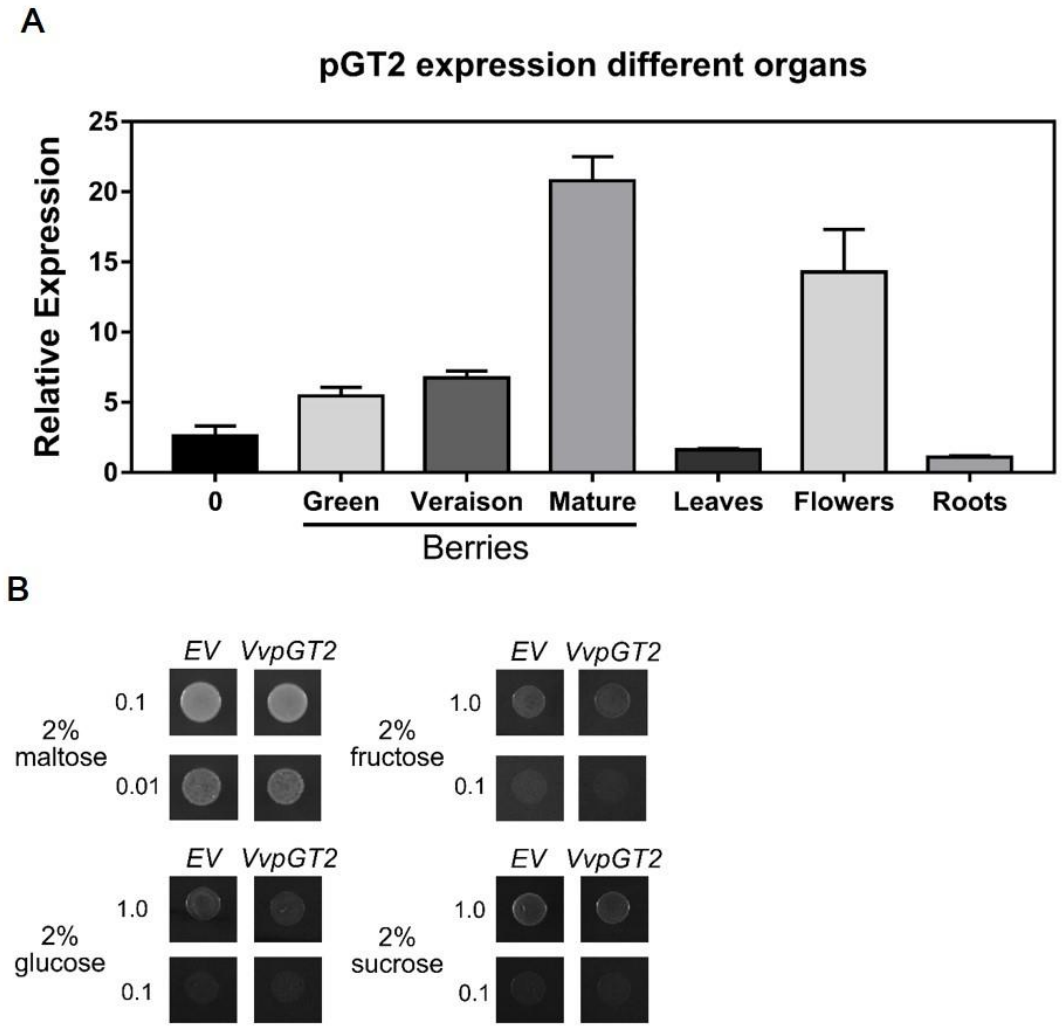

Figure 12. Expression analysis of *VvpGT2* and growth assays. Relative expression of *VvpGT2* in different *Vitis vinifera* cv. Vinhão organs (A) and growth assays in different sugars with the *Saccharomyces cerevisiae* EBY.VW4000 strain overexpressing *VvpGT2* (B). Results indicate the mean ± SD of seven independent experiments.

4. Discussion

4. Discussion

4.1 Cold reduces storage starch in grapevine woody canes

Starch is the most widespread and abundant storage carbohydrate in plants. We depend upon starch for our nutrition, exploit its unique properties in industry, and use it as a feedstock for bioethanol production (reviewed by Zeeman et al., 2010), so it is not surprising that many studies have been performed in starch crops like in cereals. Therefore, while the mechanisms of starch mobilization during germination in the dead endosperm of cereal seeds are well described, the molecular and biochemical mechanisms involved in starch storage in the heterotrophic tissues of woody plants and its utilization in spring and winter are still puzzling. The knowledge on starch storage and mobilization in woody tissues is pivotal to understand (and to optimize) some common practices in the field that modify source–sink relationships, such as pruning and defoliation, as well to understand the mechanisms of acclimation to cold (Reviewed by Noronha et al., 2018), which is the research topic we address in the present study.

In grapevine, the majority of studies examining cold-temperature effects have been performed in leaves and berry tissues (Tattersall, 2007; Yang et al., 2012; Xin et al., 2013; Xu et al., 2014; Sun et al., 2016), thus in the present work we aimed to test the hypothesis that the response of grapevine to cold also involves modifications of the sugars stored in the woody tissues. Results clearly showed that that woody canes exposed cold for up to 30 days resulted in a clear decrease in starch content, thus supporting our hypothesis. Concordantly, this decrease was associated with the induction of α-amylase gene *VvAMY5* whose expression was only detected after the onset of the cold treatment. In spite of this, other amylases may account for the degradation of starch in response to cold because the expression of the amylase genes like *VvAMY2* and *VvBAM2* was also observed, although their expression decreased during the treatment. A previous report in *Populus* also showed a decrease in starch quantity in xylem ray cells in response to cold (Sauter and Kloth, 1987). Other studies in Poplar wood have reported an increase in the biochemical activity of starch-degrading enzymes in the period of starch-sugar conversion (Elle and Sauter, 2000).

4.2. Cold promotes the accumulation of soluble sugars in woody canes and alters sucrose metabolism

Carbohydrates work as regulators of source–sink interactions in the whole plant both under normal environmental conditions and under stress conditions (Roitsch, 1999) In the present study we observed an increase in sucrose, fructose and glucose in grapevine wood in response to cold. These changes were concomitant with a transcriptional reprogramming of genes involved in sugar metabolism and transport. Thus, the steady-state transcript levels of *VvpGT2* and *VvSWEET2b* transporters, as well as

VvSusy and *VvSPS* enzymes increased along time, while the expression of the transporters *VvTMT1* and *VvSUC27* was downregulated. Our results go in agreement with previous studies showing that carbohydrates, mainly sucrose, are involved in the response to cold (Parker, 1962; Sakai, 1966; Kandler et al., 1979; Santarius et al., 1979; Höll, 1985; Sauter and Van Cleve, 1991; Nägele et al., 2012). Glucose, fructose, sorbitol, stachyose and raffinose, can also accumulate during this process (Korn et al., 2010; Findling et al., 2014), much like we observed in the present study in grapevine wood.

Previous reports have already highlighted the pivotal role of enzymes involved in sucrose synthesis, like Susy, during starch metabolism in heterotrophic tissues. Thus, lower accumulation of starch in potato tubers was observed in Susy defective mutants (Zrenner et al., 1995), and an increased starch accumulation was observed in seed endosperm of maize overexpressing Susy (Li et al., 2013). In the present study we confirmed the role of Suzy during starch degradation in grape vine wood in response to cold when its expression levels increased up to day 14.

Our results showed that the cold treatment downregulated the expression of the sucrose/H^+ symporter *VvSuc27* and the transcript levels of the plastidic monosaccharide transporter *VvTMT1*, while the expression of the vacuolar *VvSWEET2b* increased up to day 14. In agreement, increasing number of sugar transporters like SUTs have been reported to take part in the response to abiotic stress (Gong et al., 2015; Jia et al., 2015; Cai et al., 2017; Xu et al., 2018) but further experiments are still necessary to elucidate the role of sugar transporters and sucrose metabolizing enzymes during the response of grapevine wood to cold.

4.3 VvRafS5 is likely involved in the accumulation of raffinose and stachyose in grapevine wood in response to cold

In this study, it was found that the levels of raffinose and stachyose, detected by HPLC, increased during cold and that the transcript levels of genes putatively involved in the synthesis of raffinose and stachyose, particularly *VvRafS5,* increased several fold. In agreement, in poplar wood during late autumn and winter, the extent of starch consumption has also been reported to be closely related to increased levels of sucrose, raffinose and stachyose (Sauter and Kloth, 1987; Sauter and van Cleve, 1991). RFOs have been proposed to play multiple functions, from supply and storage of carbon to protection against abiotic stresses (Peterbuer and Richter, 2001). Multiple biophysical roles have been suggested for raffinose, including in reducing osmotic potential and cells water freezing point (Burke et al., 1976), and a structure-preserving effect at the time of protein binding (Steponkus, 1980).

Furthermore, accumulation of galactinol and raffinose has been postulated to function as a radical scavenger thereby helping the plant to detoxify ROS that accumulate under high salinity and low temperatures (Nishizawa et al., 2008). Besides this, raffinose and stachyose, accumulate during seed development (Ooms et al., 1993; Nishizawa-Yokoi et al., 2008).

Interestingly, in *Arabidopsis thaliana* seeds, stachyose and raffinose accumulation is regulated by *AtRS4* and *AtRS5* (Gangl et al., 2015) and another study concluded that *AtRS5* is the only RafS in Arabidopsis responsible for stress induced raffinose formation (Gangl and Tenhaken, 2016). These results suggest that *VvRafS5* could be fundamental in the observed accumulation of raffinose in response to cold in grapevine woody tissues.

Our results also suggested that *VvGolS1* and *VvGolS3* could play a role in grapevine woody tissues response to cold by providing galactinol to fuel the RFO metabolism. The observed increase of the transcript levels of *VvSusy* (following 7 and 14 days of cold storage) that catalyzes the conversion of sucrose to fructose and UDP-glucose, with the latter being a precursor of galactinol synthesis is consistent with the proposed hypothesis. GolS genes are involved in various developmental and environmental responses. Besides being described as a signal molecule, galactinol, together with sucrose, are the substrates for raffinose synthesis. Also, the addition of galactose moieties to raffinose allows the synthesis of stachyose. The mRNA expression level of *GolS* in *Arabidopsis* was found to be induced by cold (Liu et al., 1998). Up-regulation of *AtGolS1* and *AtGolS2.* in response to water and salinity stress was also reported, while *AtGolS3* is solely induced during low-temperature exposure (Taji et al., 2002). *AtGolS1* is also involved in plants response against *Botrytis cinerea* infection (Song et al., 2010). In tomato (*Lycopersicon esculentum*), dehydration caused an accumulation of LeGOLS-1 mRNA but, when under cold conditions, no alteration was observed in germinating seeds. However, both stresses induced the accumulation of LeGOLS-1 mRNA in seedling leaves (Downie et al., 2003).

As whole, our results, together with the reported studies, strongly suggested that the response of grapevine wood to cold involves the metabolism of starch towards the accumulation of compatible solutes.

4.4 What is the role of *VvpGT2* whose expression steadily increases in grapevine wood in response to cold?

The final products of starch degradation in the chloroplast during the night are mainly maltose and some glucose (Weise et al., 2004), which are exported to the cytosol by MEX (Niitylä et al., 2004) and plastidic glucose translocator pGT (Weber et al., 2000), respectively. Glucose transport across the

chloroplast envelope was first shown by Schäfer et al. (1977), from which it was lately identified the pGT (Weber et al., 2000).

In the present study we wanted to test the hypothesis that pGT2 involved in sugar export from the chloroplast could also be involved in sugar export from the amyloplast for the synthesis of soluble in response to cold. The observed steadily increase of the transcript levels of *pGT2* in grapevine wood in response to cold strongly supported our idea. To gain further knowledge into the physiological role of *VvpGT2*, the gene was cloned and expressed in a HXT-Null *Saccharomyces cerevisiae* unable to transport glucose, but no sugar transport activity was observed when growth plate assays with glucose, sucrose and fructose were performed. Currently we are checking if the protein is being correctly target to the plasma membrane using GFP fusion proteins. Further transport experiments will be performed with radioactive substrates to improve the accuracy of the analysis and also using an *Escherichia coli* mutant without sugar transporters as an expression system.

4.5 Conclusions

Carbohydrates accumulation during autumn and winter and its mobilization during the summer is essential to support a rapid seasonal growth in the coming spring of perennial woody plants. In the present study, we found that, during cold storage, there is a decrease in reserve starch concomitant with an accumulation of sugars (sucrose, glucose and fructose) and RFOs (raffinose and stachyose), which could be related to the expression of several key-genes, like *VvpGT2*, *VvGolS1*, *VvGolS3* and *VvRafS5*. These observations supported our main hypothesis that the response of grapevine to cold involves modifications of starch metabolism together with the reprograming of the expression of key genes involved in sugar metabolism towards the the accumulation of compatible solutes.

Additional studies are already in progress to consolidate some conclusions of this thesis. For instance, as reported above, although we were unable to demonstrate sugar transport mediated by *VvpGT2* we will confirm if the protein is, indeed, correctly targeted to the plasma membrane of the yeast mutant using GFP-tagged proteins. We will also use more accurate strategies to measure sugar uptake using radioactive sugars. In the case these strategies fail we will test a different expression system, such as bacteria. Another study with interest is the cloning and functional characterization of *VvRafS5* because few raffinose synthase genes have been studied in detail. Our experimental approach can also be adjusted to study the effect of other environmental constrains that affect grapevine in the context of ongoing climate changes, like heat waves, which may alter winter carbohydrate reserves and, thus, the bud-burst in the following spring.

References

References

Afoufa-Bastien D, Medici A, Jeauffre J, Coutos-Thévenot P, Lemoine R, Atanassova R, Laloi M. 2010. The *Vitis vinifera* sugar transporter gene family: phylogenetic overview and macroarray expression profiling. *BMC Plant Biology* **10**, 245.

Ainsworth E, Bush DR. 2011. Carbohydrate export from the leaf: A highly regulated process and target to enhance photosynthesis and productivity. *Plant Physiology* **155**, 64–69.

Ait Barka E, Nowak J, Clément C. 2006. Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN. *Applied Environmental Microbiology* **72**, 7246–7252.

Ali K, Maltese F, Choi YH, Verpoorte, R. 2010. Metabolic constituents of grapevine and grape-derived products. *Phytochemistry Reviews* **9**, 357–378.

Andriotis VME, Saalbach G, Waugh R, Field RA, Smith AM. 2016. The maltase involved in starch metabolism in barley endosperm is encoded by a single gene. *PLoS ONE* **11**, e0151642.

Bates TR, Dunst RM, Joy P. 2002. Seasonal dry matter, starch, and nutrient distribution in 'Concord' grapevine roots. *HortScience* **37**, 313–316.

Berrocal-Lobo M, Ibañez C, Acebo P, Ramos A, Perez-Solis E, Collada C, Casado R, Aragoncillo C, Allona I. 2011. Identification of a homolog of *Arabidopsis* DSP4 (SEX4) in chestnut: its induction and accumulation in stem amyloplasts during winter or in response to the cold. *Plant, Cell and Environment* **34**, 1693–1704.

Bertamini M, Zulini L, Muthuchelian K, Nedunchezian N. 2006. Effect of water deficit on photosynthetic and other physiological responses in grapevine (*Vitis vinifera* L. cv. Riesling) plants. *Photosynthetica* **44**, 151–154.

Bohnert HJ, Sheveleva E. 1998. Plant stress adaptations — making metabolism move. *Current Opinion in Plant Biology* **1**, 267–274.

Bonicel A, Haddad G, Gagnaire J. 1987. Seasonal variations in starch and major soluble sugars in the different organs of young poplars. *Plant Physiology Biochemistry* **25**, 451–459.

Bouard J. 1966. Recherches physiologiques sur la vigne et en particulier sur l'aoûtement des sarments, PhD thesis, University of Bordeaux (France).

Buléon A, Colonna P, Planchot V, Ball S. 1998. Starch granules: structure and biosynthesis. *International Journal of Biological Macromolecules* **23**, 85–112.

Burke MJ, Gusta LV, Quamme HA, Weiser CJ, Li PH. 1976. Freezing and injury in plants. *Annual Review of Plant Physiology* **27**, 507–528.

Burton RA, Johnson PE, Beckles DM, Fincher GB, Jenner HL, Naldrett MJ, Denyer K. 2002. Characterization of the genes encoding the cytosolic and plastidial forms of ADP-glucose pyrophosphorylase in wheat endosperm. *Plant Physiology* **130**, 1464–1475.

Cai Y, Tu W, Zu Y, Jing Y, Xu Z, Lu J, Zhang Y. 2017. Overexpression of a grapevine sucrose transporter (*VvSUC27*) in tobacco improves plant growth rate in the presence of sucrose *in vitro*. *Frontiers in Plant Science* **8**, 1069.

Cai Y, Yan J, Li Q, Deng Z, Liu S, Lu J, Zhang Y. 2019. Sucrose transporters of resistant grapevine are involved in stress resistance. *Plant Molecular Biology* **100**, 111–132.

Candolfi-Vasconcelos MC, Koblet W, Howell GS, Zweifel W. 1994. Influence of Defoliation, Rootstock, Training System, and Leaf Position on Gas Exchange of Pinot Noir Grapevines. *American Journal of Enology and Viticulture* **45**, 173–180.

Caspari HW, Lang A, Alsbach P. 1998. Effects of girdling and leaf removal on fruit set and vegetative growth in grape. *American Journal of Enology and Viticulture* **49**, 359–366.

Chen LQ, Cheung LS, Feng L, Tanner W, Frommer WB. 2015. Transport of sugars. *Annual Review of Biochemistry* **84**, 865–894.

Chen LQ, Qu XQ, Hou BH, Sosso D, Osorio S, Fernie A, Frommer W. 2011. Sucrose Efflux Mediated by SWEET Proteins as a Key Step for Phloem Transport. *Science* **335**, 207–211.

Chinnusamy V, Zhu J, Zhu JK. 2007. Cold stress regulation of gene expression in plants. *Trends in Plant Science* **12**, 444–451.

Conde A, Regalado A, Rodrigues D, Costa JM, Blumwald E, Chaves MM, Gerós H. 2015. Polyols in grape berry: transport and metabolic adjustments as a physiological strategy for water stress tolerance in grapevine. *Journal of Experimental Botany* **66**, 889–906.

Conde C, Silva P, Fontes N, Dias ACP, Tavares RM, Sousa MJ, Agasse A, Delrot S, Gerós H. 2007. Biochemical changes throughout grape berry development and fruit and wine quality. *Food* **1**, 1–22.

Copeland L, Blazek J, Salman H, Tang MC. 2009. Form and functionality of starch. *Food Hydrocolloids* **23**, 1527–1534.

Couée I, Sulmon C, Gouesbet G, El Amrani A. 2006. An involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *Journal of Experimental Botany* **57**, 449–459.

Denyer K, Johnson P, Zeeman S, Smith AM. 2001. The control of amylose synthesis. *Journal of Plant Physiology* **158**, 479–487.

Downie B, Gurusinghe S, Dahal P, Thacker RR, Snyder JC, Nonogaki H, Yim K, Fukanaga K, Alvarado V, Bradford KJ. 2003. Expression of a Galactinol Synthase gene in tomato seeds is up-regulated before maturation desiccation and again after imbibition whenever radicle protrusion is prevented. *Plant Physiology*, **131**, 1347–1359.

Duchêne E, Schneider C. 2005. Grapevine and climatic changes: a glance at the situation in Alsace. *Agronomy for Sustainable Development* **25**, 93–99.

Eifert J, Panczel M, Eifert A. 1960. Anderung des Starke und Zuckergehaltes der Rebe wahrend der Ruheperiode. *Vitis* **2**, 257–264.

Elle D, Sauter JJ. 2000. Seasonal changes of activity of a starch granule bound endoamylase and of a starch phosphorylase in Poplar wood (*Populus* × *canadensis* Moench « robusta ») and their possible regulation by temperature and phytohormones. *Journal of Plant Physiology* **156**, 731–740.

Eyéghé-Bickong HA, Alexandersson EO, Gouws LM, Young PR, Vivier MA. 2012. Optimisation of an HPLC method for the simultaneous quantification of the major sugars and organic acids in grapevine berries. *Journal of Chromatography B* **885**, 43–49.

Fege AS, Brown GN. 1984. Carbohydrate distribution in dormant Populus shoots and hardwood cuttings. *Forest Science* **39**, 999–1010.

Fernandez O, Vandesteene L, Feil R, Baillieul F, Lunn J, Clément C. 2012. Trehalose metabolism is activated upon chilling in grapevine and might participate in *Burkholderia phytofirmans* induced chilling tolerance. *Planta* **236**, 355-369.

Findling S, Zanger K, Krueger S, Lohaus G. 2014. Subcellular distribution of raffinose oligosaccharides and other metabolites in summer and winter leaves of *Ajuga reptans* (Lamiaceae). *Planta* **241**, 229–241.

Fischer K, Arbinger B, Kammerer B, Busch C, Brink S, Wallmeier H, Sauer N, Eckerskorn C, Flügge UI. 1994. Cloning and in vivo expression of functional triose phosphate/phosphate translocators from C3- and C4-plants: evidence for the putative participation of specific amino acid residues in the recognition of phosphoenolpyruvate. *Plant Journal* **5**, 215–226

Fisher DB, Oparka KJ. 1996. Post-phloem transport: principles and problems. *Journal of Experimental Botany* **47**, 1141–1154.

Gangl R, Tenhaken R. 2016. Raffinose Family Oligosaccharides Act As Galactose Stores in Seeds and Are Required for Rapid Germination of Arabidopsis in the Dark. *Frontiers in Plant Science* **7**, 1115.

Gangl R, Behmuller R, Tenhaken R. 2015. Molecular cloning of AtRS4, a seed specific multifunctional RFO synthase/galactosylhydrolase in *Arabidopsis thaliana*. *Frontiers in Plant Science* **6**, 789.

Gainza-Cortés F, Pérez R, Pérez-Castro R, Tapia J, Casaretto JA, González S, Peña Cortés H, Ruiz-Lara S, González E. 2012. Characterization of a putative grapevine Zn transporter, *VvZIP3*, suggests its involvement in early reproductive development in Vitis vinifera L. *BMC Plant Biology* **12**, 111.

Gibson S. 2005. Control of plant development and gene expression by sugar signaling. *Current Opinion in Plant Biology* **8**, 93–102.

Gong X, Zhang J, Jianbing H, Wang W, Hao W, Zhang Q, Liu JH. 2015. FcWRKY70, a WRKY protein of *Fortunella crassifolia*, functions in drought tolerance and modulates putrescine synthesis by regulating arginine decarboxylase gene: *FcWRKY70* regulates putrescine synthesis. *Plant, Cell and Environment*. **38**, 2248–2262.

Guy C, Haskell D, Neven L, Klein P, Smelser C. 1992. Hydrationstate-responsive proteins link cold and drought stress in spinach. *Planta* **188**, 265–270.

Hannah L, Roehrdanz P, Ikegami M, Shepard A, Shaw M, Tabor G, Zhi L, Marquet P, Hijmans R. 2013. Climate change, wine, and conservation. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 6907–6912.

Hardie WJ, Considine JA. 1976. Response of grapes to water-deficit stress in particular stages of development. *American Journal of Enology and Viticulture* **27**, 55–61.

Hendrickson L, Furbank R, Chow W. 2004. A Simple Alternative Approach to Assessing the Fate of Absorbed Light Energy Using Chlorophyll Fluorescence. *Photosynthesis research*. **82**, 73–81.

Hieke S, Menzel CM, Ludders P. 2002. Effects of leaf, shoot and fruit development on photosynthesis of lychee trees (*litchi chinensis*). *Tree Physiology* **22**, 955-961.

Hinesley LE, Pharr DM, Snelling LK, Funderburk SR. 1992. Foliar raffinose and sucrose in four conifer species: relationship to seasonal temperature. *Journal of the American Society for Horticultural Sciences* **117**, 852–855.

Holaday AS, Martindale W, Alred R, Brooks AL, Leegood RC. 1992. Changes in activities of enzymes of carbon metabolism in leaves during exposure of plants to low temperature. *Plant Physiology* **98**, 1105–1114.

Höll W. 1985. Seasonal fluctuation of reserve materials in the trunkwood of spruce *(Picea abies* L. Karst). *Journal of Plant Physiology* **777**, 355-362.

Huijbrechts, AML. 2008. Multifunctional starch derivatives: synthesis, characterization and properties, Doctor of Philosophy, Wageningen University, [S.l.].

Hurry VM, Strand A, Tobiaeson M, Gardeström P, Öquist G. 1995. Cold hardening of spring and winter wheat and rape results in differential effects on growth, carbon metabolism, and carbohydrate content. *Plant Physiology* **109**, 697–706.

Intergovernmental Panel on Climate Change (IPCC). 2019. Special Report: Global Warming of 1.5 °C. [online] Available at: *https://www.ipcc.ch/sr15/chapter/chapter-3/* [Accessed in 13 June 2019].

Ito J, Batth TS, Petzold CJ, Redding-Johanson AM, Mukhopadhyay A, Verboom R, Meyer EH, Millar AH, Heazlewood JL. 2011. Analysis of the *Arabidopsis* cytosolic proteome highlights subcellular partitioning of central plant metabolism. *Journal of Proteome Research* **10**, 1571–1582.

Jia X, Shen J, Liu H, Li F, Ding N, Gao C, Pattanaik S, Patra B, Li R, Yuan L. 2015. Small tandem target mimic-mediated blockage of microRNA858 induces anthocyanin accumulation in tomato. *Planta* **242**, 283–293.

Jones GV, White MA, Cooper OR, Storchmann K. 2005. Climate change and global wine quality. *Climate Change* **73**, 319–343.

Kammerer B, Fischer K, Hilpert B, Schubert S, Gutensohn M, Weber APM, Flügge UI. 1998. Molecular characterization of a carbon transporter in plastids from heterotrophic tissues: the glucose 6-phosphate/phosphate antiporter. *Plant Cell* **10**, 105–117.

Kandler O. 1979. Zellwandstrukturen bei Methan-Bakterium. Zur Evolution der Prokaryonten. *Naturwissenschaften* **66**, 95–105.

Kang F, Rawsthorne S. 1996. Metabolism of glucose-6-phosphate and utilization of multiple metabolites for fatty acid synthesis by plastids from developing oilseed rape embryos. *Planta* ***199***, 321–327.

Kasuga J, Arakawa K, Fujikawa S. 2007. High accumulation of soluble sugars in deep supercooling Japanese white birch xylem parenchyma cells. *New Phytologist* **174**, 569–579.

Kavakli IH, Slattery CJ, Ito H, Okita TW. 2000. The conversion of carbon and nitrogen into starch and storage proteins in developing storage organs: an overview. *Australian Journal of Plant Physiology* **27**, 561–570.

Keller M. 2015. Chapter 4 - Photosynthesis and respiration. in: *The Science of Grapevines* (Second Edition), Academic Press, San Diego, pp.125–143.

Kingston-Smith, Alison. 2001. Resource allocation. *Trends in Plant Science*. **6**, 48–49.

Korn M, Gartner T, Erban A, Kopka J, Selbig J, Hincha DK. 2010. Predicting *Arabidopsis* freezing tolerance and heterosis in freezing tolerance from metabolite composition. *Molecular Plant* **3**, 224–235.

Kriedemann PE, Smart RE. 1971. Effects of irradiance, temperature, and leaf water potential on photosynthesis of vine leaves. *Photosynthetica (Prague)* **213**, 6–15.

Kuipers AGJ, Jacobsen E, Visser RGF. 1994. Formation and deposition of amylose in the potato tuber are affected by the reduction of granule bound starch synthase gene expression. *Plant Cell* **6**, 43–52.

Lalonde S, Wipf D, Frommer WB. 2004. Transport mechanisms for organic forms of carbon and nitrogen between source and sink. *Annual Review of Plant Biology* **55**, 341–372.

Lebon G, Wojnarowiez G, Holzapfel B, Fontaine F, Vaillant-Gaveau N, Clément C. 2008. Sugars and flowering in the grapevine (*Vitis vinifera* L.). *Journal of Experimental Botany* **59**, 2565–2578.

Lecourieux F, Kappel C, Lecourieux D, Serrano A, Torres E, Arce-Johnson P, Delrot S. 2013. An update on sugar transport and signalling in grapevine. *Journal of Experimental Botany* **65**, 821–832.

Lemoine R, La Camera S, Atanassova R, Dédaldéchamp F, Allario T, Pourtau N, Bonnemain JL, Laloi M, Coutos-Thévenot P, Maurousset L, Faucher M, Girousse C, Lemonnier P, Parrilla J, Durand M. 2013. Source-to-sink transport of sugar and regulation by environmental factors. *Frontiers in Plant Science* **4**, 272.

Li J, Baroja-Fernández E, Bahaji A, Muñoz FJ, Ovecka M, Montero M, Sesma MT, Alonso-Casajús N, Almagro G, Sánchez-López AM, Hidalgo M, Zamarbide M, Pozueta-Romero J. 2013. Enhancing sucrose synthase activity results in increased levels of starch and ADP-glucose in maize (*Zea mays* L.) seed endosperms. *Plant and Cell Physiology* **54**, 282–294.

Li PH, Weiser CJ, Van Huystee R. 1965. Changes in metabolites of red-osier dogwood during cold acclimation. *Proceedings of the American Society for Horticultural Science* **86**, 723–730.

Lindeboom N, Chang PR, Tyler RT. 2004. Analytical, biochemical and physicochemical aspects of starch granule size with emphasis on β-granule starches: A review. *Starch-Stärke* **56**, 89–99.

Liu LY, Li H. 2013. Review: research progress in amur grape, *Vitis amurensis* Rupr. *Canadian Journal of Forest Research* **93**, 565–575.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and lowtemperature- responsive gene expression, respectively, in Arabidopsis. *Plant Cell* **10**, 1391–1406.

Lobell D, Gourdji S. 2012. The Influence of Climate Change on Global Crop Productivity. *Plant Physiology* **160**, 616–620.

Lovisolo C, Schubert A. 1998. Effects of water stress on vessel size and xylem hydraulic conductivity in *Vitis vinifera* L. *Journal of Experimental Botany* **49**, 693–700.

Lundmark M, Cavaco AM, Trevanion S, Hurry V. 2006. Carbon partitioning and export in transgenic Arabidopsis thaliana with altered capacity for sucrose synthesis grown at low temperature: a role for metabolite transporters. *Plant, Cell and Environment* **29**, 1703–1714.

Martínez-Lüscher J, Morales F, Sánchez-Díaz M, Delrot S, Aguirreolea J, Gomès E, Pascual I. 2015. Climate change conditions (elevated CO2 and temperature) and UV-B radiation affect grapevine (*Vitis vinifera* cv. Tempranillo) leaf carbon assimilation, altering fruit ripening rates. *Plant Science* **236**, 168–176.

McGovern P, Jalabadze M, Batiuk S, Callahan MP, Smith KE, Hall, GR, Lordkipanidze D. 2017. Early Neolithic wine of Georgia in the South Caucasus. *Proceedings of the National Academy of Sciences* **114**, e10309–e10318.

Moriondo M, Jones GV, Bois B, Dibari C, Ferrise R, Trombi G, Bindi M. 2013a. Projected shifts of wine regions in response to climate change. *Climatic Change* **119**, 825–839.

Moriondo M, Trombi G, Ferrise R, Giada B, Dibari C, Ammann CM, Lippi MM, Bindi M. 2013b. Olive trees as bio-indicators of climate evolution in the Mediterranean Basin. *Global Ecology and Biogeography* **22**, 818–833.

Muhlbach H, Schnarrenberger C. 1978. Properties and intercellular distribution of two phosphoglucomutases from spinach leaves. *Planta* **141**, 65–70.

Mullins MG, Bouquet A., Williams LE. 1992. *Biology of the Grapevine*. Cambridge University Press. Cambridge, UK.

Münch E. 1930. Die Stoffbewegunen in der Pflanze. Jena: Verlag von Gustav Fische.

Myers AM, Morell MK, James MG, Ball SG. 2000. Recent progress towards understanding the biogenesis of the amylopectin crystal. *Plant Physiology* **122**, 989–997.

Nägele T, Stutz S, Hörmiller I, Heyer AG. 2012. Identification of a metabolic bottleneck for cold acclimation in Arabidopsis thaliana. *Plant Journal* **72**, 102–114.

Nelson EA, Dickson RE. 1981. Accumulation of food reserves in cottonwood stems during dormancy induction. *Canadian Journal of Forest Research* **11**, 145–154.

Nenko N, Ilyina I, Kiseleva G, Yablonskaya Elena. 2018. Low-Temperature Stress Tolerance of Grapevine Varieties of Different Ecological and Geographical Origin. *Proceedings of the Latvian Academy of Sciences* **73**, 56–65.

Neuhaus HE, Henrichs G, Scheibe R. 1993. Characterization of glucose-6-phosphate incorporation into starch by isolated intact cauliflower-bud plastids. *Plant Physiology* **101**, 573–578.

Niewiadomski P, Knappe S, Geimer S, Fischer K, Schulz B, Unte US, Rosso MG, Ache P, Flügge U, Schneider A. 2005. The *Arabidopsis* plastidic glucose 6-phosphate/phosphate translocator GPT1 is essential for pollen maturation and embryo sac development. *The Plant Cell* **17**, 760–775.

Niittylä T, Comparot-Moss S, Lue WL, Messerli G, Trevisan M, Seymour MD, Gatehouse JA, Villadsen D, Smith SM, Chen J, Zeeman SC, Smith AM. 2006. Similar protein phosphatases control starch metabolism in plants and glycogen metabolism in mammals. *Journal of Biological Chemistry* **281**, 11815–11818.

Niitylä T, Messerli G, Trevisan M, Chen J, Smith AM, Zeeman SC. 2004. A previously unknown maltose transporter essential for starch degradation in leaves. *Science* **303**, 87–89.

Nishizawa A, Yabuta Y, Shigeoka S. 2008. Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiology* **147**, 1251–1263.

Nishizawa-Yokoi A, Yabuta Y, Shigeoka S. 2008. The contribution of carbohydrates including raffinose family oligosaccharides and sugar alcohols to protection of plants from oxidative damage. *Plant Signaling and Behavior* **3**, 1016–1018.

Noronha H, Araújo D, Conde C, Martins A, Soveral G, Chaumont F, Delrot S, Gerós H. 2016. The Grapevine Uncharacterized Intrinsic Protein 1 (*VvXIP1*) Is Regulated by Drought Stress and Transports Glycerol, Hydrogen Peroxide, Heavy Metals but Not Water. *PLOS ONE* **11**, e0160976.

Noronha H, Conde C, Delrot S, Gerós H. 2015. Identification and functional characterization of grapevine transporters that mediate glucose-6-phosphate uptake into plastids. *Planta* **242**, 909–920.

Noronha H, Silva A, Dai Z, Gallusci P, Rombolà AD, Delrot S, Gerós H. 2018. A molecular perspective on starch metabolism in woody tissues. *Planta* **248**, 559–568.

Ögren E. 1999. Fall frost resistance in willows used for biomass production. II. Predictive relationships with sugar concentration and dry matter content. *Tree Physiology* 19, 755–760.

Oiv.int. 2019. OIV 2018 report on the world vitivinicultural situation. [online] Available at*: http://www.oiv.int/en/oiv-life/oiv-2018-report-on-the-world-vitivinicultural-situation* [Accessed 13 June 2019].

Ollat N, van Leeuwen C, Garcia de Cortazar-Atauri I, Touzard JM. 2017. The challenging issue of climate change for sustainable grape and wine production. *OENO One* **51**, 59–60.

Ooms JJ, Leon-Kloosterziel KM, Bartels D, Koorneef M, Karssen CM. 1993. Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana*. *Plant Physiology* **102**, 1185–1191.

Parker J. 1962. Seasonal changes in cold resistance and free sugars of some hardwood tree barks. *Forest Science* **8**, 255–262.

Peterbauer T, Richter A. 2001. Biochemistry and physiology of Raffinose family oligosaccharides and galactosyl cyclitols in seeds. *Seed Science Research* **11**, 185–197.

Petrie P, Sadras V. 2008. Advancement of grapevine maturity in Australia between 1993 and 2006: Putative causes, magnitude of trends and viticultural consequences. *Australian Journal of Grape and Wine Research* **14**, 33–45.

Piispanen R, Saranpää P. 2001. Variation of non-structural carbohydrates in silver birch (*Betula pendula* Roth) wood. *Trees* **15**, 444–451.

Pillet J, Egert A, Pieri P, Lecourieux F, Kappel C, Charon J, Gomès E, Keller F, Delrot S, Lecourieux D. 2012. *VvGOLS1* and *VvHsfA2* are Involved in the Heat Stress Responses in Grapevine Berries. *Plant and Cell Physiology* **53**, 1776–1792.

Ramos M, Jones G, Martínez-Casasnovas J. 2008. Structure and trends in climate parameters affecting winegrape production in northeast Spain. *Climate Research* **38**, 1–15.

Ray DK, Gerber JS, MacDonald GK, West PC. 2015. Climate variation explains a third of global crop yield variability. *Nature Communications* **6**, 59–89.

Reid KE, Olsson N, Schlosser J, Peng F, Lund ST. 2006. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC Plant Biology* **6**, 27.

Rodrigo J, Hormaza JI, Herrero M. 2000. Ovary starch reserves and flower development in apricot (*Prunus armeniaca*). *Physiologia Plantarum* **108**, 35–41.

Roitsch T. 1999. Source-sink regulation by sugar and stress. *Current Opinion in Plant Biology* **2**, 198–206.

Ruelland E, Zachowski A. 2010. How plants sense temperature. *Environmental and Experimental Botany* **69**, 225–232.

Saito M, Yoshida M. 2011. Expression analysis of the gene family associated with raffinose accumulation in rice seedlings under cold stress. *Journal of Plant Phisiology* **168**, 2268–2271.

Sakai A, Larcher W. 1987. Frost survival of plants. Springer-Verlag, New York.

Sakai A. 1966. Studies of frost hardiness in woody plants. II. Effect of temperature on hardening. *Plant Physiology* **41**, 353–359.

Santarius K, Müller M. 1979. Investigation on heat resistance of spinach leaves. *Planta* **146**, 529–538.

Sauter JJ, Kloth S. 1987. Changes in carbohydrates and ultrastructure in xylem ray cells of Populus in response to chilling. *Protoplasma **137***, 45–55.

Sauter JJ, van Cleve B. 1991. Biochemical and ultrastructural results during starch-sugar conversion in ray parenchyma cells of *Populus* during cold adaptation. *Journal of Plant Physiology* **139**, 19–26.

Sauter JJ, van Cleve B. 1994. Storage, mobilization and interrelations of starch, sugars, protein and fat in the ray storage tissue of poplar trees. *Trees* **8**, 297–304.

Sawicki M, Ait Barka E, Clément C, Vaillant-Gaveau N, Jacquard C. 2015. Cross-talk between environmental stresses and plant metabolism during reproductive organ abscission. *Journal of Experimental Botany* **66**, 1707–1719.

Schäfer G, Heber U, Heldt HW. 1977. Glucose transport into spinach chloroplasts. *Plant Physiology* **60**, 286–289.

Schneider T, Keller F. 2009. Raffinose in chloroplasts is synthesized in the cytosol and transported across the chloroplast envelope. *Plant Cell Physiology* **50**, 2174–2182.

Scholefield PB, Neales TP, May P. 1978. Carbon balance of the Sultana vine (*Vitis vinifera* L.) and the effects of autumn defoliation by harvest pruning. *Australian Journal of Plant Physiology* **5**, 561–570.

Schultz HR, Jones GV. 2010. Climate Induced Historic and Future Changes in Viticulture, *Journal of Wine Research* **21**, 137–145.

Schulz A, Beyhl D, Marten I, Wormit A, Neuhaus E, Poschet G, Büttner M, Schneider S, Sauer N, Hedrich R. 2011. Proton-driven sucrose symport and antiport are provided by the vacuolar transporters SUC4 and TMT1/2. *Plant Journal* **68**, 129–136.

Schünemann D, Borchert S, Flugge U, Heldt H. 1993. ADP/ATP Translocator from Pea Root Plastids (Comparison with Translocators from Spinach Chloroplasts and Pea Leaf Mitochondria). *Plant Physiology* **103**, 131–137.

Slattery CJ, Kavakli IH, Okita TW. 2000. Engineering starch for increased quantity and quality. *Trends in Plant Science* **5**, 291–298.

Smith AM, Denyer K, Martin C. 1997. The synthesis of the starch granule. *Plant Molecular Biology* **48**, 67–87.

Smith AM, Zeeman SC. 2006. Quantification of starch in plant tissues. *Nature Protocols* **1**, 1342–1345.

Song MC, Kang EY, Kim MS, Yoo SJ, Im YJ, Kim YC, Yang KY, Kim KY, Kim KS, Choi YS, Cho BH. 2010. Jasmonate-dependent expression of a galactinol synthase gene is involved in priming of systemic fungal resistance in *Arabidopsis thaliana*. *Botany* **88**, 452–461.

Steponkus PL. 1980. Cold acclimation and freezing injury from a perspective of plasma membrane. In: Katterman F, ed *Environmental injury to plants*. San Diego: Academic Press, 1-16.

Striegler RK, Howell GS, Flore JÁ. 1993. Influence of rootstock on the response of seyval grapevines to flooding stress. *American Journal of Enology and Viticulture* **44**, 313–319.

Sun L, Song W, Geng Q, Du Y, Zhai H, Qin W. 2016. Comparison of cold hardiness in the leaves of various grape cultivars based on photochemical reflectance index. *Vitis –Geilweilerhof* **55**, 107–112.

Swanson CA, El Shishiny ED. 1958. Translocation of sugars in the Concord grape. *Plant Physiology* **33**, 33–37.

Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K. 2002. Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. *The Plant journal: for cell and molecular biology* **29**, 417–426.

Tattersall EA, Grimplet J, Deluc L, Wheatley MD, Vincent D, Osborne C, Ergül A, Lomen E, Blank RR, Schlauch KA, Cushman JC, Cramer GR. 2007. Transcript abundance profiles reveal larger and more complex responses of grapevine to chilling compared to osmotic and salinity stress. *Functional and Integrative Genomics* **7**, 317–333.

Tester RF, Karkalas J, Qi X. 2004. Starch—composition, fine structure and architecture. *Journal of Cereal Science* **39**, 151–165.

Theocharis A, Clément C, Ait Barka E. 2012. Physiological and molecular changes in plants at low temperatures. *Planta* **235**, 1091–105.

Van Dingenen R, Dentener FJ, Raes F, Krol MC, Emberson L, Cofala J. 2009. The global impact of ozone on agricultural crop yields under current and future air quality legislation. *Atmospheric Environment* **43**, 604–618.

Walker MD, Ingersoll RC, Webber PJ. 1995. Effects of interannual climate variation on phenology and growth of two alpine forbs. *Ecology* **74**, 1067–1083.

Wardlaw IF. 1990. Tansley Review No. 27: The control of carbon partitioning in plants. *New Phytologist* **116**, 341–381.

Weber A, Servaites JC, Geiger DR, Kofler H, Hille D, Groner F, Hebbeker U, Flügge UI. 2000. Identification, purification, and molecular cloning of a putative plastidic glucose translocator. *Plant Cell* **12**, 787–801.

Weise S, Weber APM, Sharkey TD. 2004. Maltose is the major form of carbon exported from the chloroplast at night. *Planta* **218**, 474–482.

Weschke W, Panitz R, Gubatz S, Wang Q, Radchuk R, Weber H, Wobus U. 2003. The role of invertases and hexose transporters in controlling sugar ratios in maternal and filial tissues of barley caryopses during early development. *Plant Journal* **33**, 395–411.

Weschke W, Panitz R, Sauer N, Wang Q, Neubohn B, Weber H, Wobus U. 2000. Sucrose transport into barley seeds: molecular characterization of two transporters and implications for seed development and starch accumulation. *Plant Journal* **21**, 455–467.

White M, Diffenbaugh N, Jones G, Pal J, Giorgi F. 2006. Extreme heat reduces and shifts United States premium wine production in the 21st century. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 11217–11222.

Wingenter K, Schulz A, Wormit A, Wic S, Trentmann O, Hoermiller II, Heyer AG, Marten I, Hedrich R, Neuhaus HE. 2010. Increased activity of the vacuolar monosaccharide transporter TMT1 alters cellular sugar partitioning, sugar signaling, and seed yield in *Arabidopsis*. *Plant Physiology* **154**, 665–677.

Winkler AJ, Williams WO. 1938. Carbohydrates metabolism of *Vitis vinifera*. *Plant Physiology* **20**, 412–432.

Wischmann B, Nielsen TH, Moller BL. 1999. In vitro biosynthesis of phosphorylated starch in intact potato amyloplasts. *Plant Physiology* **119,** 455–462.

Xin, H.P., Zhu, W., Wang, L., Xiang Y, Fang L, Li J, Sun X, Wang N, Londo JP, Li S. 2013. Genome wide transcriptional profile analysis of *Vitis amurensis* and *Vitis vinifera* in response to cold stress. *PLoS One* **8**, e58740.

Xu J, Xie J, Yan C, Zou X, Ren D, Zhang S. 2014. A chemical genetic approach demonstrates that MPK3/MPK6 activation and NADPH oxidase-mediated oxidative burst are two independent signaling events in plant immunity. *Plant Journal* **77**, 222–234.

Xu QY, Chen SY, Ren YJ, Chen SL, Liesche J. 2018. Regulation of sucrose transporters and phloem loading in response to environmental cues. *Plant Physiology* **176**, 930–945.

Yang YS, Hori Y, Ogata R. 1980. Studies on retranslocation of accumulated assimilates in 'Delaware' grapevines. II. Retranslocation of assimilates accumulated during the previous growing season. *Tohoku Journal of Agricultural Research* **31**, 109–119.

Yang YS, Hori Y. 1979. Studies on retranslocation of accumulated assimilates in 'Delaware' grapevines. I. Retranslocation of C-assimilates in the following spring after 14C feeding in summer and autumn. *Tohoku Journal of Agricultural Research* **30**, 43–56.

Yang, Y., He, M., Zhu, Z., Li, S., Xu, Y., Zhang, C., Singer, S.D. and Wang, Y. (2012) Identification of the dehydrin gene family from grapevine species and analysis of their responsiveness to various forms of abiotic and biotic stress. *BMC Plant Biology* **12**, 140.

Zapata C, Magné C, Deléens E, Brun O, Audran JC, Chaillou S. 2001. Grapevine culture in trenches. I. Root growth and dry matter partitioning. *Australian Journal of Grape and Wine Research* **7**, 127–131.

Zapata M, Jeffrey SW, Wright SW, Rodríguez F, Garrido J, Clementson L. 2004. Photosynthetic pigments in 37 species (65 strains) of Haptophyta. *Marine Ecology-Progress Series* **270**, 83–102.

Zeeman SC, Kossmann J, Smith AM. 2010. Starch: its metabolism, evolution, and biotechnological modification in plants. *Annual Review of Plant Biology* **61**, 209–234.

Zeeman SC, Rees TA. 1999. Changes in carbohydrate metabolism and assimilate export in starch-*excess* mutants of *Arabidopsis*. *Plant, Cell and Environment* **22**, 1445–1453.

Zheng Y, Tian L, Liu H, Pan Q, Zhan J, Huang W. 2009. Sugars induce anthocyanin accumulation and flavanone 3-hydroxylase expression in grape berries. *Plant Growth Regulators* **58**, 251–260.

Zrenner R, Salanoubat M, Willmitzer L, Sonnewald U. 1995. Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum*). *The Plant Journal* **7**, 97–107.