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Analyses of Anthocyanin Biosynthesis in healthy and Berry Shrivel Berries of Zweigelt at different developmental Stages

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Analyses of Anthocyanin Biosynthesis in healthy and Berry Shrivel Berries of Zweigelt at different developmental Stages

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Markus Eitle Tulln an der Donau, July 2013

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I Introduction of the Master Thesis

Berry growth of *Vitis vinifera* L. can be described as a number of complex series of biochemical and physical changes (DELUC et al. 2007). It's important for winegrowers to monitor and manage the berry growth and development in order to achieve an optimal basis for the resulting wine. Grape berry growth is separated in three stages (COOMBE and MCCARTHY 2000): Stage I (seed formation and cell division), Stage II (seed maturation) and Stage III (cell expansion and sugar accumulation). Phytohormones like cytokinins, auxins, gibberellins, ethylene, abscisic acid and brassinosteroids (KELLER 2010) as well as changes in gene expression levels stimulate and coordinate the linked biochemical and physical processes during the three growth stages. A huge number of different compounds are synthesized or imported from the mother vine into the berries.

The "big story" (KENNEDY 2002) in the berry growth stages II and especially III is the sugar accumulation into the berries. Source organs like mature leaves export assimilates of photosynthesis (mainly sucrose) via the phloem to sink organs. Once sucrose arrived in the sink organs e.g. berries, apoplastic phloem unloading starts. Firstly sucrose is hydrolyzed to glucose and fructose by invertases in different cell compartments: Cell wall invertases are bound to cell membranes in the apoplast (HAYES et al. 2007), neutral invertases act mainly in the cytosol (NONIS et al. 2008) and vacuolar invertases cleave sucrose inside the vacuoles (ZHANG et al. 2006). Cleaved to monosaccharides, they are translocated by hexose transporters into the cytosol of the sink cells (AFOUFA-BASTIEN et al. 2010). If the monosaccharides are not used for respiration or metabolisation, they cross the tonoplast membrane mediated by tonoplast monosaccharide transporters (ÇAKIR and GIACHINO 2012) to be stored in the vacuoles of mesocarp parenchyma cells.

A second biochemical process initiating after veraison is the accumulation of anthocyanins. Their biosynthesis is one branch of the complex phenylpropanoid pathway. In the beginning of berry development flavonols, then proanthocyanidins and in the end anthocyanins accumulate (CZEMMEL et al. 2012). Anthocyanin accumulation leads to veraison, which is expressed by the color change from green to red or black. These temporal shifts in the flavonoid biosynthesis are regulated and controlled on the one hand by changes in the expression levels of genes of specific enzymes. Nine enzymes are directly involved in the biosynthesis of anthocyanins: PAL, C4H, 4CL, CHS, CHI, F3H, DFR, LDOX and UFGT. Two enzymes shift the anthocyanin composition: F3'H and F3'5'H. On the other hand there are enzymes competing for identical substrates: FLS, ANR and LAR (CZEMMEL et al. 2012). Recently several MYB R2R3 transcription factors: MYB5b (DELUC et al. 2008), MYBF1 (CZEMMEL et al. 2009), MYBPA1/2 (CZEMMEL et al. 2012) and MYBA1/2 (JEONG et al. 2004; KOBAYASHI et al. 2013) have been isolated and characterized for grapevine. They enhance gene expression levels or the enzyme activity and so regulate the phenylpropanoid pathway.

Berry shrivel (BS) is an increasing problem and a threat for the Austrian and worldwide viticulture. An increasing number of BS cases has been described from South Tyrol and Friuli (Italy; RAIFER cited in RIEDEL 2008), Baden (Germany; BACHTELER and RIEDEL 2011), the

US (KELLER 2008) and recently China (FANG et al. 2011). *Vitis vinifera* L. cultivar Zweigelt (Blaufränkisch x St. Laurent) is the most important red wine cultivar in Austria and at the same time very susceptible against BS (KNOLL et al. 2010). Berry shrivel is assumed to be a physiological disorder, since scans for phytoplasms and viruses were negative (KRASNOW et al. 2009). For the winegrowers BS symptoms like lower sugar content of berries (KRASNOW et al. 2009; GRIESSER et al. 2012[1]), a delayed veraison and small flaccid berries (BONDADA and KELLER 2012[2]) are difficult to foresee and thought to be physiologically initiated before they are visible. Former research has shown that year, vineyard site and position of the plant in the vineyard doesn't correlate with BS (Griesser 2012[2]). Compositional changes in the berries caused by BS such as decreased sugar and anthocyanin content, acid and bitter taste (BACHTELER and RIEDEL 2011) combined with low cell viability (BONDADA and KELLER 2012[1]) limits their use for winemaking, so affected clusters have to be considered as yield loss.

Problem

Berry shrivel becomes a serious threat for the current and for the future viticulture. The main problem is the lack of information and knowledge of winegrowers and scientists so far. An estimation of KRASNOW et al. 2010 says that US winegrowers suffer yield losses of 1-5% annually, but particular vineyards can lose up to 50% of the berries due to BS. Research is directed to many scientific fields, trying to find explanations, earlier symptoms or management solutions against this physiological disorder.

Hypothesis

In this research article we investigated the transcriptional modifications of BS concerning the sugar accumulation and anthocyanin biosynthesis in berries of *Vitis vinifera* L. cultivar Zweigelt in the berry growth period 2011:

Hypothesis 1: BS affects the expression levels of genes related to sugar accumulation in grape berries negatively. Modified genes might be coding for 1) invertases: cell wall invertase (cwINV), neutral invertase (NI) and vacuolar invertases (vacINV1 and vacINV2); 2) hexose transporter proteins: hexose transporters (HT1 and HT3) and 3) tonoplast monosaccharide transporters (TMT1, TMT2 and TMT3). They are key factors of the sugar accumulation from the phloem unloading until the sugar storage in the vacuoles. We analyzed them and expected to find down regulations of at least one gene comparing healthy control and BS affected berries. This would partially explain the decreased sugar content in berries.

Hypothesis 2: We assumed that expression levels of genes coding for key enzymes of the phenylpropanoid pathway were inhibited by BS. Key enzymes of the biosynthetic pathway are 1) biosynthetic enzymes: PAL CHS, CHI, F3H, DFR; LDOX, UFGT, F3'H, F3'5'H; 2) competitive enzymes: FLS, LAR, ANR and 3) regulatory transcription factors: MYB5b, MYBPA1, MYBA1/2. We expected to find one or more down regulations of biosynthetic genes or an up regulation of competing enzymes or transcription factors. This could partially explain the decreased color pigmentation of BS berries.

Objectives
Therefore the objectives of this study were to:

1) Find changes in expression levels of genes coding for enzymes and transporter proteins involved in sugar transport in BS affected berries.
2) Find changes in expression levels of genes coding for enzymes and transcription factors related to the phenylpropanoid pathway in BS affected berries.

Structure of the Thesis
After the Introduction of the Master Thesis, the Literature Review will present and update the relevant topics correlated with this research work: Berry Growth and Development, *Vitis vinifera* L. cultivar Zweigelt, Sugar Transport into Berries, Anthocyanin Biosynthesis and Berry Shrivel. After this part the actual Publication Format will include: Abstract, Introduction, Material and Methods, Results, Discussion, References and Supplementary Material. The advantage of this format is the possibility to publish this research. The final parts are the Summary in English as well as a Zusammenfassung in and References for the whole Thesis.

II Literature Review

II. 1 Berry Growth and Development

II.1.1 Grape Berries

Berries of grapevine *Vitis vinifera* L. are the basis for winemaking. Every winegrower is supposed to monitor, understand and take care of berry growth and development of the clusters on the vines. One of the most important viticultural goals is to produce a uniformly ripened crop with a high level of colour and aroma compounds. The harvest date should coincide with the stage of optimal maturity (KENNEDY 2002; DELUC et al. 2007). This is rather difficult to manage due to the large ripening heterogeneity among vineyard sites, plants within one vineyard, clusters on each plant and even berries within the same cluster (DELUC et al. 2007). KENNEDY 2002 considers every single grape berry as an independent biochemical factor, because they have the potential to accumulate and synthesize a number of primary and secondary metabolites. Viticultural strategies and management practices for example site selection, irrigation, canopy management and balancing cropping levels are focused to reach a common and optimal grape maturity (KENNEDY 2002). To estimate the berry harvest time the veraison is a critical determent (DELUC et al. 2007). Veraison is defined as the onset of ripening or in other words it marks the beginning of the ripening phase. This phenological event varies from year to year and from berry to berry (COOMBE AND MCCARTHY 2000). In case of red wine cultivars like *Vitis vinifera* L. cultivar Zweigelt veraison is marked by a color change due to accumulation of anthocyanins in the grape berry skin. Other metabolic changes like berry softening and the ceasing of the xylem occur during or right after veraison (KENNEDY 2002; ZHANG et al. 2006; DELUC et al. 2007). For this research article it's necessary to understand the berry structure, berry growth and berry development over time,

because the physiological reasons and the initial time point of berry shrivel aren't known so far (GRIESSER et al. 2012[2]).

The following Figure 9 models the berry structure:

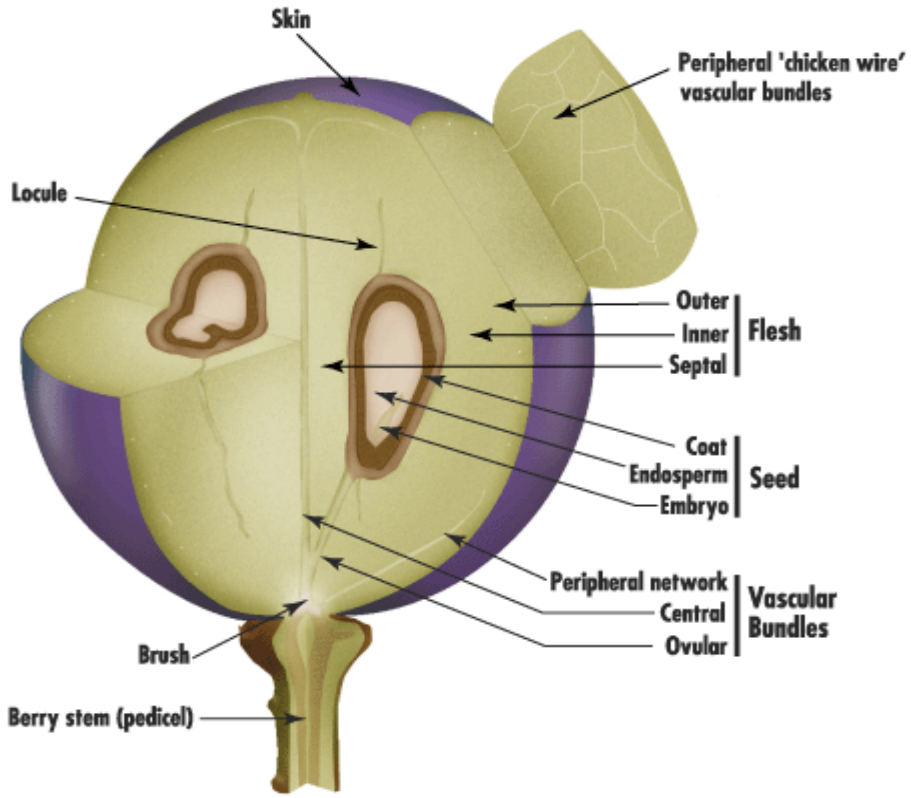


Figure 9: Grape berry structure; Structure of a ripe grape berry partially sectioned on the long and central axis to show internal parts. (COOMBE 2001 and KOUTROUMANIDIS 2002 reviewed by KENNEDY 2002)

Grape berries consist of four mayor parts: the seed, the flesh, vascular bundles and the skin. The variations in their composition and extensions contribute to the final wine style. Theoretically one grape berry contains four seeds, but conditions concerning the fertilization, environment and nutritional status of the plant during bloom and berry development usually decrease the number of seeds per berry (KENNEDY 2002). Seeds consist of the seed embryo, endosperm and the seed coat. The berry is connected to the vascular system of the rachis and the mother vine through the pedicel. The vascular system in the berry starts with the central and ovular bundles, but extends to a peripheral "chicken wire" network (COOMBE 2001 and KOUTROUMANIDIS 2002 reviewed by KENNEDY 2002). Different nutrients, storage compounds and precursors are transported into berries during their growth and development. Particularly carbohydrates in form of sucrose are important, because they are the main source of energy. The accumulation sites of sugars and water in the berries are the parenchyma cells of the berry flesh (mesocarp). The berry flesh can be separated due to its compositional different layers: the inner zone contains more malic acid and the central zone more tartaric acid. (KENNEDY 2002). The skin (exocarp) protects the berry against transpiration, UV-light and

pathogenic attacks (DIXON et al. 2004; DELUC et al. 2008), but at the same time it attracts mammals and birds to disperse the seeds. Therefore the grape berry skin contains a number of phenolic compounds for example flavonols and anthocyanins in red cultivars (CZEMMEL et al. 2012).

II.1.2 Growth and Development

Nowadays it's believed that grape berry growth consists of two successive sigmoid growth periods separated by a lag phase (KENNEDY 2002). Berry development can be described as a number of complex series of biochemical and physical changes regulated and coordinated by specific gene expression patterns. A broad study (DELUC et al. 2007) analyzed twenty different gene expression patterns of dynamic metabolic, transport and control processes considering berry growth and ripening. Generally scientists and winegrowers divide the berry growth in three stages (COOMBE and MCCARTHY 2000): Stage I (seed formation and cell division), Stage II (lag phase) and Stage III (cell expansion and sugar accumulation). The following Figure 10 (COOMBE and KOUTROUMANIDIS 2002 reviewed by KENNEDY 2002) links berry growth, defined as increase in berry size, with phenological events and physiological changes on a time scale after flowering.

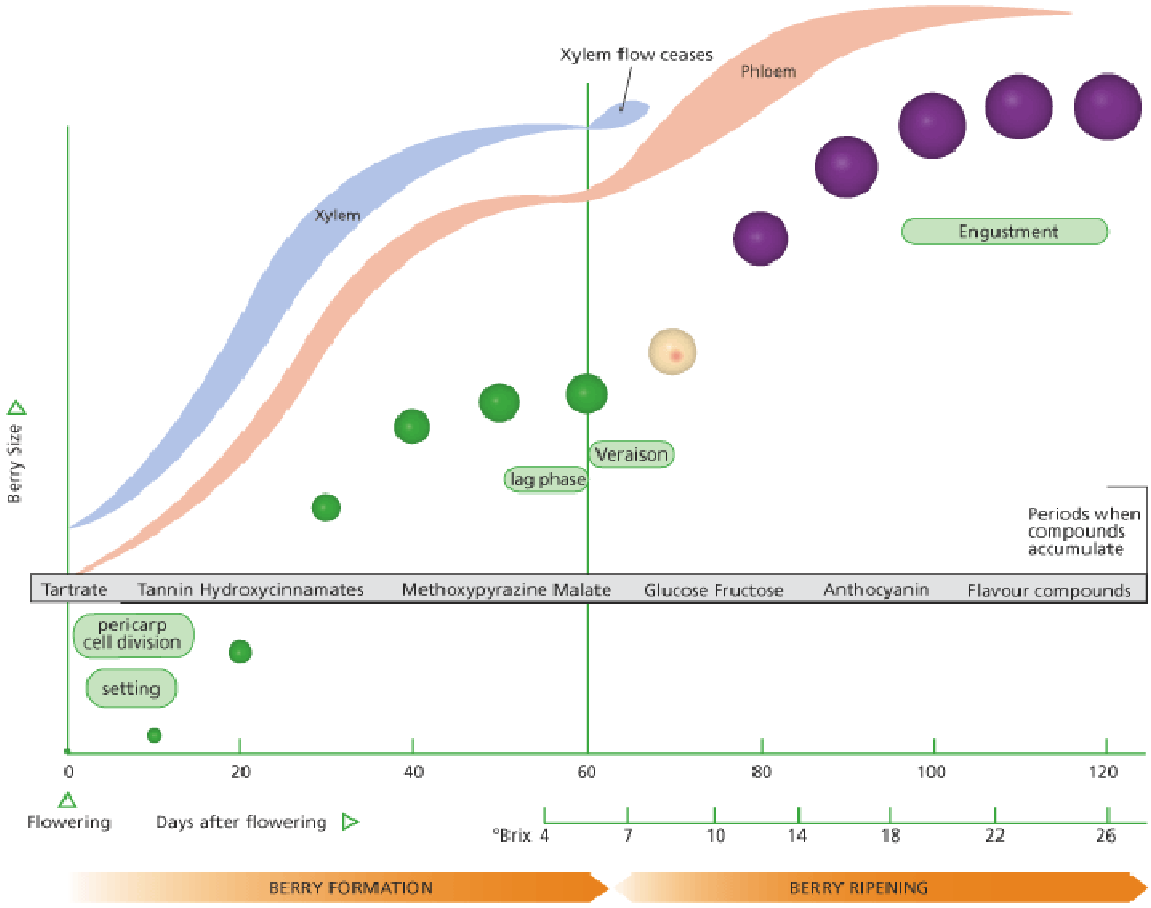


Figure 10: Berry growth model; Showing relative size and color of berries at 10-day intervals after flowering, passing through major developmental events (rounded boxes). Also shown are the periods when compounds accumulate, the levels of juice °Brix, and an indication of the rate of inflow xylem and phloem vascular saps into the berry (COOMBE 2001 and KOUTROUMANIDIS 2002 reviewed by KENNEDY 2002).

Stage I

The first stage of berry development lasts from the bloom until approximately 60 days afterwards (KENNEDY 2002). In the first weeks the green and hard seeds are formed by embryogenesis (embryo formation) and endosperm growth (KELLER 2010). The number of seeds is decisive for the final size of the berry. After fertilization the cell number is stimulated and fixed by phytohormones. Specifically cytokinins, auxins and gibberellins, synthesized by the seed embryos or imported from the mother vine, stimulate the rate and duration of cell division. Imported abscisic acid prevents seed and berry abortion (KELLER 2010). The expansion in volume can be explained by the increasing cell number and the accumulation of tartaric and malic acid (KENNEDY 2002; DELUC et al. 2007; KELLER 2010). Additionally hydroxycinammic acids, tannins, methoxypyrazines, minerals, amino acids, micronutrients and other aroma compounds are accumulated in stage I (KENNEDY 2002). Particularly hydroxycinnamic acids are precursors for the phenylpropanoid pathway, whereas tannins contribute to the bitter flavor, astringency and the color stability of the resulting wine (DELUC et al. 2007). Summarizing Stage I is the first rapid increase of the seed volume and it fixes the cell number of the berry (KELLER 2010).

Stage II

Stage II is termed: Lag phase. It initiates 1-6 weeks before veraison and ends with the beginning of veraison. Late cultivars of *Vitis vinifera* L. undergo a more extended lag phase compared to early fruiting ones (KELLER 2010). In this stage there is a slow or no increase in berry fresh weight or size (DELUC et al. 2007). The seeds enter their maturation stages by processes like the lignifications of the seed coats, which restrict further seed expansion (KELLER 2010). The transcriptional background of processes, occurring in stage II, hasn't been well characterized so far (DELUC et al. 2007). An important physiological process in the second stage is the decreasing activity and the final ceasing of the xylem connection after veraison. Initially the xylem transported water, minerals, growth regulators and nutrients from the vine into the berry. After veraison the phloem becomes the primary ingress for water and nutrients (KENNEDY 2002). The breakdown of the xylem is supposed to be cultivar dependent and a mechanism to avoid backflow of sugars from the berry to the vine due to the higher sugar concentration in the berry in the third growth stage (KELLER et al. 2006). In the lag phase the accumulation of sugars into the fleshy parenchyma cells starts (DELUC et al. 2007).

Stage III

The third berry growth stage initiates right after veraison. It's marked by a number of processes, which result in berry ripening. The first process is the initial rapid increase in berry volume due to cell expansion. It slows down towards maturity, because of evaporative water loss of the berries (KELLER 2010). The expansion, achieved by water and sugar accumulation in vacuoles, is accompanied by a disassembly of the mesocarp cells, which lead to berry softening. The mesocarp cell walls remain functional, but are weaker, more open, less flexible and more hydrated (KELLER 2010). Another physiological process is the reduction of malic acid, which depends on many factors. Especially the influence of the climate is thought to be important, as berries of warmer regions tend to have less malic acid (KENNEDY 2002). Seed tannins are reduced, whereas skins tannins remain stable after veraison, but can be modified or complexed with each other, anthocyanins or pectins (KENNEDY 2002). Aroma compounds

like methoxypyrazines decline in stage III. Especially *Vitis vinifera* L. cultivars like Cabernet Sauvignon or Sauvignon Blanc contain initially high numbers of methoxypyrazines, but reduce them in the presence of sun light (KENNEDY 2002). Color pigment formation in the berry skin of red cultivars is due to anthocyanin biosynthesis and accumulation. KELLER 2010 reports a decrease of chlorophyll and the transformation of chloroplasts to chromoplasts. However the "big story" (KENNEDY 2002) in the third stage of berry growth is the accumulation of monosaccharides in the berries. Imported sucrose is hydrolyzed to glucose and fructose and they are stored in vacuoles of parenchyma cells (KENNEDY 2002; KELLER 2010). In the later part of the third stage - called engustment (COOMBE and MCCARTHY 2000) or gustation (KENNEDY 2002) - volatile flavor compounds are accumulated depending on the cultivar and environmental conditions. For example different classes of glycolized terpenoids, which are precursors of fruit and flower aromas, are localized in the berries of *Vitis vinifera* L. cultivars Riesling and Muscat (KENNEDY 2002). Summarized in stage III the berry increases in size, softens and accumulates compounds, which influence the wine style significantly.

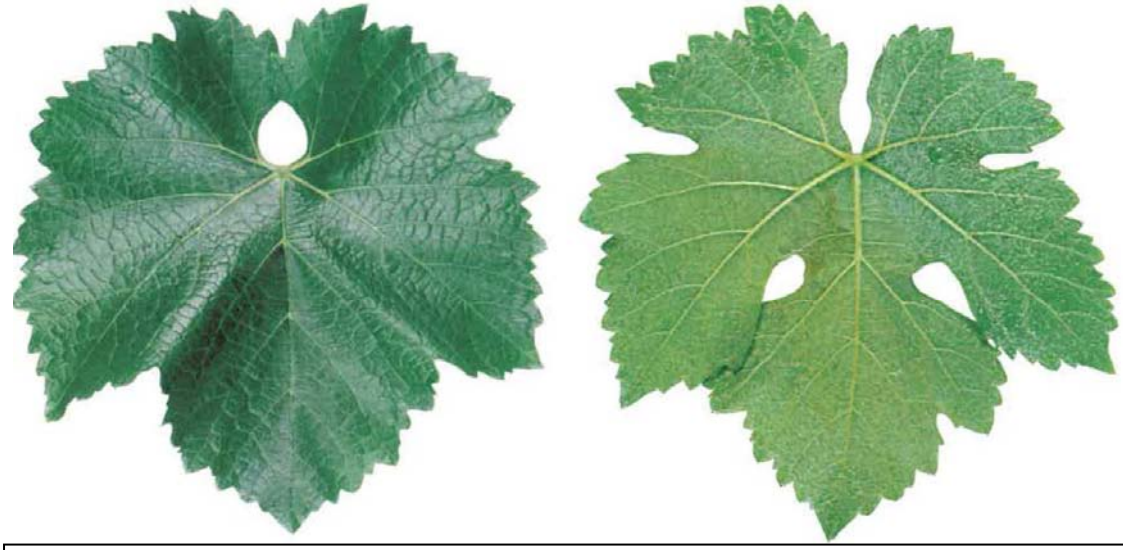
II.1.3 Conclusion and Future Perspectives

The primary role of grape berries is to attract birds and mammals for seed dispersal. The actual fruit can be seen as "ticket price to cover the cost of transportation" (KELLER 2010). Therefore a homogeneous ripening of berries haven't been a primary natural selection criteria. On contrary one of the goals of modern winemakers is to achieve homogeneous mature berries by cultural management practices. It's essential for them to understand berry development and "the what, when and how of berry manipulation" (KENNEDY 2002). Applying the knowledge, that the berry growth stage I determines cell number and stage II and III the cell expansion and accumulation of solutes, we can assume that efforts in cultivar selection and breeding should force stage I, whereas management practices are more efficient in the stages II and III (KENNEDY 2002). In the context of the anthocyanin biosynthesis and sugar accumulation in berry shrivel it is important to define their synthesis and accumulation period. On the one hand changes in gene expression levels of directly involved enzymes should be detectable prior or during growth stage III. On the other hand it's necessary to give an overview of other physiological processes, because they might influence each other.

II.2 *Vitis vinifera* L. cultivar Zweigelt

Vitis vinifera L. cultivar Zweigelt is the most important red wine cultivar in Austria. Additionally it's one of the main affected cultivars by berry shrivel (KNOLL et al. 2010). For these reasons it has been selected to be the cultivar used in this research and a short description is added in the following. According to REGNER et al. 2008 there are 6500 hectares planted in the country. Zweigelt was bred by Fritz Zweigelt in the Lehr- und Forschungszentrum für Wein- und Obstbau (Education and Research Center for Viticulture and Pomology) in Klosterneuburg in 1922 (AMBROSI et al. 1998). It's a cross of Blaufränkisch x St. Laurent (REGNER et al. 2008). Synonyms are Rotburger in Austria and Zweigeltrebe in Czech Republic and Slovakia (AMBROSI et al. 1998). In the vineyard Zweigelt vines show an upright to medium upright shoot with a medium anthocyanin color. On the upside young leaves are green, copper-colored and reddish. On the downside they show few trichomes near the vascular bundles. During berry development and ripening the round shaped, pentagonal

mature leaves have three to five lobes with a flat profile and the toothing in the edge of the leaves is round shaped or straight (REGNER et al. 2008). Images 1 and 2 of Zweigelt leaves are attached here:



Images 1 and 2: Zweigelt leaves; upside (left) and downside (right) mature leaves of *Vitis vinifera* L. cultivar Zweigelt (REGNER et al. 2008).

The rachis is approximately 5-7 cm long. The cylindrical and dense clusters have a medium size of 14-18 cm often with one to three shoulders. Single berries are round with a diameter ranging between 14-20 mm and weight ~2 g. The berry color is classified as blue to black (REGNER et al. 2008). The berry skin is thick with a juicy flesh compared to other cultivars (HOFÄCKER 2004). Image 3 of a Zweigelt cluster and Image 4 of the berries are attached here:

Images 3 and 4: Zweigelt cluster *(left)* and berries *(right)* (REGNER et al. 2008)

Furthermore Zweigelt is an early shooting and flowering cultivar with a good frost resistance (HOFÄCKER 2004; REGNER et al. 2008). It adapts to many soil types. In general the cultivar is vigorous and very fertile. Therefore it's necessary to have a proper leaf management and to thin the fruits to avoid overcropping (AMBROSI et al. 1998; REGNER et al. 2008), which would result in decreased color in the wine (HOFÄCKER 2004). Besides berry shrivel the cultivar might develop potassium deficiencies (REGNER et al. 2008). Fungal diseases may affect Zweigelt. It's medium tolerant against downy mildew – *Plasmopara viticola* and Botrytis – *Botrytis cinerea*, but low tolerant against powdery mildew – *Uncinula necator* (REGNER et al. 2008). The wine profile is described to be strong in tannins with a full and long body. The major aroma is sour cherry and the wines often are stored in wood. The wine color is violet to red (REGNER et al. 2008).

II.3 Sugar Transport into Berries

The phloem is the transport channel for many essential compounds, which are the basis for nutrition, growth and development in all higher plants (TAIZ and ZEIGER 2002) like:

- Water
- Carbohydrates (e.g. sucrose)
- Phytohormones (e.g. auxins, gibberellins, abscisic acid and cytokinins)
- Amino acids (e.g. glutamate, aspartate and their respective amides glutamine and asparagine)
- Inorganic minerals (preferentially phosphates, chlorides, magnesium and potassium)

Carbohydrate flow in higher plants generally is directed from source organs with a high photosynthetic activity (e.g. mature leaves) to sink organs, which require energy supply to maintain their biological functions. Sink organs can be structurally separated in vegetative growing organs (e.g. root tips and young leaves), storage organs (e.g. roots or stems) and organs for reproduction and dispersal (e.g. seeds or fruits).

In *Vitis vinifera* L. the accumulation of carbohydrates in form of monosaccharides in the berry flesh during ripening is one of the most important quality parameters. Firstly sweetness in table grape berries is a very important gustative quality parameter for fresh consumption. Secondly in the context of winemaking, sugar concentration determines the alcoholic graduation by yeast fungi during fermentation. Thirdly sugar is important for the augmentation of specific flavour profiles in the wine (HAYES et al. 2007). Therefore monitoring and increase of sugar accumulation in the berries over their developmental period is a traditional goal of viticultural management practices and one criterion to choose the harvesting time (BINDON et al. 2013). Furthermore in cool climate areas sugar accumulation is often the limiting factor for viticulture (NONIS et al. 2008). From a physiological point of view the carbohydrates are not only nutrients, which are the basis for plant growth, but also important osmotic and signalling molecules (AFOUFA-BASTIEN et al. 2010). The carbohydrate transport in *Vitis vinifera* L. from source to sink organs can be separated in three physiological mechanisms: phloem loading, phloem unloading and storage of monosaccharides in vacuoles of berry flesh cells (DAVIES and ROBINSON 1996). In the context

of berry shrivel in *Vitis vinifera* L. the respective sink organs to consider are grape berries, because affected berries have decreased sugar content (KRASNOW et al. 2009; GRIESSER et al. 2012[1]). The following part of the literature review about sugar transport will sum up phloem loading, focus on phloem unloading and then characterize specific enzymes and proteins involved in sugar transport from the phloem unloading until the storage of monosaccharides in vacuoles of the fleshy parenchyma cells of grape berries.

II.3.1 Phloem Loading

The first step of the phloem loading is the formation of triose phosphates by photosynthesis during the day. Transported from the chloroplasts to the cytosol triose phosphates are transformed to nonreducing sugars (OPARKA et al. 1992 reviewed by TAIZ and ZEIGER 2002). This transformation is necessary, because reducing monosaccharides contain chemically reactive groups. For example monosaccharides like D-glucose contain an aldehyde group or D-fructose contains a ketone group. After the transformation the ketone and aldehyde groups are either converted to less reactive alcohol (-OH) groups or they are eliminated by polymerization with other sugar molecules (TAIZ and ZEIGER 2002). Sucrose is the most common translocated sugar in the phloem (AFOUFA-BASTIEN et al. 2010). In the first short-distance-transport sucrose is transported from the producing source cell to other source cells and finally to a companion cell – sieve element complex. This process named sieve element loading can be symplastic or apoplastic.

Symplastic phloem loading is driven by diffusion. It requires a sucrose gradient decreasing from the exporting source cell to the sieve element. Open plasmodesmata are the connections for this passive cell to cell translocation of assimilates. Depending on the plant species sucrose might be polymerized to longer sugar molecules for example raffinose or stachyose to avoid back diffusion from sieve elements to parenchyma cells. This form of phloem loading is often observed in trees, shrubs and vines (TAIZ and ZEIGER 2002).

Apoplastic phloem loading is energy dependent and therefore defined as an active process. It takes place, when the concentration of sucrose in the companion cell - sieve element complex is higher than in the exporting mesophyll cells. It requires ATP dependent H^+ pumps, which import H^+ protons from the apoplastic space into the cytosol of mesophyll cells against an H+ concentration gradient. Then sucrose H^+ symporters export sucrose molecules together with H^+ protons from the cytosol into the apoplastic space. There the sucrose molecules are taken up by specific SUT1/2/4 membrane transporters directly into the sieve elements. Another way could be the indirect import of sucrose from the aploplastic space into companion cells by other specific membrane transporters like SUC2. From the companion cells the sucrose diffuses through plasmodesmata into the sieve elements (TAIZ and ZEIGER 2002). Interestingly the synthesis of the SUT1/2/4 membrane transporter proteins of sieve elements is believed to take place in the companion cells, where mRNA coding for the SUT1/2/4 proteins was found (TAIZ and ZEIGER 2002). The long-distance-transport in the phloem starts once sucrose or other nonreducing sugars arrived in the sieve elements and are exported to the sink organ driven by a concentration gradient (OPARKA et al. 1992 reviewed by TAIZ and ZEIGER 2002).

II.3.2 Phloem Unloading

The second step of the carbohydrate transport is phloem unloading. Once nonreducing sugars are transported via the phloem to the sink organs, they need to be downloaded. Many of the processes of phloem unloading are thought to be simply the reverse mechanisms of phloem loading. Generally phloem unloading can be summed up to three processes: sieve element unloading, short distance transport and storage or metabolism (TAIZ and ZEIGER 2002). In the sieve element unloading transport sugars exit the sieve elements in sink organs (e.g. grape berries). Equivalent to the phloem loading, phloem unloading can be symplastic or apoplastic. The symplastic unloading is usually observed in berry growth stage I (ZHANG et al. 2006). Sucrose arrives in the multiplying and developing cells of the berries, because the carbohydrates are quickly metabolized or respired. It's a passive process driven by a decreasing concentration of transport sugars from the sieve element to the sink cells. No membranes are crossed, because the sugars are transported via open plasmodesmata from cell to cell (TAIZ and ZEIGER 2002). During the berry development stages II and especially III apoplastic sieve element unloading occurs (ZHANG et al. 2006). The vine stuffs monosaccharides in the berry vacuoles in order to attract seed dispersers. Therefore the sugar concentration in the sink cells becomes higher than in the sieve elements. To avoid backflow of sugars the plasmodesmata of the symplastic pathway are closed. ZHANG et al. 2006 observed that in grapevine berries approximately 10-20% of the plasmodesmata are closed after veraison. The first step is the cleavage of sucrose to glucose and fructose by invertases in different cell compartments. Cell wall invertases are bound to the cell membranes in the apoplast (HAYES et al. 2007), Neutral invertases act mainly in the cytosol (NONIS et al. 2008) and Vacuolar invertases cleave sucrose inside the vacuoles (ZHANG et al. 2006). Cleaved to monosaccharides, these are translocated by energy dependent hexose transporters e.g. HT1 and HT3 into the cytosol of the sink cells (AFOUFA-BASTIEN et al. 2010). If the monosaccharides aren't used for respiration or metabolisation, they cross the tonoplast membrane mediated by another species of hexose transporter proteins for example TMT1, TMT2 or TMT3 (ÇAKIR and GIACHINO 2012) to be stored. Other fruit model species like tomato - *Solanum lycopersicum* L., shows a similar shift from the symplastic to apoplasmic phloem unloading (RUAN and PATRICK 1995), whereas the phloem unloading in apple - *Malus domestica* Bork., remains apoplastic (ZHANG et al. 2004).

II.3.3 Proteins involved in the Phloem Unloading

One of the central topics of the present research project is the analysis of gene expression levels of selected enzymes and transporter proteins involved in the phloem unloading, short distance transport and storage of monosaccharides in the vacuoles in berries of grapevine. Therefore in the following part some of these enzymes and transporter proteins are briefly characterized to have an idea about their specific functions, requirements and additionally to give hints to more detailed literature. As mentioned before phloem unloading is driven by the cleavage of sucrose to glucose and fructose (DAVIES and ROBINSON 1996) by different invertases. They increase the steepness in the sucrose concentration gradient from source to sink organs, so that the import of sucrose into sink organs is accelerated (PATRICK 1997). Three putative invertases are described in the following:

Cell wall Inv – cwINV (HAYES et al. 2007)

After the import of sucrose from the phloem in the berries the first cleaving enzyme is cell wall invertase (AFOUFA-BASTIEN et al. 2010). It's bound to cell membranes on the aploplastic side and has an acid pH optimum. Genes coding for cell wall invertase are expressed through the whole berry development with a maximum after veraison, when monosaccharides start to accumulate in vacuoles. Therefore cwINV is assumed to play an important role in the apoplastic phloem unloading (HAYES et al. 2007).

Neutral Invertases – NIs (NONIS et al. 2008)

Another group of enzymes cleaving sucrose to glucose and fructose are neutral invertases. In comparison to other invertases the investigation of NIs in fruit development has remained in the background. NONIS et al. 2008 identified nine different neutral invertases in grapevine tissues, of which five are expressed in the exocarp and mesocarp cells of grape berries during ripening. NIs are localized inside of mesocarp cells. MURAYAMA and HANDA 2007 assume that β neutral invertases are present in the cytosol, whereas α1 neutral invertases localize inside of mitochondria and α2 neutral invertases in chloroplasts in rice - *Oryza sativa* L.. Generally the expression of genes coding for NIs correlates with the sugar accumulation in stage III of berry development (NONIS et al. 2008).

Vacuolar invertases – vacINV

Vacuolar invertases or soluble invertases play a major role in symplastic phloem unloading to hydrolyze sucrose in the beginning of berry development. ZHANG et al. 2006 confirmed the observations of DAVIES and ROBINSON 1996 that the activity of soluble invertases decreases from fruit set until fruit ripening. They localized vacuolar invertases in the vacuoles of companion cells and fleshy parenchyma cells in grape berries before veraison. DAVIES and ROBINSON 1996 reported that soluble vacuolar invertases are synthesized much earlier than hexose accumulation occurs. They concluded that on a physiological level the grape vine prepares the machinery of phloem unloading.

After invertase cleavage from sucrose to monosaccharides, they have to be transported through different membranes firstly into the cytosol and secondly in the vacuoles of fleshy parenchyma cells. Developmental and environmental factors influence the expression of the genes coding for transporter proteins (Afoufa-Bastien 2012). Two groups of transmembrane transporters are characterized briefly in the following.

Hexose transporters – HT

There are several hexose transporters identified in *Vitis vinifera* L., but HT1 and HT3 are reported to be higher expressed in grape berry tissues (HAYES et al. 2007). Their assumed biological function is to transport monosaccharides cleaved by the invertases from the apoplastic space into the cytosol. A study of AFOUFA-BASTIEN et al. 2010 revealed a high molecular similarity to the sugar transporter protein family of Arabidopsis thaliana (AtSTP). There are some differences between the specific requirements and functions of the hexose transporters. Some reports focusing on HT1 were summed up in the research article of AFOUFA-BASTIEN et al. 2010: Observations showed that HT1 is strongly expressed in sink organs like berries and leaves (FILLION et al. 1999 and ATANASSOVA et al 2003). On the

contrary the observations of AFOUFA-BASTIEN et al. 2010 revealed that HT1 is poorly expressed in berries, but in vegetative growing sink organs. These different scientific results might be explained by different regulatory factors of the gene expression like sucrose, monosaccharides and abscisic acid for *Vitis vinifera* L. cultivar Cabernet Sauvignon (VIGNAULT et al. 2005). Another explanation could be that HT1 plays an important role in symplastic phloem unloading before veraison in berry growth stage I, when energy is required (VIGNAULT et al. 2005). This might be supported by findings of HAYES et al. 2007, which reported a peak in the gene expression of HT1 and a decline towards the sugar accumulation stage III. Simultaneously he pointed out, that the expression of HT3 is stable in berry tissues during their development, concluding that HT3 plays a crucial role in hexose transport in the sugar accumulation phase.

Tonoplast monosaccharide transporters – TMT (ÇAKIR and GIACHINO 2012)

Very few tonoplast transporters have been isolated and characterized. Therefore knowledge about biological functions is limited. TMTs are localized in the tonoplast membrane and mediate the transport of monosaccharides from the cytosol in the vacuolar storage. ÇAKIR and GIACHINO 2012 found a strong homology of TMTs in grapevine with those of other species. Three TMTs are characterized for grapevine so far: TMT1, TMT2 and TMT3. Although TMT1 and TMT2 are expressed in vegetative organs, their expression was found to be higher in berries. During berry development TMT1 and TMT2 expressions were analyzed to be minimal in fruit set, but after veraison they increased. Therefore they might play a crucial role in the last step of hexose accumulation (AFOUFA-BASTIEN et al. 2010). Particularly TMT2 is thought to be regulated by sugars, abiotic stresses and phytohormones, since in its promoter region an ABA motif was identified, but the experimental verification remains to be tested (ÇAKIR and GIACHINO 2012).

II.4 Anthocyanin Biosynthesis

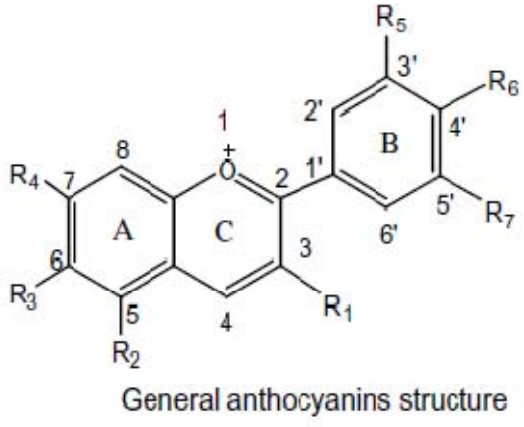
II.4.1 General Facts

Flavonoids belong to the secondary plant metabolites, which are necessary for the survival of the individual plant in the environment. There are thousands of flavonoid compounds distributed through the whole plant kingdom. Out of these anthocyanins, flavonols, lignin and proanthocyanidins are the most abundant ones (DELUC et al. 2006). Flavonoids have diverse and multiple functions depending on the plant species and its environment. For example flavonols and proanthocyanidins serve as UV protections (DELUC et al. 2008). Proanthocyanidins also called condensed tannins protect plants against insects, larger herbivores and microbial pathogens (DIXON et al. 2004). Lignin compounds are essential structural elements in a variety of plant species. Anthocyanins are colored pigments in flower petals, ranging from red to blue, to attract pollinators, whereas in fruits they attract the dispersers of seeds (CZEMMEL et al. 2012). Flavonoids are very beneficial for human nutrition and health. A number of scientific research articles proofs the antioxidant capacity to prevent for example cardiovascular diseases (BAGCHI et al. 2000). Also anti-inflammatory and antiproliferative effects of flavonoids have been postulated (HAVSTEEN 2002 reviewed by CZEMMEL et al. 2009). TRAKA AND MITHEN 2011 consider flavonoids to comprise a future potential as medicament for therapies against various disease treatments, after having tested

them carefully. Particularly anthocyanins e.g. malvidin 3-O-ß-glucoside were found to have a high antioxidant capacity (MÉRILLON et al. 1997). Besides the health promoting properties flavonoids influence taste and food quality. In *Vitis vinifera* L. proanthocyanidins contribute to bitterness and astringency and stabilize the color of red wines (DELUC 2006). Skin color of grape berries was one of the most important criteria for vine selection (AZUMA et al. 2008). In red wine varieties anthocyanins are the primary color pigment. White grape varieties lack anthocyanins due to single mutations for example in Riesling, Semillon or Chardonnay (BOSS et al. 1996[1]) or retrotransposonal activity along the biosynthetic pathway (AZUMA et al. 2008). Anthocyanins and flavonols are located in the grape berry skin. They generally accumulate more in the inner layer of grape skin than in the outer one. Particularly anthocyanins co-localize between theses layers (ADAMS 2006 reviewed by CZEMMEL et al. 2012). The accumulation of anthocyanins starts at veraison and continues through the ripening period (JEONG et al. 2004). Besides other physiological changes described in the Berry Growth and Development part, veraison is marked by the color change from green to red or black. In this project we focus on the gene expression background of anthocyanin biosynthesis. More detailed we measure specific gene expression levels of berry shrivel affected clusters, which might be the reason for a delayed veraison and an overall decreased content of anthocyanins at harvest (GRIESSER et al. 2012[2]).

II.4.2 Chemical Structure

The aglycons of anthocyanins are called anthocyanidins. They consist of two aromatic Rings A and B bounded to a heterocyclic ring C, which contains oxygen (Figure 11). Linked to a sugar moiety, they form anthocyanins. There is a huge variety of anthocyanins present in nature (CASTAÑEDA-OVANDO et al. 2009). A study focusing on metabolic profiling (MATTIVI et al. 2006) shows the relative composition of anthocyanins for *Vitis vinifera* L. cultivar Zweigelt: Malvidin 3-glucoside > delphinidin 3-gucoside > peonidin 3-glucoside > petunidin 3-glucoside > cyanidin 3-glucoside.



General anthocyanins structure

Figure 11: General anthocyanin structure (CASTAÑEDA-OVANDO et al. 2009)

Name	Substitution pattern						
	R1	R2	R3	R4	R5	R6	R7
Cyanidin	OH	OH	H	OH	OH	OH	H
Delphinidin	OH	OH	H	OH	OH	OH	OH
Malvidin	OH	OH	H	OH	OMe	OH	OMe
Peonidin	OH	OH	H	OH	OMe	OH	H
Petunidin	OH	OH	H	OH	OMe	OH	OH

Table 1: Substitution patterns of anthocyanins: Specific for *Vitis vinifera* L. (modified from CASTAÑEDA-OVANDO et al. 2009)

Once anthocyanins are synthesized they are susceptible for degradation. Several factors influence their chemical stability: pH, storage temperature, chemical structure, light, oxygen and presence of enzymes, other flavonoids, proteins and metallic ions (REIN 2005).

II.4.3 Phenylpropanoid Pathway

Anthocyanins and other flavonoid compounds derive from the phenylpropanoid pathway, which was studied in many plant species. The pioneer species, of which enzymes involved in flavonoid biosynthesis were isolated and characterized, are: snapdragon - *Antirrhinum majus* L., maize - *Zea mays* L. and petunia - *Petunia hybrida* (BELD et al.1989; HOLTON and CORNISH 1995)**.** Later approaches also investigated the anthocyanin biosynthesize in fruit species such as apple - *Malus domestica* Bork. (TAKOS et al. 2006*)* and bilberry - *Vaccinium myrtilus* L. (JAKKOLA et al. 2002). For the model plant grapevine a number of research articles focusing on the gene regulation of the phenylpropanoid pathway have been published. The majority of them are dealing with red cultivars of *Vitis vinifera* L. (BOSS et al. 1996[1], DELUC et al. 2008, HICHRI et al. 2010 and CZEMMEL et al. 2012). Others include the interspecific hybrid *Vitis x labruscana* (AZUMA et al. 2008; 2012). The complete phenylpropanoid pathway is complex as there are diverse enzymatic branches included, which compete for identical substrates. To introduce and simplify this complex pathway, the first following scientific model (Figure 12; JEONG et al. 2004) focuses on the single enzymatic branch resulting in anthocyanins. A second scientific model (Figure 13; CZEMMEL et al. 2012) adds other important side branches resulting in stilbenes, lignins, flavonoles and proanthocyanidins.

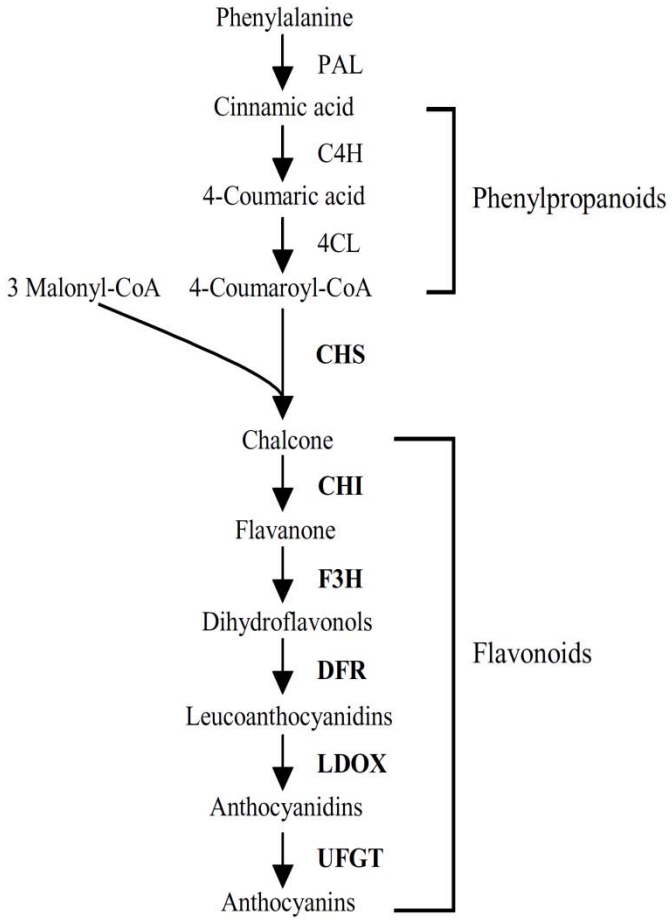


Figure 12: Simplified phenylpropanoid pathway for anthocyanins (JEONG et al. 2004)

CHS	Chalcone synthase
CHI	Chalcone isomerase
F3H	Flavanone-3β-hydroxylase
DFR	Dihydroflavonol 4-reductase
LDOX	Leucoanthocyanidin dioxygenase
UFGT	Flavonoid-3O-glucosyltransferase
F3'H	Flavonoid-3'-hydroxylase
F3'5'H	Flavonoid-3',5'-hydroxylase
FLS	Flavonol synthase
LAR	Leucoanthocyanidin reductase
ANR	Anthocyanidin reductase

Right: **Table 2: Abbreviations of enzymes involved in phenylpropanoid pathway** (modified from CASTAÑEDA-OVANDO et al. 2009)

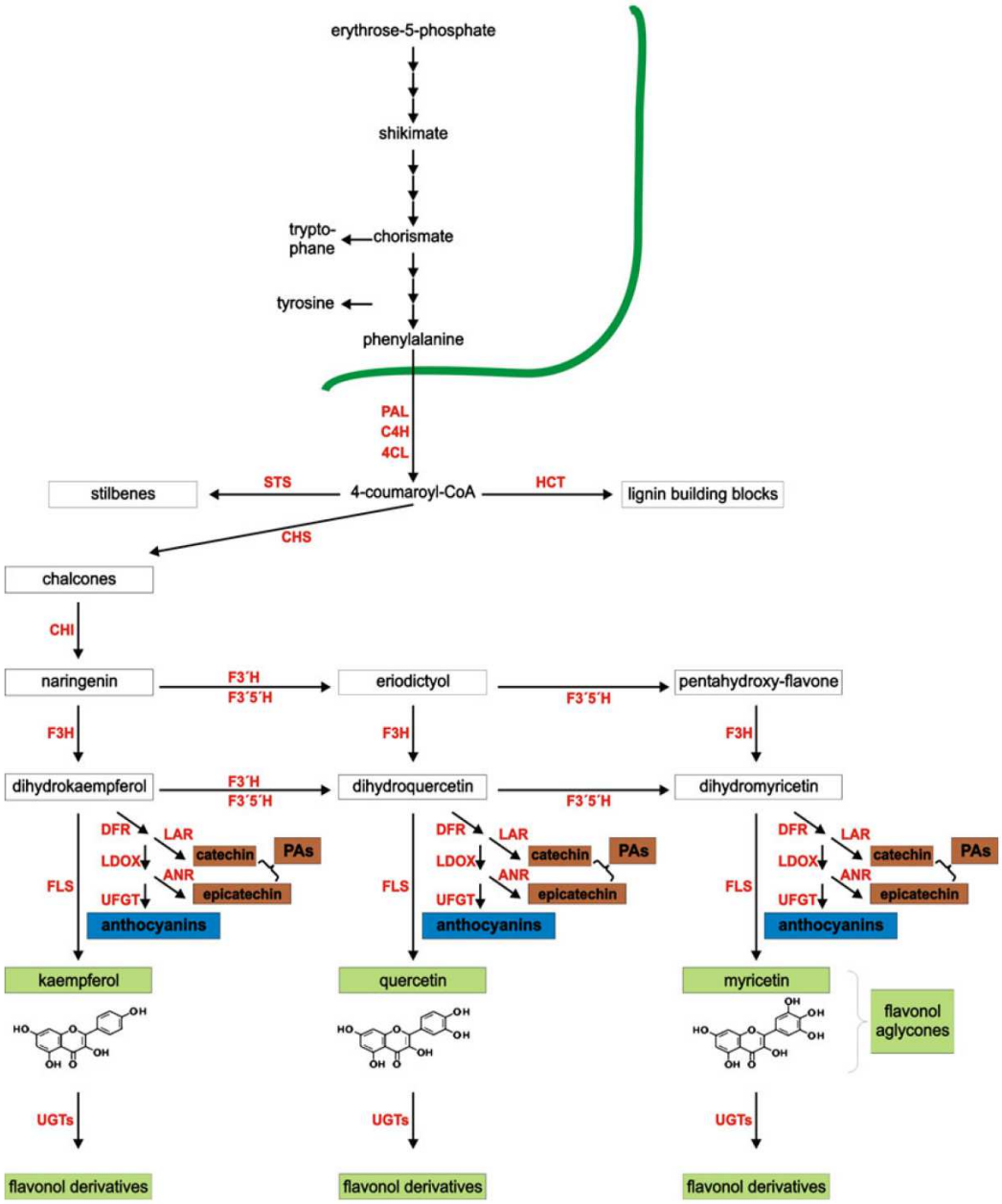


Figure 13: General simplified phenylpropanoid pathway; "Simplified presentation of the phenylpropanoid biosynthesis in *V. vinifera* highlighting the flavonoid-specific branch of the pathway. The chloroplast harboring the precursory Shikimate pathway is bordered by a *green* line. Note that biosynthetic steps involved in the production of the aromatic amino acids phenylalanine, tryptophane and tyrosine are not indicated. The classes of phenylpropanoids are indicated in boxes including the major flavonoid subclasses: flavonols (*light green*), anthocyanins (*blue*) and proanthocyanidins (*PAs brown*). Abbreviations for enzymatic steps (in red) (…)" (CZEMMEL et al. 2012).

The starting point of the phenylpropanoid pathway of all flavonoids is the aromatic amino acid phenylalanine deriving from the Shikimate pathway in chloroplasts. This step shifts the primary to the secondary plant metabolism. It's assumed that approximately 20% of the carbon fixed by photosynthesis is used to form aromatic amino acids and the major part of them is phenylalanine (CZEMMEL et al. 2012). The stepwise transformation from phenylalanine to anthocyanins is done by nine enzymes (Figure 12). These directly involved enzymes of the anthocyanin biosynthesize can be categorized in early and late synthesizing enzymes and will be characterized briefly in the following.

Early synthesizing enzymes:

- PAL - Phenylalanine ammonia-lyase, C4H - Cinnamate 4-hydroxylase and 4CL - 4-coumarate CoA-ligase
  PAL, C4H and 4CL are the first key enzymes in the phenylpropanoid pathway. They shift the primary to the secondary plant metabolism, transforming the aromatic amino acid phenylalanine stepwise to cinnamic acid, 4-coumaric acid and finally to 4-coumaryl-CoA.
- CHS - Chalcone synthase
  CHS transforms 4-coumaryl-CoA and 3-malonyl-CoA to chalcones. Chalcones are the first precursors for all intermediates of the flavonoid pathway (CZEMMEL et al. 2012). According to JEONG et al. 2004 there are at least three different chalcone synthases (*Chs1*, *Chs2* and *Chs3*) for grapevine. *Chs2* and *Chs3* are the main enzymes of the enzyme family present in grape berry skin.
- CHI - Chalcone isomerase
  CHI transforms rapidly chalcones to flavanones for example to naringenin. It was observed by HOLTON and CORNISH 1995 that even in the absence of CHI chalcones isomerizes spontaneously to naringenin.
- F3H - Flavanone-3β-hydroxylase
  F3H transforms flavanones enzymatically to dihydroflavonoles. JEONG et al. 2004 analyzed two different flavanone-3β-hydroxylases called *F3h1* and *F3h2*. Dihydroflavonoles are common substrates for three competitive enzymatic branches resulting not just in anthocyanins, but also in flavonols. The third enzymatic branch would lead to compositionally diverse dihydroflavonols.

Late synthesizing enzymes:

- DFR - Dihydroflavonol 4-reductase
  DFR transforms dihydroflavonols (dihydrokaempferol, dihydromyrcetin or dihydroquercetin) to leucoanthocyanidins. DFR competes with flavonol synthase (FLS) for the same substrates. DFR favors the biosynthesis of anthocyanins and proanthocyanidins and limits the biosynthesis of flavonols.
- LDOX - Leucoanthocyanidin dioxygenase
  LDOX competes for with LAR for leucoanthocyanidins. It limits the synthesis of proanthocyanidins by the transformation of leucoanthocyanidins to chemically unstable anthocyanidins (CZEMMEL et al. 2012).
- UFGT - Flavonoid-3O-glucosyltransferase
  UFGT stabilizes the final anthocyanins. The glycolisation of anthocyanidins to anthocyanins by flavonoid-3O-glucosyltransferase inhibits the transformation of anthocyanidins to proanthocyanidins like catechin or epichatechin by ANR. Flavonoid-3O-glucosyltransferase was solely detected in skin tissues of red grape berries (BOSS et al. 1996[2]).

Enzymes changing the composition:

Two enzymes are responsible for the shift in the composition of anthocyanins. The following Figure 14 (GUTHA et al. 2010) visualizes stepwise the biosynthetic changes in the composition of different anthocyanins for grapevine. Naringenin flavanone or dihydrokaempferol can be transformed on the one side to eriodyctol or dihydroquercetin resulting in cyanidin and peonidin derivates. On the other side pentahydroxyflavanone or dihydromyrcetin result in delphinidin, petunidin or malvidin derivates.

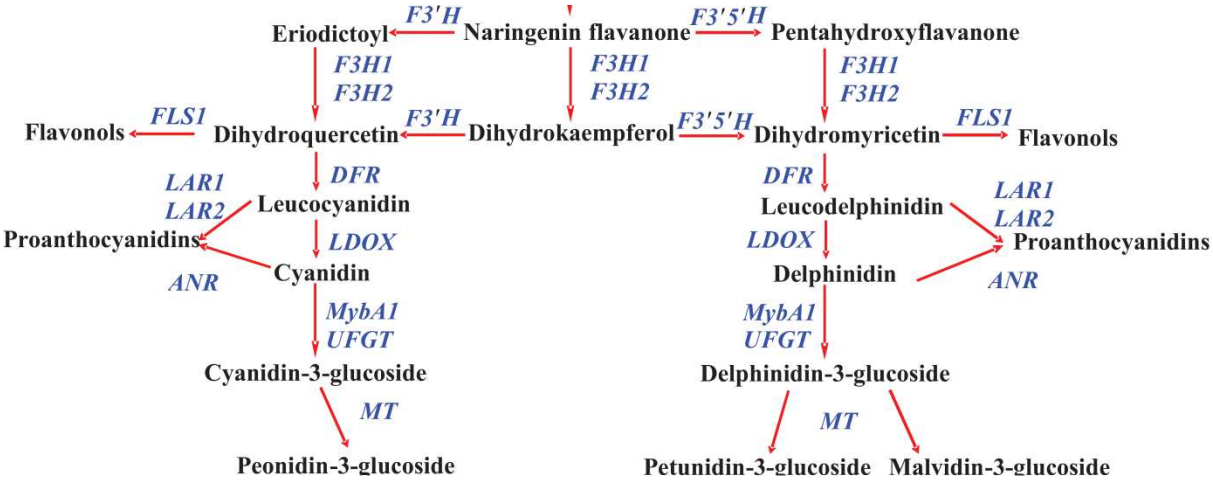


Figure 14: Phenylpropanoid pathway; focusing on compositional differences in anthocyanins (GUTHA et al. 2010)

- F3'H - Flavonoid-3'-hydroxylase
  F3'H is essential to shift the composition either from naringenin flavanone to eriodyctol or from dihydrokaempferol to dihydroquercetin. This results in a different anthocyanin composition towards more cyanidin-3-glucoside and peonidin-3-glucosides.

- F3'5'H - Flavonoid-3',5'-hydroxylase
  F3'5'H transforms either naringenin flavanone to pentahydroxyflavanone or dihydrokaempferol to dihydromyrcetin. These are important precursors to synthesize delphinidin-3-glucoside, petunidin-3-glucoside and malvidin-3-glucoside derivates in the end of the phenylpropanoid pathway.

Competing enzymes:

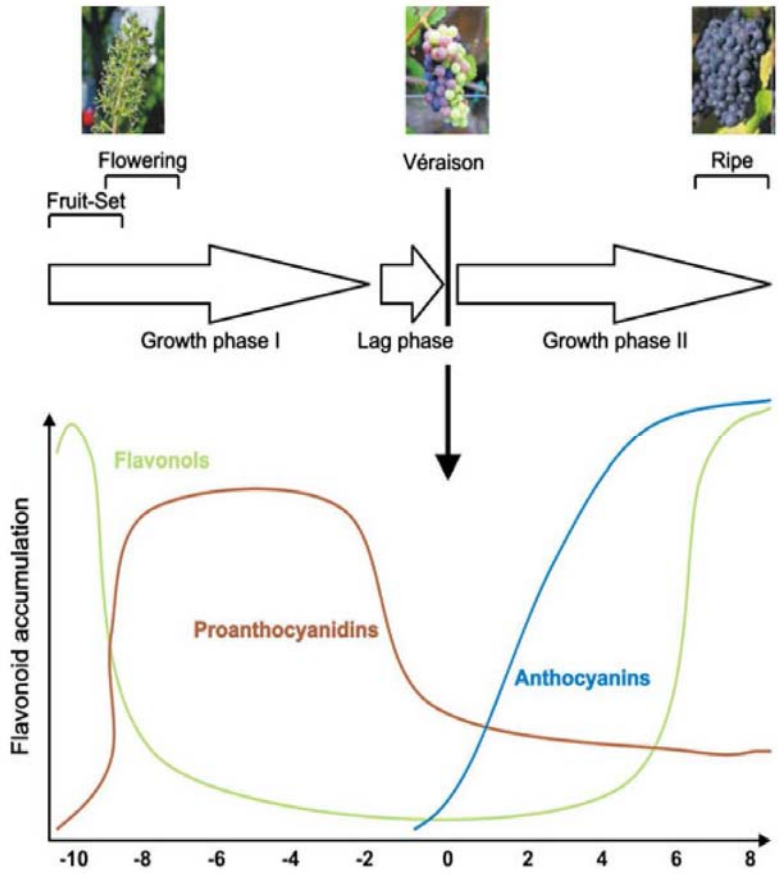
HOLTON and CORNISH 1995 pointed out that the accumulation of particular flavonoids depends on the amount of competing enzymes and their relative substrate specifities. The major competing enzymes involved in the synthesis of other flavonoids than anthocyanins are briefly characterized in the following, because they might be important to consider in this research project.

- FLS - Flavonol synthase
  FLS transforms dihydroflavonols directly to flavonols like kaempferol, quercetin and myrcetin. FUJITA et al. 2006 described five potentially different flavonol synthases: *Fls1*, *Fls2*, *Fls3*, *Fls4* and *Fls5* for grapevine.

- LAR - Leucoanthocyanidin reductase and ANR – anthocyanidin reductase
  LAR and ANR shift the accumulation from anthocyanins and flavonols to proanthocyanidins (condensed tannins). LAR acts earlier and takes leucoanthocyanidins as substrates to transform them to catechin. It competes for the same substrate with DFR. The enzyme family of LAR consists of at least two enzymes: *Lar1* and *Lar2* (LACAMPAGNE et al.2010). ANR competes with LDOX for the unstable anthocyanindins and transforms them to epicatechin.

During berry development the vine synthesizes and accumulates different secondary metabolites by for example changing the gene expressions of the related competing enzymes FLS, LAR and ANR. In Graph 1 CZEMMEL et al. 2012 describe the temporally shift in accumulations of flavonols, proanthocyanidins and anthocyanins. Flavonols start to

accumulate before anthesis and increase during flowering to protect the florescence and the pollen against UV light (CZEMMEL et al. 2009). Eight weeks before veraison until veraison proanthocyanidins accumulate preferentially in the berries. After veraison the phenylpropanoid pathway is shifted towards the biosynthesis of anthocyanins.



Graph 1: Flavonoid accumulation; "Schematic representation of the accumulation of flavonoids in grape skin during berry development. Flavonoid accumulation during berry development is color-coded with flavonols in light green, proanthocyanidins in brown and anthocyanidins in blue. Note that flavonol and proanthocyanidin accumulation and underlying gene expression profiles have been measured earliest 10 weeks before the onset of ripening which is indicated by an arrow, leaving open the possibility that both compounds accumulate earlier. As only the flavonoid class proanthocyanidins is present in seeds, only comparative flavonoid accumulation patterns in skins are shown (…)" (cited from CZEMMEL et al. 2012)

Transcription factors

The regulatory mechanism behind this spatiotemporal accumulation of diverse flavonoids in grape berries is partially based on the expression of transcription factors. Recently several of them have been isolated and characterized for grapevine (CZEMMEL et al. 2012). R2R3-MYB transcription factors were found to activate or enhance the expression of specific genes coding for enzymes of the phenylpropanoid pathway. Once expressed the R2R3-MYB transcription factors interact with WD repeat proteins (DELUC et al. 2008, CZEMMEL et al. 2012) and basic helix-loop-helix (bHLH) proteins, which are general regulators of several cell fate scenarios (HICHRI et al. 2010). Only this interaction leads to an enhanced expression of particular genes. In the case of grape berries four different types of transcription factors are important for the regulation of the phenylpropanoid pathway:

- MYB5b
  This transcription factor activates the gene expression of enzymes involved in the synthesis of proanthocyanidins and anthocyanins: LAR, ANS, CHI, ANR, F3'5'H. It doesn't affect the activity of UFGT, the last enzyme of the anthocyanin biosynthesis. Therefore it's generally considered to favor proanthocyanidin accumulation (DELUC et al. 2008). Furthermore DELUC et al. 2008 observed that MYB5b is higher expressed in young tissues of leaves and roots. Within the berries its concentration is increased in the skin, showing a concentration maximum before and right after veraison.

- MYBPA1/2
  On the one hand these two transcription factors control the expression of general genes coding for enzymes of the phenylpropanoid pathway like CHS or CHI. On the other hand they are considered to enhance gene expression of enzymes specific for proanthocyanidin production: LDOX, LAR and ANR (CZEMMEL et al. 2012).

- MYBA1/A2
  MYBA1 and MYBA2 are putative transcription factors for the anthocyanin biosynthesis (JEONG et al. 2004). They regulate specifically the expression of the genes coding for UFGT, which stabilizes the anthocyanins. KOBAYASHI et al. 2004 showed that a loss of function of the MYBA1 resulted in white cultivars of *Vitis x labruscana*.

II.5 BERRY SHRIVEL

II.5.1 History and General Facts

Berry shrivel (BS) was first mentioned referring to observations of a phenomenon in the 20th century. JENSEN 1970 (reviewed by KRASNOW et al. 2009) described flaccid shaped berries of red table grapes with low sugar accumulation and poor coloration. For Europe the first incidence of berry shrivel was termed "Zweigeltkrankheit" (REISENZEIN and BERGER 1997) referring to the susceptible *Vitis vinifera* L. cultivar Zweigelt (KNOLL et. al 2010), but then renamed to "Traubenwelke", as it soon affected other grapevine cultivars like Grüner Veltliner, St. Laurent, Neuburger or Zierfandler (RIEDEL 2008; HALL 2010[1]). Other synonyms SAD "Sugar accumulation disorder" or SOUR "Suppression of uniform ripening" (BONDADA AND KELLER 2012[2]) exist in literature. Nowadays BS is distinguished from other shrivels. Increasing numbers of BS cases are described in scientific literature worldwide. In Austria, where Zweigelt is the economically most important red wine cultivar, BS started in the area of Neusiedlersee in Burgenland and has been spread to other important viticultural areas e.g. to Weinviertel in Lower Austria (BESSER 2010). In the US especially Cabernet Sauvignon, Sauvignon Blanc, Semillon and Pinot Gris are affected (KELLER 2008). In Northern Italy BS occurs in South Tyrol (Alto Adige) and in Friuli for Sauvignon Blanc and Pinot Blanc (RAIFER cited in RIEDEL 2008). The first German report dates in Baden 2007 (BACHTELER and RIEDEL 2011). In Switzerland SCHUHMACHER et al. 2007 reported BS for Blauburger (Pinot Noir) and Gutedel (Chasselas). Chinese Scientists observed BS recently for Cabernet Sauvignon, Merlot, Pinot Noir and Sauvignon Blanc in Xiangning County in the Shanxi

province (FANG et al. 2011). Although intensive international research has been done, the exact causes of BS aren't known yet (GRIESSER et al. 2012[2]). Grapevine *V. vinifera* L. is the only known fruit species with incidences of BS (BONDADA and KELLER 2012[2]). BS is assumed to be a physiological disorder (GRIESSER et al. 2012[1]; BONDADA and KELLER 2012[2]), since scanning for phytoplasms and known viruses among them closteroviruses, fanleaf-viruses, nepoviruses and fleck complex viruses of affected plant material were negative (KRASNOW et al. 2008; KRASNOW et al. 2009).

A number of other possible causes have been investigated. An initial hypothesis was based on the assumption that the xylem hydraulic connectivity after veraison in some cultivars was partially maintained. Water is imported with the carbohydrate transport from the vine to the berry. Via the connected xylem excessive water flows back to the vine. In BS affected clusters this backflow was thought to be blogged and excessive water remained in the fruit inhibiting the import of additional carbohydrates (KELLER et al. 2006, KELLER 2008). Later trials inhibiting the phloem flow by girdling of berry pedicels (ROGIERS et al. 2006) revealed similar shriveling symptoms like BS. The phloem imports carbohydrates and other nutrients into berries. The fact that in affected berries sugar accumulation slows down and ceases (KRASNOW et al. 2009; GRIESSER et al. 2012[2]), indicates a phloem inhibition. Callose depositions, as a reaction of environmental influences, could cause a spontaneous reduction of phloem flow on a cluster basis (KELLER 2008), but research on transcriptional gene regulation revealed no detected up regulation of genes related to the callose formation in rachis tissues of BS affected clusters (GRIESSER and FORNECK 2010[1]). Other approaches of BS deal with the vineyard management. Lack of available potassium in the soil is discussed, but contradictory results were obtained. On the one hand FARDOSSI 2001 postulated that a low potassium/magnesium ratio increased the incidences of berry shrivel. A trial applying threefold amount of K foliar fertilizer to Pinot Blanc and Zweigelt reduced the incidences of berry shrivels, however it didn't eliminate it (BACHTELER AND RIEDEL 2011). On the other hand no correlation between potassium and BS was observed, applying foliar K fertilizer over two seasons. Moreover the K fertilizer limited photosynthesis and stomata conductance (KNOLL et al. 2007). Rootstock selection influenced partially berry shrivel incidences in field trials. Vines propagated on vigour inducing rootstocks are more susceptible to BS (SCHUMACHER et al. 2006). Specifically rootstock SO4 could promote BS, because of its lower potential for water and magnesium uptake on critical vineyard sites (RIEDEL 2008). The Obst- und Weinbauschule Krems in Lower Austria didn't observe rootstock differences, but unfavourable seasonal weather conditions promoting the berry shrivel phenomenon. Extremer weather conditions in the same and the previous year increased cases of BS (KÜHRER and GABLER 2011). Furthermore they postulated a low leaf area/fruit load ratio to increase BS. Trials 2009/2010 in Lower Austria showed that higher leaf walls and an early fruit reduction by dividing clusters decreased BS significantly in *V. vinifera* L. cultivar Zweigelt (KÜHRER and GABLER 2011; KÜHRER and GABLER 2013). Contradictory results were found in Washington State. Water stress, overheating or abrupt coldness had no significant influence on BS occurrence (KELLER 2008). Although "it seems that as soon as a cause has been suggested, a contradictory result is found" (cited from HALL 2010[1]), three temporary defined categories to fight BS on a grower's level were emphasized (REDL 2008):

- short term solution: balanced leaf-area, adjusting fruit number early by cluster division and optimization of the irrigation system
- midterm solution: proper root development and avoid soil compensation
- long term solution: replanting, cultivar selection and fertilization

II.5.2 Symptoms

The occurrence of BS is difficult to foresee. Research has shown that year, vineyard site and position of the plant in the vineyard doesn't correlate with BS (Griesser 2012[2]). An estimation of the US says that annually 1-5% of the fruits are lost due to BS, but particular vineyards bare up to 50% of the berries due to BS (KRASNOW et al. 2010). There is an ongoing discussion about the spatial basis of BS. Some American scientists consider the whole plant to be affected by this physiological disorder. KRASNOW et al. 2009 analyzed all the clusters of a BS affected grapevine plant. The novelty was that visually not affected clusters had intermediate compositional differences for example in sugar and anthocyanin contents compared to visually affected BS and healthy control clusters. They called these intermediate clusters LTS "likely to shrivel". Whereas in Europe BS is cluster based (GRIESSER et al. 2012[2]). Some compositional differences were observed among clusters on the same affected plant, but visually healthy clusters can still be used economically for winemaking (BESSER 2010). In the flat arch system Zweigelt clusters on the distal third of the shoots were more affected than the proximal ones (BESSER 2010; GRIESSER et al. 2012[1]). Distal berries showed more symptoms than centric ones on the same cluster (HALL 2010[1]). Temporary detection of BS symptoms depends on many factors. BS symptoms were found earliest 50-60 DAA (days after anthesis) in Austria (BESSER 2010). In the US the detection was possible after 112-126 DAA (KRASNOW et al. 2009). Nowadays there are several known BS symptoms. The most common ones are:

1. Smaller berries with reduced berry weight due to water loss after veraison (BONDADA and KELLER 2012[2], GRIESSER 2012 et al.[2]).
2. Flaccid berries, reminiscing "deflated soccer balls" with numerous skin folds (BONDADA and KELLER 2012[2]).
3. Peduncle and rachis appear visibly green and healthy (BONDADA and KELLER 2012[2])
4. Pedicels of affected clusters have a thinner pedicels (GRIESSER et al. 2012[2])
5. Less or delayed coloration in red varieties. In Cabernet Sauvignon berries show a pink coloration (BONDADA and KELLER 2012[1]). In white cultivars berries exhibit a green-grey colour (RIEDEL 2008).
6. Acid, bitter and unripe taste of berries (RIEDEL 2008).

Photos provided by the Obst- und Weinbauschule Krems (KÜHRER 2010) demonstrate visible symptoms of BS on the clusters for Zweigelt in Lower Austria. Cell viability of mesocarp cells in berries declined down to 15% after the visual onset of the symptoms stained with FDA fluorescein diacetate (KRASNOW et al. 2008). Dead cells in the collapsed mesocarp contributed to off-flavour development during winemaking (BONDADA and KELLER 2012[1]).

Image 5: Beginning of berry shrivel; Heterogeneous coloration of berries (KÜHRER 2010)

Image 6: Berry shrivel cluster; affected cluster later in season (KÜHRER 2010)

II.5.3 Compositional Effects

The physiological mechanisms of BS are considered to be initiated before visual symptoms and compositional differences occur. However differences in the composition could be a hint for growers to foresee potential BS threats in their vineyards. Several differences were analyzed and demonstrated. Phloem connectivity seems to be a crucial factor of BS. One of its main tasks is the import of carbohydrates from source organs for example leafs to sink organs in this case berries. In BS berries the carbohydrate transport initially appears to develop

regularly. Two weeks before symptoms were visible, the carbohydrate transport slowed down and ceased dramatically. This cessation can be demonstrated comparing the lower soluble solid contents of affected and control berries (KRASNOW et al. 2009; GRIESSER et al. 2012[1]). The pH increased along berry development, but in the end of the period BS berries had lower pH than control berries (KRASNOW et al. 2009, GRIESSER et al. 2010[2]). Amounts of malate and tartrate per berry were similar in the end of the period (KRASNOW et al. 2009), but titrable acidity was higher (GRIESSER et al. 2010[2]). This can be explained by the fact that healthy berries contain more water than BS berries. Therefore the same amount of organic acids per berry is diluted in less water in BS berries. An analysis concerning the amino acid composition revealed that thirteen amino acids are reduced and two are enhanced (GRIESSER et al. 2012[1]). Hydroxyproline, one of the two enhanced amino acids, might be related to general stress responses (KRASNOW et al. 2010). Total anthocyanins were lower in BS berries than in control berries, whereas flavonols increased in the berry skin (KRASNOW et al. 2009). Mineral composition analyses showed that potassium was lower in BS berries (BONDADA and KELLER 2012[1], HALL et al. 2010[2]) due to the potential loss in phloem functionality. The values for calcium in the rachis and berries are elevated (KRASNOW et al. 2009; BACHTELER and RIEDEL 2011; GRIESSER et al. 2010[2]) or not significantly different (BONDADA and KELLER 2012[1]). Crystals of calcium oxalate were observed in the collapsed mesocarp of BS berries (BONDADA and KELLER 2012[1]). Summarized the compositional changes such as low carbohydrate, low potassium content, acid and bitter taste (BACHTELER and RIEDEL 2011) combined with low cell viability in BS berries limits their use for winemaking and so affected clusters have to be considered as yield loss.

II.5.4 Other physiological Disorders

In Pomology many fruit species can develop physiological disorders, but the detailed mechanisms often aren't elucidated yet. Grapevine is a model plant for fleshy fruits to investigate for example physiological mechanisms. BS is one of five common physiological disorders in grapevine. It's essentially to differentiate them on a winegrower's level in order to collect reliable data about incidences and spread. A summary distinguishing the main characteristics of other physiological disorders in grapevine: sunburn, prolonged dehydration, late and early season bunch necrosis is listed in the following.

1) Sunburn (KRASNOW et al. 2010)
   Sunburn occurs if berries are exposed intensively to UV radiation and high temperatures. Additionally wind speed accelerates transpiration and increases sunburn risk. It's possible that just the exposed side of a cluster is affected. In case of white or red cultivars before veraison sunburn causes brownish discolorations on the berry skin. In case of red cultivars sunburn leads to less colored berry skins, which appear shiny or pinkish. Due to the degradation of the crystalline structure of the epicuticular wax, water transpires uncontrolled (BONDADA and KELLER 2012[1]) and berries are likely to crack. In extreme cases berries appear like raisins.

2) Prolonged dehydration – PD (KRASNOW et al. 2010)
   PD is typically observed in the cultivars Shiraz and Cabernet Sauvignon two weeks before harvest. Diverse symptoms are observed, but the reduction in berry volume and concentration of sugars are constant. The exact mechanism was thought to be an

unbalanced situation between phloem influx of water and transpirational efflux in the late ripening stage. New results discuss a cultivar dependent intact xylem connection of the berries after veraison (KELLER et al. 2006), through which water flows back excessively to the vine, drying out the berries additionally.

3) Late season bunch stem necrosis – LSBN (KRASNOW et al. 2008; KRASNOW et al. 2010; CHRISTENSEN and BOGGERO 1985 rewieved by KRASNOW et al. 2010)
LSBN has diverse names such as: Stiellähme (Germany), palo negro (Chile), dessichimiento della racchide (Italy) or waterberry (California). Similar to BS this disorder affects solely *Vitis vinifera* L. (BONDADA and KELLER 2012[2]). Many cultivars are affected, but Cabernet Sauvignon shows a high susceptibility. LSBN starts with small black necrotic spots and develops towards the distal end of the rachis. Entire clusters or berries downwards this necrotic zone are affected. The reasons aren't identified, but assumptions focus on xylem conductivity, imbalance of minerals, amino acid metabolism and light interception. LSBN occurs after veraison concentrating carbohydrates in berries.

4) Early bunch stem necrosis – EBSN (BONDADA and KELLER 2012[2])
EBSN is similar to LBSN. The main difference is that it occurs during flowering and before ripening. Clusters are partially affected by necrotic areas, but remaining berries develop similar to healthy ones.

To additionally demonstrate differences in the berry composition between BS and other described physiological disorders, BONDADA and KELLER[2] 2012 published a table, which is attached in the following. It demonstrates that BS berries had lowest calcium, total soluble solid (TSS) and sugar contents concerning glucose and fructose in g/berry.

Fruit composition of berries afflicted with different ripening disorders

	TSS (g/berry)	Glu + Fru (g/berry)	Tartaric acid (mg/berry)	Malic acid (mg/berry)	Ca (µg/berry)
H	0.28	0.30	4.63	0.91	56.7
SB	0.20	0.22	3.08	0.46	53.1
PD	0.25	0.27	3.09	0.97	100.9
LBSN	0.23	0.26	1.21	0.56	97.4
BS	0.05	0.05	4.60	0.44	51.8
LSD (d.f. = 14)	0.025	0.035	0.737	0.259	12.75

Table 3: Fruit compositional differences; for five types of shrivels (from BONDADA and KELLER[2] 2012)

III Publication Format (English)

Analyses of Anthocyanin Biosynthesis in healthy and Berry Shrivel Berries of Zweigelt at different developmental Stages

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Abstract

Berry shrivel (BS) becomes an increasing threat for the Austrian and worldwide viticulture, limiting yield and grape quality. The knowledge on the causes of this physiological disorder is sparse. Former experiments revealed that sugar and anthocyanin content of BS affected berries were decreased at harvest. Phloem unloading of sucrose form the sieve element into the mesocarp cells is a crucial process of the sugar accumulation in berries. Anthocyanin biosynthesis is one branch of the complex phenylpropanoid pathway. Both physiological processes are regulated by gene expression of specific enzymes, transporter proteins and transcription factors. The aim of our study was to find differences in the gene expression levels of specific enzymes, transporter proteins or transcription factors due to BS. Healthy and BS affected berries of *Vitis vinifera* L. cultivar Zweigelt were sampled on six days during the berry growth period 2011 in Lower Austria. Real Time quantitative Polymerase Chain Reactions were performed with RNA extracted from berries to evaluate the expression of genes involved in sugar accumulation (cwINV, NI, vacINV1, vacINV2, HT1, HT3, TMT1, TMT2 and TMT3) and anthocyanin biosynthesis (PAL, CHS, CHI, F3H, F3'H, F3'5'H, FLS, DFR, LAR, LDOX, ANR, UFGT, MYB5b, MYBPA1, MYBA1/2). The analysis revealed a significantly reduced expression of two monosaccharide transporters (TMT1 and TMT2) in BS affected berries as well as a reduced expression of the anthocyanin biosynthetic enzymes CHS and UFGT and their respective transcriptions factors MYBPA1 and MYBA1/2. These novel findings confirm the gene regulatory effects of BS and explain partially the inhibition of the sugar accumulation and anthocyanin biosynthesis in grape berries.

Key words: Berry shrivel, *Vitis vinifera* L., Zweigelt, anthocyanin biosynthesis, sugar accumulation

Abbreviations:

BS	Berry shrivel	F3H	Flavanone-3β-hydroxylase
cwINV	Cell wall invertase	F3'H	Flavonoid-3'-hydroxylase
NI	Neutral invertase	F3'5'H	Flavonoid-3',5'-hydroxylase
vacINV1/2	Vacuolar invertase 1/2	FLS	Flavonol synthase
HT1/3	Hexose transporter 1/3	DFR	Dihydroflavonol 4-reductase
TMT1/2/3	Tonoplast monosaccharide transporter 1/2/3	LAR	Leucoanthocyanidin reductase
PAL	Phenylalanine ammonia-lyase	LDOX	Leucoanthocyanidin dioxygenase
CHS	Chalcone synthase	ANR	Anthocyanidin reductase
CHI	Chalcone isomerase	UFGT	Flavonoid-3O-glucosyltransferase

III Publikations Format (Deutsch)

Analyse der Anthocyanbiosynthese in Gesunden und von Traubenwelke befallenen Beeren von Zweigelt in verschiedenen Entwicklungsstadien

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Abstract

Traubenwelke wird zu einer wachsenden Bedrohung für den österreichischen und weltweiten Weinbau, dabei führt sie zu Ertragseinbußen und Minderung der Traubenqualität. Das Wissen über die Auslöser dieser physiologischen Störung ist spärlich. Frühere Experimente zeigten, dass die Zucker- und Anthocyangehalte, der von Traubenwelke befallenen Beeren, zur Ernte geringer waren. Das Entladen von Saccharose aus den Siebröhren des Phloems in die Zellen des Mesokarps ist ein entscheidender Prozess der Zuckeranreicherung in den Beeren. Die Anthocyaninbiosynthese ist ein Ast des Phenylpropanoidsyntheseweges. Beide physiologischen Prozesse sind durch Genexpression spezifischer Enzyme, Transporterproteine und Transkriptionsfaktoren reguliert. Das Ziel der Studie war es von Traubenwelke hervorgerufene Unterscheide in der Genexpression der spezifischen Enzyme, Transporterproteine und Transkriptionsfaktoren zu finden. Gesunde und von Traubenwelke befallene Beerenproben von *Vitis vinifera* L. cv Zweigelt wurden an sechs Tagen der Beerenwachstumsperiode 2011 in Niederösterreich gesammelt. Die RNA wurde extrahiert und es wurden quantitative Real Time Polymerase Kettenrekationen mit extrahierter Beeren-RNA getestet, um die Expression von Genen der Zuckeranreicherung (cwINV, NI, vacINV1, vacINV2, HT1, HT3, TMT1, TMT2 and TMT3) und der Anthocyanbiosynthese (PAL, CHS, CHI, F3H, F3'H, F3'5'H, FLS, DFR, LAR, LDOX, ANR, UFGT, MYB5b, MYBPA1, MYBA1/2) zu bewerten. Die Analysen ergaben sowohl eine signifikant unterregulierte Expression zweier Monosaccharid Transporter (TMT1 und TMT2) in von Traubenwelke befallenen Beeren als auch eine unterregulierte Expression der Enzyme der Anthocyaninbiosynthese CHS und UFGT und deren Transkriptionsfaktoren MYBPA1 und MYBA1/2. Diese neuen Ergebnisse bestätigen den Einfluss der Traubenwelke auf die Genexpression und erklären teilweise die geminderte Zucker- und Anthocyananreicherung in Weintrauben.

Schlüsselwörter: Berry shrivel, *Vitis vinifera*, Zweigelt, Anthocyanbiosynthese, Zuckeranreicherung

Abkürzungen:

BS	Traubenwelke	F3H	Flavanon-3β-Hydroxylase
cwINV	Zellwandinvertase	F3'H	Flavonoid-3'-Hydroxylase
NI	Neutrale Invertase	F3'5'H	Flavonoid-3',5'-Hydroxylase
vacINV1/2	Vakuoläre Invertase 1/2	FLS	Flavonolsynthase
HT1/3	Hexose Transporter 1/3	DFR	Dihydroflavonol 4-Reduktase
TMT1/2/3	Tonoplast Monosaccharid Transporter 1/2/3	LAR	Leucoanthocyanidinreduktase
PAL	Phenylalanin Ammoniak-Lyase	LDOX	Leucoanthocyanidindioxygenase
CHS	Chalconsynthase	ANR	Anthocyanidinreduktase
CHI	Chalconisomerase	UFGT	Flavonoid-3O-Glucosyltransferase

Introduction

Berry growth of *Vitis vinifera* L. can be described as a number of complex series of biochemical and physical changes (DELUC et al. 2007). It's separated in three stages (COOMBE and MCCARTHY 2000): Stage I (seed formation and cell division), stage II (seed maturation) and stage III (cell expansion and sugar accumulation). Phytohormones like cytokinins, auxins, gibberellins, ethylene, abscisic acid and brassinosteroids (KELLER 2010) as well as changes in gene expression levels stimulate and coordinate these processes. During berry growth stage II and especially III glucose and fructose are accumulated in the berry flesh (ZHANG et al. 2006). Source organs e.g. mature leaves export mainly sucrose via the phloem to sink organs. In stage III sucrose is downloaded by apoplastic phloem unloading into the berries. Firstly sucrose is hydrolyzed to glucose and fructose by invertases in different cell compartments: Cell wall invertases are bound to cell membranes in the apoplast (HAYES et al. 2007), neutral invertases act mainly in the cytosol (NONIS et al. 2008) and vacuolar invertases cleave sucrose inside the vacuoles (ZHANG et al. 2006). Cleaved to monosaccharides, they are translocated by hexose transporters like HT1 and HT3 into the cytosol of the sink cells (AFOUFA-BASTIEN et al. 2010). If the monosaccharides are not used for respiration or metabolisation, they cross the tonoplast membrane mediated by tonoplast monosaccharide transporters like TMT1, TMT2 or TMT3 (ÇAKIR and GIACHINO 2012) to be stored in the vacuoles of mesocarp parenchyma cells. A second biochemical process initiating after veraison is the accumulation of anthocyanins. Their biosynthesis is one branch of the complex phenylpropanoid pathway. In the beginning of berry development flavonols, then proanthocyanidins and in the end anthocyanins accumulate (CZEMMEL et al. 2012). This temporal shift in the flavonoid biosynthesis is regulated and controlled on the one hand by changes in the expression levels of genes coding for biosynthetic enzymes. Nine enzymes are directly involved in the biosynthesis of anthocyanins: PAL, C4H, 4CL, CHS, CHI, F3H, DFR, LDOX and UFGT. Two enzymes shift the anthocyanin composition: F3'H and F3'5'H. On the other hand there are enzymes competing for identical substrates: FLS, ANR and LAR (CZEMMEL et al. 2012). Recently several MYB R2R3 transcription factors: MYB5b (DELUC et al. 2008), MYBF1 (CZEMMEL et al. 2009), MYBPA1/2 (CZEMMEL et al. 2012) and MYBA1/2 (JEONG et al. 2004; KOBAYASHI et al. 2013) have been isolated and characterized for grapevine. They regulate specific gene expressional patterns or the activity of specific enzymes of the phenylpropanoid pathway.

The interplay of processes during grape berry ripening can be influenced by physiological ripening disorders and plant stress. BS, a physiological ripening disorder, is an increasing problem and potential threat for the Austrian and worldwide viticulture. An increasing number of BS cases is described from South Tyrol and Friuli (Italy; RAIFER cited in RIEDEL 2008), Baden (Germany; BACHTELER and RIEDEL 2011), the US (KELLER 2008) and lately China (FANG et al. 2011). *Vitis vinifera* L. cultivar Zweigelt (Blaufränkisch x St. Laurent) is the most important red wine cultivar in Austria and at the same time very susceptible against BS (KNOLL et al. 2010). Berry shrivel is considered as physiological disorder, since scans for phytoplasms and viruses were negative (KRASNOW et al. 2009). For the winegrowers BS symptoms are difficult to foresee and thought to be physiologically initiated before they are detectable. Compositional changes in berries caused by BS such as decreased sugar and

anthocyanin content, acid and bitter taste (BACHTELER and RIEDEL 2011, GRIESSER et al 2012[1]) combined with low cell viability (BONDADA and KELLER 2012[1]) limits their use for winemaking, so affected clusters have to be considered as yield loss. An analysis concerning the amino acid composition revealed that thirteen amino acids are reduced and two are enhanced (GRIESSER et al. 2012[1]).

A number of possible causes have been investigated. An initial hypothesis was based on the assumption that the xylem hydraulic connectivity after veraison in some cultivars was partially maintained (KELLER et al. 2006) was discarded, because later trials inhibiting the phloem flow by girdling of berry pedicels (ROGIERS et al. 2006) revealed similar shriveling symptoms like BS. The phloem imports carbohydrates and other nutrients into berries. The fact that in affected berries sugar accumulation slows down and ceases (GRIESSER et al. 2012[2]), indicates a phloem inhibition. Callose depositions, as a reaction of environmental influences, could cause a spontaneous reduction of phloem flow on a cluster basis (KELLER 2008), but research on transcriptional gene regulation revealed no detected up regulation of genes related to the callose formation in rachis tissues of BS affected clusters (GRIESSER and FORNECK 2010). Other approaches of BS deal with the vineyard management. On the one hand lack of available potassium in the soil is discussed (FARDOSSI 2001, BACHTELER AND RIEDEL 2011), but contradictory results were obtained. On the other hand no correlation between potassium and BS was observed, applying foliar K fertilizer over two seasons (KNOLL et al. 2007). Rootstock selection affected partially berry shrivel incidences in field trials. Vines propagated on vigourous rootstocks are more susceptible to BS (SCHUMACHER et al. 2007). The Obst- und Weinbauschule Krems in Lower Austria observed unfavourable seasonal weather conditions promoting the berry shrivel phenomenon (KÜHRER and GABLER 2011). Furthermore they postulated a low leaf area/fruit load ratio to increase BS (KÜHRER and GABLER 2013). Contradictory results were found in Washington State, US. Water stress, overheating or abrupt coldness had no significant influence on BS occurrence (KELLER 2008).

The objectives of this research project are to obtain more information about two major physiological processes affected by BS: sugar accumulation and anthocyanin biosynthesis. We hypothesize that expression levels of specific genes related to sugar accumulation and anthocyanin biosynthesis are down regulated. In contrast, expression levels of genes coding for competing enzymes and transcription factors in the anthocyanin biosynthesis are assumed to be up regulated.

Material and Methods

Plant material and sampling

Berries of *Vitis vinifera* L. cultivar Zweigelt propagated on Kober 5BB and planted in 1974 were collected from a commercial vineyard in Mailberg in the viticultural region of Weinviertel in Lower Austria in 2011. The trellising system was a vertical shoot positioning (VSP) with bilateral canes and each plant had 4.2 m^2 of space. Yield was limited to 20 clusters per plant or 15.000 - 17.000 kg per hectare. Berry samples were taken on six sampling dates: 15.07./22.07.; 29.07.; 4.08.; 11.08.; 17.08. and 24.08.2011 in order to cover the berry ripening period. The detached berries were immediately snap frozen in liquid nitrogen to avoid RNA degradation or other physiological stress reactions. As it wasn't

possible to foresee the berry shrivel in the young clusters, the sampling process included the marking of the remaining parts of the sampled clusters on the vine. In the end of the berry growth period, when berry shrivel was visible, eight healthy control and eight affected clusters from different plants were selected for each sampling date. The samples were stored at -80°C.

Total anthocyanins and soluble solids

Four berries per samples were ground in liquid nitrogen and 200 mg of powdered plant tissue was used to determine total anthocyanin concentration. A pH differential method (WROLSTAD et al. 2005) was applied. This protocol (Details in supplementary material Table S1) compared spectroscopically the absorbance at 530 nm and 700 nm of a purified methanol extract of the berry tissue at pH 1.0 with the absorbance at pH 4.5. The difference in the absorbance was transformed by an equation to mg/L of the equivalent cyanidin-3-glucoside and further transformed to a final concentration of mg/g dry weight. The same berry material was used to determine the soluble solids with a refractometer (Pocket Refractometer, ATAGO,Tokyo, Japan) in °Brix. The applied statistical method was a t-Test ($p<0.05$)

RNA extraction and reverse transcription

Gloves, surfaces, technical devices and tools were sprayed with ethanol (70%) and RNase away (Molecular BioProducts, San Diego, USA) prior and during the RNA extraction. All used chemicals were prepared with DEPC water. DEPC water is double distilled water incubated for 24 hours with the RNase degrading compound diethylpyrocarbonate (Carl Roth, Karlsruhe, Germany) and then autoclaved. Eight healthy and eight BS probes per sampling date were selected and combined to four biological replicates each consisting of four berries. Kernels of frozen berries were removed. The frozen pulp and skin were ground to a fine powder by a Retsch Mill (Retsch MM 400, Haan, Germany). For RNA extraction an optimized protocol for RNA extraction for grapevine berries was applied (REID et al. 2006). The protocol is described in detail in the Supplementary Material (Table S2). The concentration and the purity of the RNA were measured spectroscopically by NanoDrop 2000c (Thermo Scientific, Wilmington, USA). For this experiment we considered the RNA concentration to be minimum 100 ng/µl and the two integrity ratios to be higher than 1.8 (FLEIGE and PFAFFL et al. 2006, PENNA et al. 2011). To determine the RNA integrity electrophoreses were run on a 1.5 % Agarose gel stained with GelRed (Biotum, Hayward, USA) for 40 min at 100 V. Reverse transcription was performed using the QuantiTec Reverse Transcription Kit (Qiagen, Hilden Germany) and 1000 ng total RNA per sample. Reaction conditions were: 42°C for 30 min; 95°C for 3 min and cooling down to 8°C (Mastercycler Gradient, Eppendorf, Hamburg, Germany). Prior to the sample analysis, it was necessary to find two reference genes for a reliable statistical evaluation (HELLEMANNS et al. 2007). Four candidate genes: actin (VIT_04s0044g00580), ef1 (VIT_06s0004g03220), gadph (VIT_17s0000g10430) and ubiquitin (VIT_16s0098g01190) were tested with cDNA samples of three sampling dates: 22.07 (early); 11.08 (veraison) and 24.08 (late). The obtained Ct values were processed with Normfinder (LINDBJERG et al. 2004) and the most stable reference genes, actin and ubiquitin, were used for further analyses.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The quantitative Real Time Polymerase Chain Reaction (qPCR) technology is a simple, specific and sensitive method to compare RNA levels (FLEIGE and PFAFFL 2006). qPCR reactions (12 µl volume, 300 nM Primer each) was performed using the KAPA SYBR Fast qPCR Universal Kit (Peqlab Biotechnology, Erlangen, Germany) and the Rotor-Gene-Q PCR cycler (Qiagen, Hilden, Germany). PCR efficiency of all primers was determined with 1:3 dilution series and calculated according to E = [10(-1/slope)]-1 (PFAFFL 2006). The expression of 22 genes was determined: Nine genes coded for sequences of enzymes and proteins involved in the sugar transport from the phloem into the berries and thirteen genes coded for sequences of enzymes and transcription factors involved in the anthocyanin biosynthesis. In this research all primers were designed online using the primer design tool of the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/). A table of the primers used for the analysis and the pipette scheme for the qPCR loading are attached in the Supplementary Material (Table S3 and S4). A gene maximation principle (HELLEMANNS et al. 2007) was applied for qPCR runs. Each run consisted of two healthy control and two berry shrivel samples diluted 1:4 of an identical sampling date to minimize differences between runs. The cycling program consisted of 40 cycling events. Cycling conditions were: one cycle for 4 min at 95 ºC, 40 cycles for 5 s at 95 ºC, 20 s at 60 ºC, 15 s at 72 ºC and 5 s at 75 ºC, where the fluorescence signal was measured. Relative quantification of gene expression was calculated as normalized relative quantities according to HELLEMANNS et al. 2007.

Results

Soluble solids and total anthocyanin concentration in grape berries

Sugar content as soluble solids is an important fruit quality parameter of grapevine at harvest. Soluble solids of all labeled grape clusters were measured at six sampling dates during the growing season 2011 (KURRLE and SOMMER 2013). Soluble solids in healthy berries are increasing during ripening phase, whereas the accumulation of sugars in BS affected berries stops after veraison (Figure. 1). Specifically in the later sampling dates the differences becomes significant, because sugar accumulation of BS berries ceased completely after the 17.08.2011.

The concentration of total anthocyanins was determined with the pH differentiation method and the results are shown in Figure 2. In healthy berries the total anthocyanin content increases after veraison, whereas BS affected berries show significant lower anthocyanin accumulation. Similar to the accumulation of soluble solids the biosynthesis of anthocyanins seems to be stopped during BS symptom development.

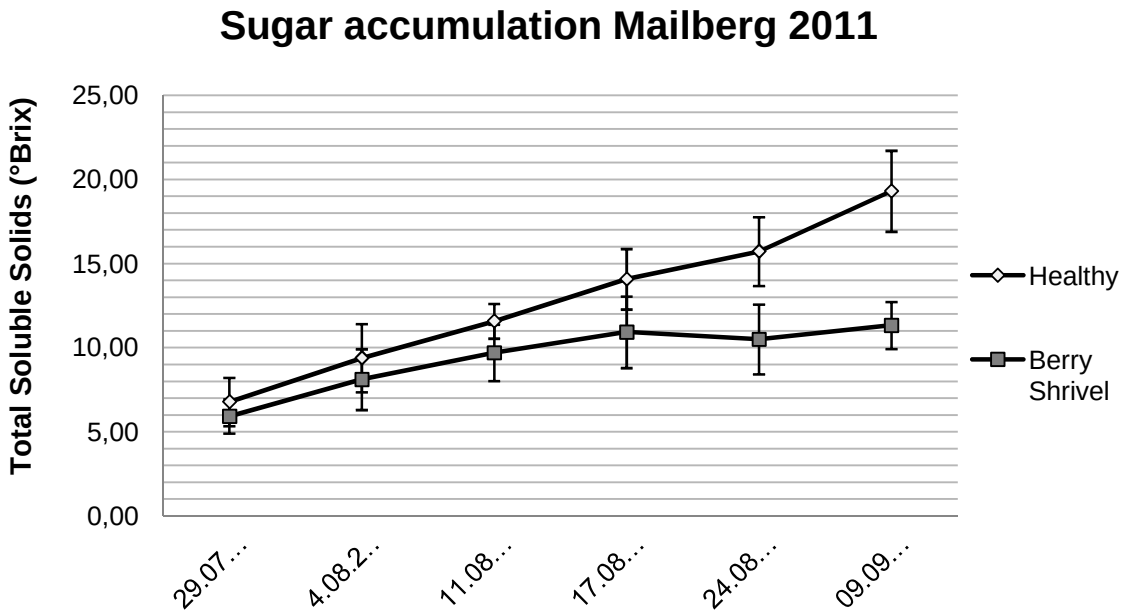


Figure 1: Summary of measured soluble solids (°Brix) during grape berry ripening in healthy and BS grapes (N=8 each). Mean values with standard deviations. Statistically significant differences were obtained for 24.08.2011 and 09.09.2011 (p>0.5).

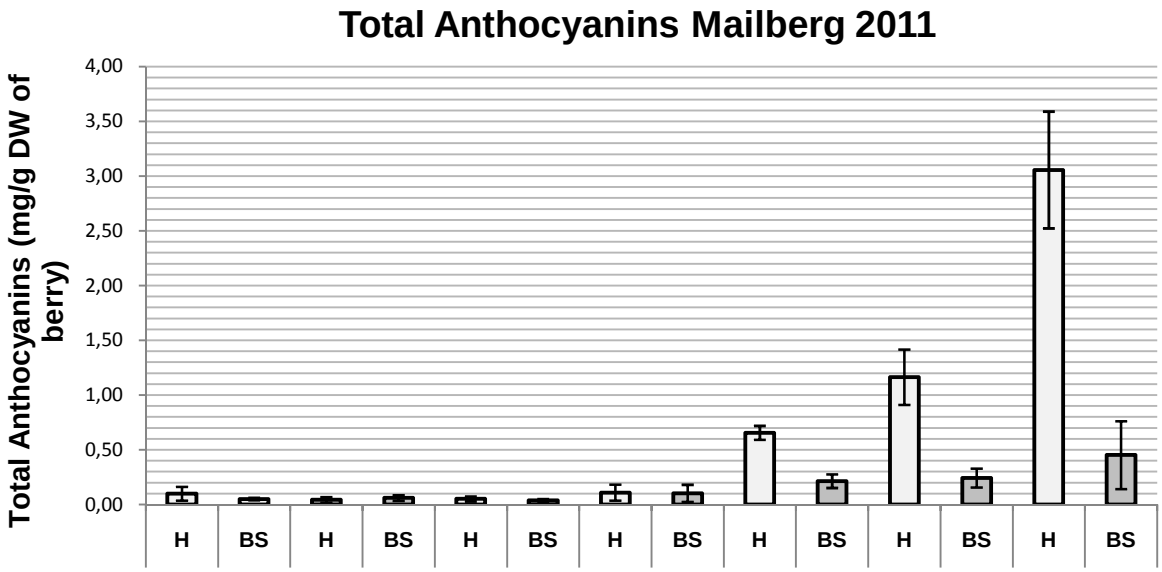


Figure 2: Concentration of total anthocyanins in healthy and BS affected berries (mg/g DW of berries**)** determined with the pH Differential Method. Mean values with standard deviation (N=4 each). Statistical significant differences were obtained starting from 17.08.2011.

It can be confirmed that after veraison anthocyanins start to accumulate in healthy and BS affected berries. Anthocyanin accumulation indicates veraison. Therefore the pigments are not detectable before and increase towards the end of the berry development period. BS affected berries show significantly lower anthocyanin contents compared to the healthy samples after veraison. It seems that their accumulation stopped in BS affected berries.

Transcriptional regulation of genes of the sugar transport and sugar metabolism

The relative expression of four genes involved in sucrose metabolism and five genes involved in sugar transport have been analyzed. The results of cell wall invertase, neutral invertase, vacuolar invertase 2, TMT1 and TMT2 are shown as normalized quantities in Figure 3 and Figure 4. Results obtained for vacuolar invertase 1, HT1 and HT3 are presented in the Supplementary Material (Table S5). In our experiment the overall gene expression of cell wall invertase is low at all sampling dates in healthy berries as well as in BS berries. There are no significant differences comparing healthy with BS affected berries. Similar results are observed for the neutral invertase. A significant and consistent difference between healthy and BS berries over the developmental period doesn't exist. The overall gene expression levels of vacuolar invertase 2 are higher in both healthy and BS berries as the before mentioned enzymes. The expression levels of vacuolar invertase 2 are increased right after veraison (4.08 and 11.08) in BS affected berries compared to healthy berries (Figure 3).

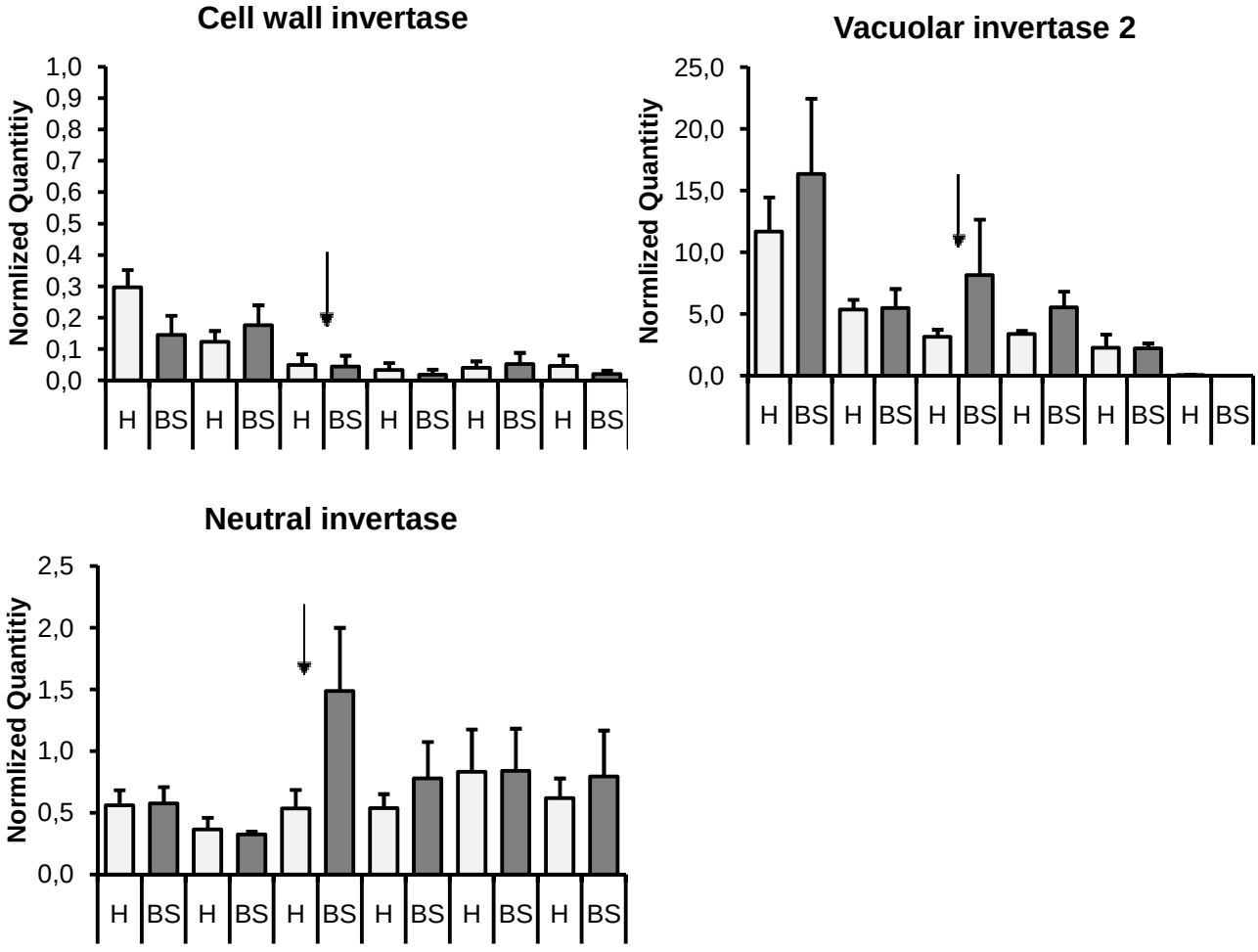


Figure 3: Relative expression of the genes cell wall invertase (GSVIVT00034185001), vacuolar invertase 2 (GSVIVT00004764001) and neutral invertase (GSVIVT01034944001) as normalized quantities in healthy and BS affected grape berries. Data represent mean values and standard deviation (N=4 each) calculated according to HELLEMANNS et al. 2007. H= healthy, BS = berry shrivel. Arrow indicates veraison.

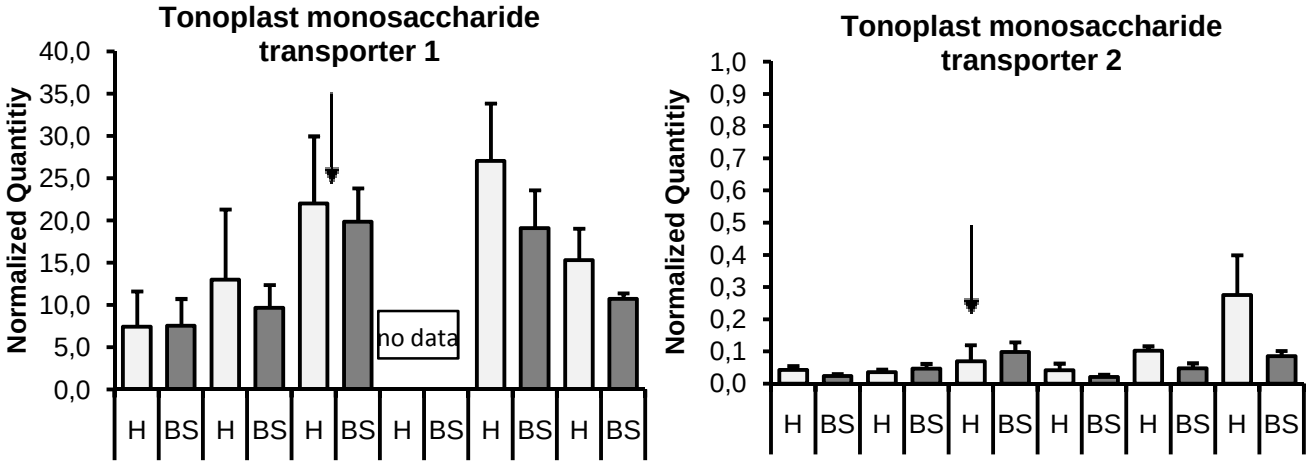


Figure 4: Relative expression of the genes TMT1 (GSVIVT01013414001) and **TMT2** (GSVIVT00019321001) as normalized quantities in healthy and BS affected grape berries. Data represent mean values and standard deviation (N=4 each) calculated according to HELLEMANNS et al. 2007. H= healthy, BS = berry shrivel. Arrow indicates veraison.

Hexose transporters as well as tonoplast monosaccharide transporter were analyzed with qPCR. Both hexose transporters HT1 and HT3 show low expression levels and no significant gene expression trend between the healthy and BS affected berries can be observed. Therefore both graphs are attached to the Supplementary Material S5.

Three tonoplast monosaccharide transporters were tested in this experiment. TMT1 is highly expressed in the tested plant material, whereas TMT2 has a low, but interesting gene expression. Unfortunately the analyses of TMT1 for the sampling date 11.08.2011 did not work, due to unknown reasons and will have to be repeated. Furthermore the gene expression levels of TMT1 of BS affected berries are lower compared to the healthy ones after veraison. Also the expression of TMT2 is lower in BS berries, but later as TMT1. TMT3 shows low gene expression levels (Supplementary Material S5).

Transcriptional regulation of genes of the anthocyanin biosynthesis

Relative expression of eleven genes coding for enzymes and two transcriptions factors related to the anthocyanin have been analyzed. The relative expression of PAL, the first enzyme of the flavonoid biosynthesis pathway is shown in Figure 5. A significant increase in its expression after veraison towards ripening could be determined in healthy as well as BS berries. Comparing healthy and BS affected berries no significant difference is observed with exception of the last date (24.08). In this experiment a primer within a conserved coding region of the genes *Chs1*, *Ch2*, and *Chs3* was designed to analyse their general gene expression. CHS is highly expressed in berries especially at later ripening stages (Figure 5). Unfortunately the date from 17.08. is missing and analyses have to be repeated. BS affected berries after veraison show a reduced expression level. CHI is generally expressed in the berry material of this experiment (Figure 5). CHI gene expression levels increase after veraison significantly towards ripening. BS doesn't modify the expression levels of the gene coding for CHI significantly. However the last date (24.08) shows a significantly decreased gene

expression of CHI. Figure 5 demonstrates that genes coding for F3H are generally expressed in the berries of the experiment during all development stages. After veraison there is an increasing trend of the expression levels towards ripening. Berries affected by BS show no significant differences in the expression level of F3H. Exceptionally the last date (24.08) has a significantly lower expression of this enzyme in BS affected berries. The gene expression of F3'H (Figure 5) is stable in the berry samples, but slightly increased towards the last sampling day (24.08). BS down regulates F3'H significantly at the last sampling date (24.08). General gene expression of DFR in Figure 5 is detectable in the berry samples. Although there is no data for the last day (24.08) there is evidence to assume an increasing trend of the gene expression towards ripening. No significant difference between healthy and BS affected berries is observed. The overall gene expression of LDOX during the berry growth period is also detectable (Figure 6). Gene expression levels increase after veraison. There is no visible difference between healthy and BS samples, but the last date (24.08). For this specific date the expression level of LDOX in BS affected berries is tendencially lower than in the healthy samples. Gene expression levels of UFGT increase after veraison (Figure 6). Around veraison (4.08 and 11.08) a significant lower expression of UFGT due to BS is found. The gene expression levels of the transcription factors MYBA1 and MYBA2 are detected in the plant material of the present research in Figure 6. Furthermore after veraison they show an increasing trend towards ripening. At veraison (4.08) there is a significant lower expression for the BS affected berries. Primers within a conserved coding region of the genes for MYB5b, FLS and F3'5'H weren't established in this experiment and have to be designed again. Gene expression levels of two enzymes of the LAR family were tested separately: LAR1 and LAR2. Figure 7 considering LAR1 is attached in the Supplementary Material S5, because of low overall expression levels indicated on the y-axis. Figure 7 presents the results of the gene expression levels related to LAR2. Firstly the expression of LAR2 is present in the berry samples. In the earlier berry growth stages, there is a higher gene expression than in the end. Particularly after veraison the gene expression levels of LAR2 drop. Figure 7 shows low, but still detectable overall gene expression levels for ANR in the berry samples. The expression trend for ANR is higher in the beginning until veraison, but postveraison it decreases. There is no significant influence on ANR gene expression by BS observed in this experiment. The results of the transcription factor MYBPA1 in Figure 7 demonstrate low overall gene expression levels. BS down regulates the expression levels of MYBPA1 after veraison tendencially.

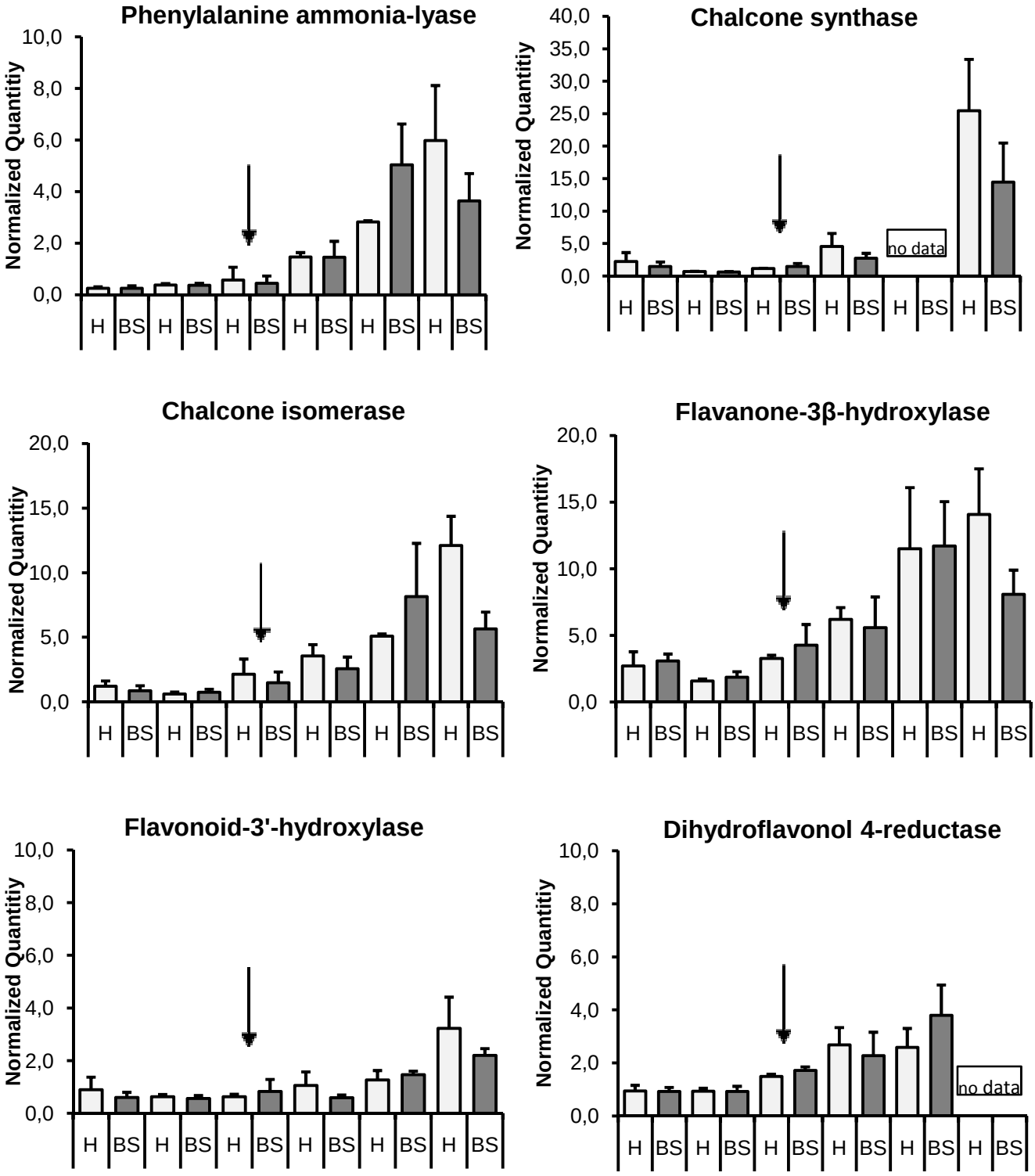


Figure 5: Relative expression of the genes PAL (GSVIVT00018175001), **CHS** (GSVIVT00037967001, GSVIVT01032968001, GSVIVT01000521001), **CHI** (GSVIVT00029513001), **F3H** (GSVIVT01018781001), **F3'H** (GSVIVT00016217001) and **DFR** (GSVIVT01009743001) as normalized quantities in healthy and BS affected grape berries. Data represent mean values and standard deviation (N=4 each) calculated according to HELLEMANNS et al. 2007. H= healthy, BS = berry shrivel. Arrow indicates veraison.

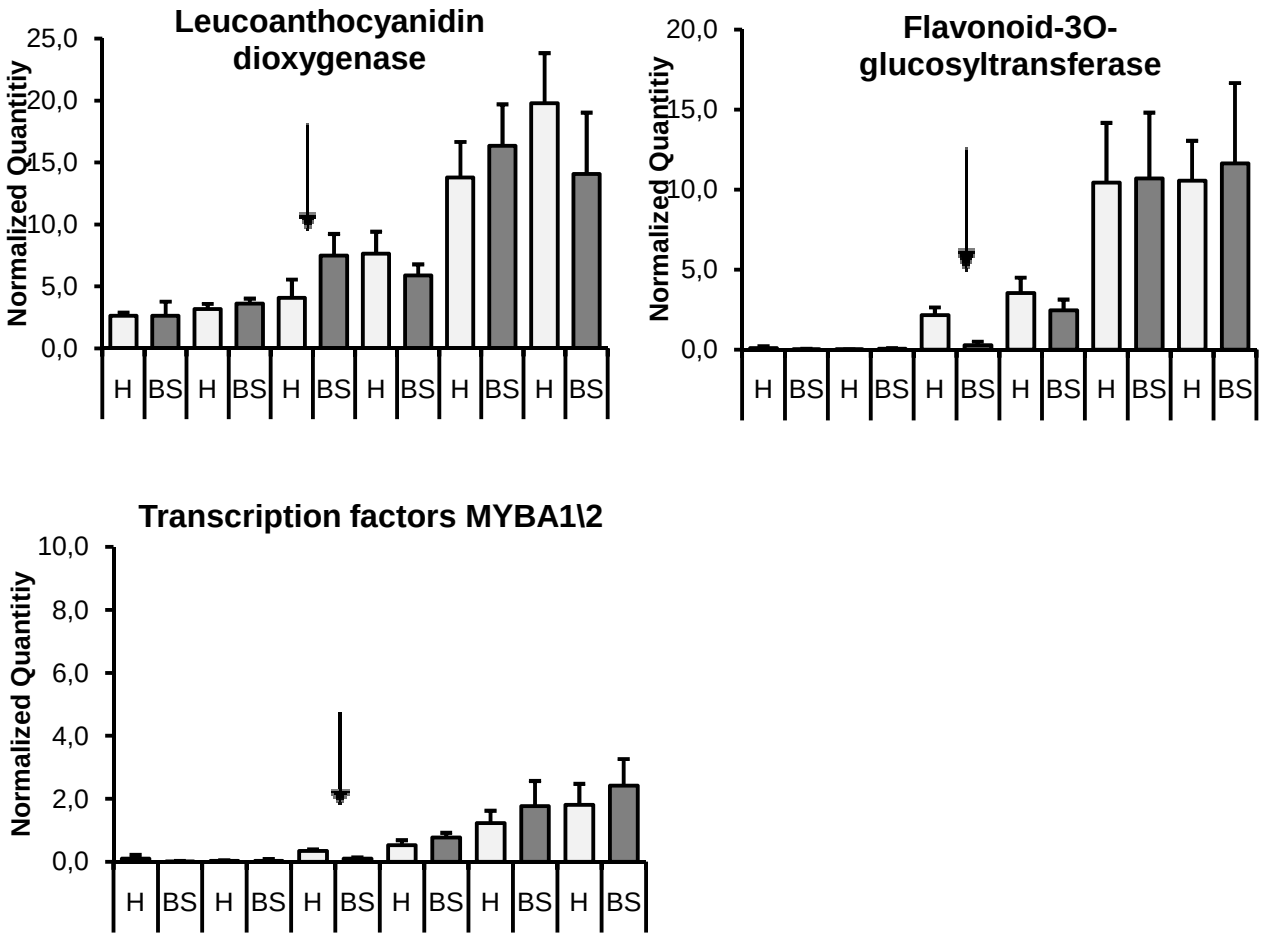
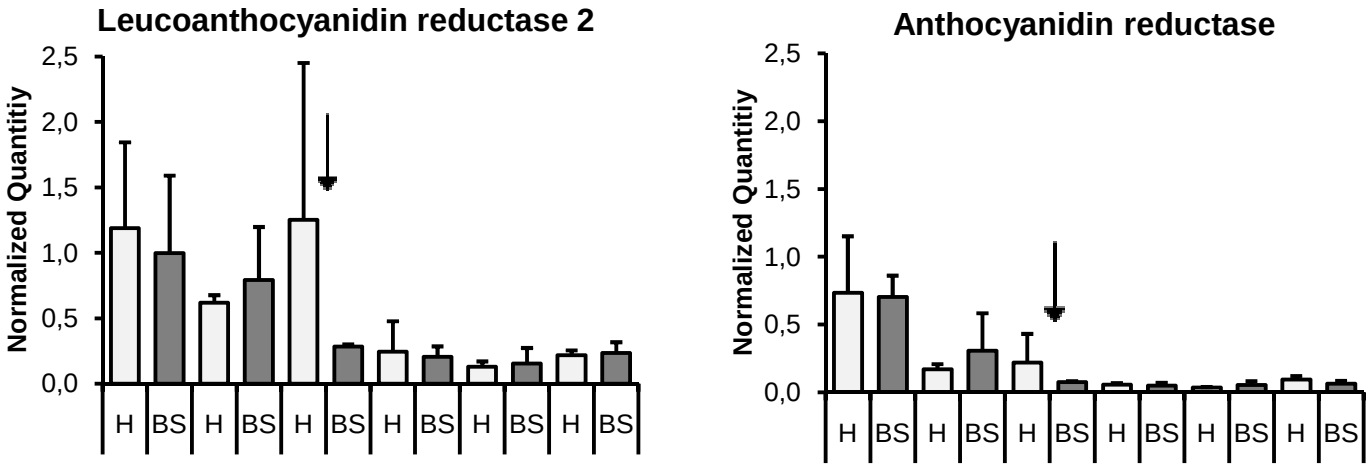


Figure 6: Relative expression of the genes LDOX (GSVIVT01019892001), **UFGT** (GSVIVT01024419001) **and MYBBA1/2** (GSVIVT01022659001) as normalized quantities in healthy and BS affected grape berries. Data represent mean values and standard deviation (N=4 each) calculated according to HELLEMANNS et al. 2007. H= healthy, BS = berry shrivel. Arrow indicates veraison.



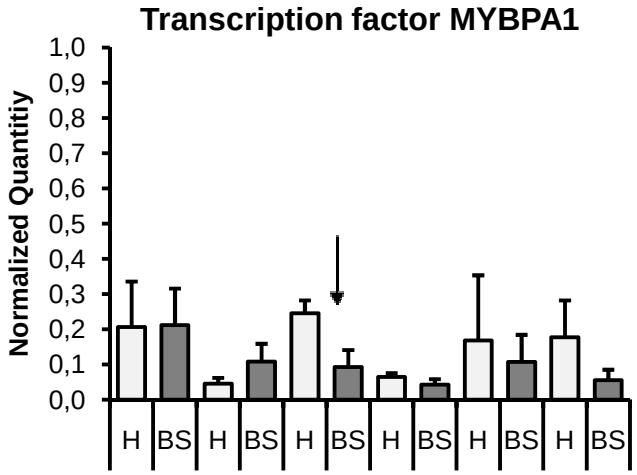


Figure 7: Relative expression of the genes LAR2 (GSVIVT01008238001), **ANR** (GSVIVT01006396001), **and MYBPA1** (GSVIVT01025452001) as normalized quantities in healthy and BS affected grape berries. Data represent mean values and standard deviation (N=4 each) calculated according to HELLEMANNS et al. 2007. H= healthy, BS = berry shrivel. Arrow indicates veraison.

Discussion

The present study aimed to add more knowledge about the effects of BS on grapes and their physiological and gene regulatory background. Changes in the expression level of sugar transport and sugar metabolism genes as well as genes along the anthocyanin biosynthesis pathway were analyzed.

BS modifications related to the sugar accumulation

One of the most important BS symptoms described is the decreased sugar accumulation after veraison (e.g. KRASNOW et al. 2009; GRIESSER et al. 2012[2]). The stop of sugar accumulation was confirmed significantly in our analyses. Cell wall invertase and neutral invertase are thought to play important roles in the sugar transport cleaving sucrose to glucose and fructose (NONIS et al. 2008; AFOUFA-BASTIEN et al. 2010). Genes coding for cell wall invertase are reported to be expressed through the whole berry development with a maximum after veraison (HAYES et al. 2007).We could not confirm these results, since our samples showed low overall gene expression levels of cell wall invertase. Other sucrose cleaving enzymes like neutral invertase were found to be higher expressed in the berry samples of this experiment. Therefore neutral invertase seemed to play a crucial role in the sugar transport in Zweigelt berries. Our results confirmed tendencially the increase in the gene expression of neutral invertase after veraison described in literature (NONIS et al. 2008). The genes coding for vacuolar invertase 1 were low expressed, so we considered them to play a minor role for the sugar transport. On the contrary genes coding for vacuolar invertase 2 were high expressed with a decreasing trend towards veraison. The decreasing trend towards veraison of vacuolar invertase 2 was consistent with findings in literature (DAVIES and ROBINSON 1996), which assume this enzyme to be more important in the early stages of berry growth. BS didn't modify the gene expression levels of the analyzed invertases in this experiment. However it is

not possible to exclude the invertases of the berry shrivel phenomenon, because in this experiment we didn't analyse their enzyme activity and role of cofactors, which might be influenced by BS. Furthermore biotic and abiotic influences of the planting site might have regulated the gene expression of invertases.

After invertase cleavage from sucrose to monosaccharides, they have to be transported through different membranes into the vacuoles of fleshy parenchyma cells. The first analyzed sugar transporter family were the hexose transporters. Hexose transporter 1 played a minor role in the sample material due to its low expression levels. This finding was in line with AFOUFA-BASTIEN et al. 2010, which postulated a poor gene expression of HT1 in berry tissue. The results of VIGNAULT et al. 2005, telling that hexose transporter 1 is important for the symplastic phloem downloading in the early berry development stages, couldn't be confirmed by our findings in the observed period. On the contrary hexose transporter 3 is described to be stable expressed in grape berry tissue (HAYES et al. 2007). This is true for our data, concluding that HT3 plays a crucial role in hexose transport (HAYES et al. 2007). Considering BS modifications of the gene expression of the hexose transporters, we didn't detect significant up- or down regulations. A second important sugar transporter family are the tonoplast monosaccharide transporters (TMT). Genes coding for TMT1 were highly expressed in the berry tissue, whereas the expression of TMT 2 was lower, but still relevant. Gene expression of TMT3 was nearly not detected. These results are in line with findings of AFOUFA-BASTIEN et al. 2010, which reported significant elevated expression levels of TMT1 and TMT2 in grape berries. Furthermore they postulated an increased gene expression after veraison. In our experiment this was true for TMT1, which showed a peak at veraison and for TMT2 which, despite of a low overall gene expression levels, showed an increasing trend after veraison. Interestingly BS was able to down regulate the gene expression of TMT1 and TMT2 significantly. These novel findings indicate that BS is able to down regulate key transporter proteins involved in the sugar transport and accumulation in grape berries and could be responsible factors for the limited sugar content of BS affected berries. However it is important to have in mind that probably much more mechanism are modified negatively by BS and our findings are contributing factors to the overall decreased sugar accumulation. Future research work dealing with BS should be directed to gain more information about the inhibition of the sugar accumulation by e.g. testing additional enzymes and transporters or analysing enzyme activities and the role of their cofactors.

BS modifications related to anthocyanin biosynthesis

A second known symptom of BS is the decreased color of affected berries at harvest due to lower anthocyanin contents in the grape berry skin (KRASNOW et al. 2009; GRIESSER et al. 2012[1]). In the current experiment this was significantly confirmed. The analysis of the gene expression levels of key enzymes involved in the anthocyanin biosynthesis revealed that PAL, CHI, F3H, F3'H, DFR and LDOX were higher expressed in the berry tissue after veraison. This was expected and is observed in literature (CZEMMEL et al. 2012), since these enzymes are responsible for anthocyanin biosynthesis and accumulation. A novel finding was that BS down regulated the gene expression levels of all of before mentioned enzymes significantly in the last sampling date, with exception of DFR - due to missing data. According to this, we

assumed that BS might have down regulated even later stages of these key enzymes of the anthocyanin biosynthesis. The down regulation of PAL, CHI, F3H, F3'H, DFR and LDOX could explain the lack of anthocyanins in the berry skin of BS affected berries at harvest. Future research related BS modifications of the anthocyanin content should consider and include samples of later berry development stages. Besides these results concerning the general anthocyanin biosynthetic pathway, we found two enzymes specifically modified by BS. CHS showed a typical gene expression starting with initially low levels, but after veraison they increase. This gene expression was compatible with findings in literature (CZEMMEL et al. 2012). The novelty is that BS was able to down regulate CHS gene expression significantly after veraison. Interestingly the expression of CHS is reported to be linked to the transcription factor MYBPA1, which is supposed to enhance the gene expression of several genes and among them CHS (CZEMMEL et al. 2012). Comparing the gene expression levels of this transcription factor, we found a similar down regulation after veraison synchronized to the inhibited gene expression of CHS. These findings firstly confirmed the regulatory effect of MYBPA1 for the gene expression of CHS (CZEMMEL et al. 2012) for *Vitis vinifera* L. Zweigelt. Secondly it proofed that BS has the potential to down regulate directly key enzymes of the anthocyanin biosynthesis like CHS and/or indirectly their regulatory transcription factors. Another similar example, found in this study, was UFGT. Flavonoid-3O-glucosyltransferase is described to be the last enzyme of the anthocyanin biosynthesis. Its gene expression levels in the healthy berry samples of the experiment correlated with the ones described literature (BOSS et al. 1996[2]). We could observe that BS down regulated the expression levels of UFGT in veraison significantly. One of the first symptoms of BS for winegrowers is a delayed color change of the berries. Veraison is marked by the color change of the berries (DELUC et al. 2007). Linking this fact with the findings, the delayed veraison in BS affected berries can be explained scientifically by the down regulation of UFGT in veraison. In particular: BS down regulated the gene expression of UFGT in veraison, therefore less flavonoid-3O-glucosyltransferase was present, so less anthocyanins were synthesized, less color pigments were accumulated in the berry skin and therefore color change happened later compared to healthy grapes on the same plant. Additionally the transcription factors MYBA1 and MYBA2, which are reported to enhance specifically UFGT expression (CZEMMEL et al. 2012), showed a synchronized down regulation in veraison due to BS. Similar to the findings of CHS, this confirmed firstly the regulatory effects of MYBA1/2 on the gene expression of UFGT for our plant material. Secondly it proofed that BS was able to down regulate the gene expression of key enzymes related to the anthocyanin biosynthesis and/or their regulatory transcription factors.

The gene expression analysis of the competing enzymes of the anthocyanin biosynthesis LAR and ANR demonstrated initially higher gene expression levels with a decrease towards veraison. This trend was also described in former articles (LACAMPAGNE et al. 2010) and is expected since they are supposed to synthesize proanthocyanidins prior veraison. However, we didn't find a significant modification of their gene expression due to BS in this experiment. So assumptions that BS shifts the phenylpropanoid pathway towards other biosynthetic side branches than the anthocyanins weren't confirmed.

Summarizing the experiment revealed that BS was able to influence the expression of two specific key enzymes and their transcription factors related to the anthocyanin biosynthesis. The modification of UFGT explained the delayed veraison, whereas the down regulation of CHS potentially contributed to the decreased anthocyanins at harvest. Additionally there were hints that BS down regulated the gene expression of more key enzymes in later berry growth stages. It has to be mentioned that this article focused on gene expression levels, but future research might also consider other parameter like enzyme activity, the role of cofactors and accumulation of other products of the phenylpropanoid pathway e.g. proanthocyanidins and flavonols in order to fully understand the effects of BS on the anthocyanin biosynthesis.

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Supplementary Material

Table S1

pH Differential Method

to extract and determine Total Anthocyanins (WROLSTAD et al. 2005)

MeOH Extract preparation:

1) Fill whole berry in precooled mixing container with 1 spatula of ascorbic acid.
2) Mill samples at 30 Hz for 30 sec.
3) Weigh 1.25 g of each sample.
4) Add 12.5 ml acidified MeOH (0.1 % HCl v/v).
5) Extract with ultrasonic assistance for 30 min.
6) Centrifuge for 5 min at maximum speed.
7) Transfer supernatant in new tube and determine volume.
8) Add the removed volume of MeOH (0.1 % HCl v/v).
9) Repeat extraction step (5 to 9).

Total Anthocyanin determination:

10) Prepare a potassium chloride (0.025 M) buffer solution:
    - Mix 1.86g KCl in 980 ml distilled water.
    - Adjust the pH to 1.0 with HCl
    - Dilute in 1 L of distilled water
11) Prepare a sodium acetate (0.4 M) buffer solution:
    - Mix 54.43g CH_3CO_2Na x 3 H_2O with 960 ml distilled water
    - Adjust the pH to 4.5 with HCl
    - Dilute in 1 L distilled water
12) Add 1 ml of sample extract in 9 ml (dilution: 1:10) of each buffer and let it equilibrate for 15 min.
13) Measure at the wavelength for maximum absorbance (λvis max) for cyanidin-3-glucoside (for HCl in MeOH): 530 nm for both buffers.
14) Calculate:

    (1) $A = (A530–A700)pH1.0 – (A530–A700)pH4.5$

15) For the monomeric anthocyanin pigment concentration in the original sample calculate:

    (2) monomeric anthocyanin pigment (mg/L)= (A*MW*DF*1000)/(ε*1)

    A: Absorbance of equation (1)
    MW: Molecular weight of cyaniding-3-glucoside (= 449.2)
    DF: dilution factor (= 10)
    ε: Molar absorptivity for cyanindin-3-glucoside (= 26900)

Table S2

Modified RNA extraction protocol

(REID et al. 2006)

a. Preparation of Extraction buffer (200 ml):
    300mM Tris HCl (pH 8.0)
    25 mM EDTA

2 M NaCl
2% CTAB powder
2% PVPP; 0.05% spermidine trihydrochoride
4.5% ME ß-mercaptoethanol.
Filled up with DEPC water to 200 ml

b. The prewarmed buffer (65°C) was mixed with the deep frozen sample and incubated for 15 – 20 min at 65°C under continuous vortexing.

c. Two chloroform:isoamyl alcohol (1 vol.; 24:1) centrifugation steps at 10000 rpm for 10 min at 4°C extracted the mRNA further.

d. An additional centrifugation step at 13000 rpm for 5 min at 4°C separated the insoluble components.

e. After adding Na Acetate (0.1 vol.; 3M; 5.2 pH) and isopropanol (0.7 vol.) samples were stored at -80°C for 30 min.

f. An additional centrifugation at 10.000 rpm for 20 min at 4 °C precipitated the nucleic acids to a pellet. This pellet was washed with Ethanol (70%) and dissolved in DEPC water.

g. A DNA digest Kit (Sigma-Aldrich, St. Louis, USA) was used.

   a) Master mix was added containing RNA free DNAse (3µl), reaction buffer (5µl) and RiboLock (1µl). Samples were incubated at room temperature for 15 min.

   b) To deactivate the DNAse a stop solution (5µl) containing EDTA was added and the sample was heated up to 70°C for 10 min to additionally inactivate the DNAse thermically.

h. LiCl (0.33 vol.; 8M) was added to precipitate selectively the mRNA in an overnight step on ice.

i. A last centrifugation at 13.000 rpm for 30 min at 4 °C separated a transparent RNA pellet. The pellet was washed twice with Ethanol (70%) and dissolved in 50µl DEPC water.

Table S3

Pipette Scheme for Reverse cDNA transcription

Following the KAPA^{TM} SYBR FAST protocol (peqlab, Biotechnolgie GmbH, Erlangen, Germany)

Master Mix (µl)	
SYRB Green*	6
Forward Primer 100µM (1:10)	0.4
Reverse Primer 100µM (1:10)	0.4
Double distilled Water	3.2
cDNA Sample (1:4)	2
Total	**12**

Table 4: Master mix for qRT-PCR; KAPA^{TM} SYBR FAST (peqlab, Biotechnolgie GmbH, Erlangen,

Table S4

Primers used for quantification of gene expression levels by qRT-PCR

Gene		Gene name	Acession number	Forward sequence (5'-3')	Reverse sequence (5'-3')	R^2	Primer efficiency (PFAFFL 2006)
Reference genes							
act		**actin**	GSVIVT00034893001	TGTGCTTAGTGGTGGGTCAA	ATCTGCTGGAAGGTGCTGAG	0.999	1.00
ubi		**ubiquitin**	GSVIVT00037199001	TTGATGCAATTGGCTAGGAA	TGTAACACTGCATGCACCAA	0.999	1.00
Sugar transport proteins							
βIN		Neutral β invertase	GSVIVT01034944001	TTCATAGGCAAGCAGTCACG	TAGTCCTCTTCCCAAGCCAA	0.991	0.93
cwINV		Cell wall invertase	GSVIVT00034185001	AACCCACCAGCCTTACAGAA	CTGACCAGCAGCCATTGATA	0.990	0.91
HT1		Hexose transporter 1	GSVIVT00004559001	GAATTTCTGGTGGGGTCACGTCCAT	AGGCCACCAGCGACGAGAGA	0.997	1.00
HT3		Hexose transporter 3	GSVIVT00005628001	GCGGGCCGAAGAAGACCACTAC	CGACCCGAAAGAAGCATCGCCA	0.995	1.00
vacINV1		Vacuolar invertase 1	GSVIVT00029907001	GAGGGAAGAGGGGTGGCTCAGG	CAGGCAAACATGGCGGTAGTCCAA	0.994	0.98
vacINV2		Vacuolar invertase 2	GSVIVT00004764001	ACGCCTCACTTGTGTTTTCA	CAACGAGGTTTCCAACGG	0.996	1.00
TMT1		Tonoplast monosaccharide transporter 1	GSVIVT01013414001	GCTCCCTGAAACGGGAAACTACGC	ATGGGAAGGAGGGGGCACCA	0.997	1.00
TMT2		Tonoplast monosaccharide transporter 2	GSVIVT00019321001	GGCTGCTGCTGACGACGCTA	CGGCCCAAAGGCCATGACGA	0.999	0.98
TMT3		Tonoplast monosaccharide transporter 3	GSVIVT00019321001	GGCCTTTGGGCCGATTCCCA	CCAGCAAGGCCAACAGAAGAGAGC	0.995	0.89
Anthocyanin biosynthetic enzymes							
a) Early synthetizing enzymes							
PAL2		Phenylalanine ammonia-lyase	GSVIVT00018175001	AATCTGTCGGGTGGCCGCAAT	TGTGTTGCTGCGCTCTGGAC	0.994	0.98
CHS mix	CHS1	Chalcone synthase 1	GSVIVT00037967001				
	CHS2	Chalcone synthase 2	GSVIVT01032968001	ATGATGTACCAACAGGGCTGC	CAGCTGTGATTTCAGAGCAGAC	0.996	1.00
	CHS3	Chalcone synthase 3	GSVIVT01000521001				
CHI		Chalcone isomerase	GSVIVT00029513001	ACGCTCGCCGTCAAGTGGAA	GCGACCCGTCAAAGGCAAAATCG	0.992	1.00
F3H 1		Flavanone-3β-hydroxylase	GSVIVT01018781001	TTATCTGAGCAATGGGAGGTTCA	GCTCATCTTCCTCCTGTACATCT	0.988	0.97

Gene		Gene name	Acession number	Forward squence (5'-3')	Reverse sequence (5'-3')	R^2	Primer efficiency (PFAFFL 2006)
b) Late syntehtizing enzymes							
DFR		Dihydroflavonol 4-reductase	GSVIVT01009743001	CTGAGCAAGCTGCATGGAAG	CAGTGATCGGGGAAAGAGCA	0.998	1.00
LDOX		Leucoanthocyanidin dioxygenase	GSVIVT01019892001	ACCGTGTTAAGGTTGCTGGA	GTCCTCCCACTCAAGCTGTC	0.999	1.00
UFGT		Flavonoid-3O-glucosyltransferase	GSVIVT01024419001	CCTAAGGGACAAGGCAAGGG	CCCAACTGCCTCATGTGCTA	0.995	0.99
c) Enzymes changing anthocyanin composition							
F3'H		Flavonoid-3’-hydroxylase	GSVIVT00016217001	AGGCCGACGAGTTCAAAGAG	TCAAACCGAGCATGGAGCTT	0.997	1.00
F3'5'H		Flavonoid-3’,5’-hydroxylase	GSVIVT00007868001	GTTCAAGGACATGGTGGTGGA	TCCTATGTAAATGCTCCATCCCG	n.e.*	n.e.*
d) Competing enzymes							
FLS mix	FLS1	Flavonol synthase 1	GSVIVT01008913001				
	FLS2	Flavonol synthase 2	GSVIVT01008914001	TGCAAGCCATTGCCTTTTC	ATGAAGGGTTGTGATGGCAGG	n.e.*	n.e.*
	FLS4	Flavonol synthase 4	GSVIVT01008907001				
LAR1		Leucoanthocyanidin reductase 1	GSVIVT01011958001	TCTATTGATGGCCCGGAGGA	GGTCGGCTGCTTTTCCTCTA	0.977	1.00
LAR2		Leucoanthocyanidin reductase 2	GSVIVT01008238001	TCGCTTCATTTCCGACCTCC	TTCTTCCACGGTTACACGGG	0.997	0.99
ANR		Anthocyanidin reductase	GSVIVT01006396001	CTTGATGGGACAGGTCTGGT	TGTCTTGGAGGCAGGATAGC	0.997	0.98
e) Transcription factors							
VvMYB5b		Transcription factor MYB5b	GSVIVT01027182001	TCTTCAAAGGCAAAAGCTGAAG	TCGGTGCCACAGTTAAGGTC	n.e.*	n.e.*
VvMYBPA1		Transcription factor MYBPA1	GSVIVT01025452001	TATTGGGGTTGACGGGGTTG	TCGCTCAAGCAGTTGCAGAT	0.977	1.00
VvMYBA1/2		Transcription factors MYBA1/2	GSVIVT01022659001	TAGTCACCACTTCAAAAAGG	GAATGTGTTTGGGGTTTATC	0.997	1.00

Table 5: Primer table; used for quantification of gene expression levels by qRT-PCR. *n.e. = Primer not established

Table S5

Gene expression levels

Invertases, sugar transporters and anthocyanin biosynthetic enzymes

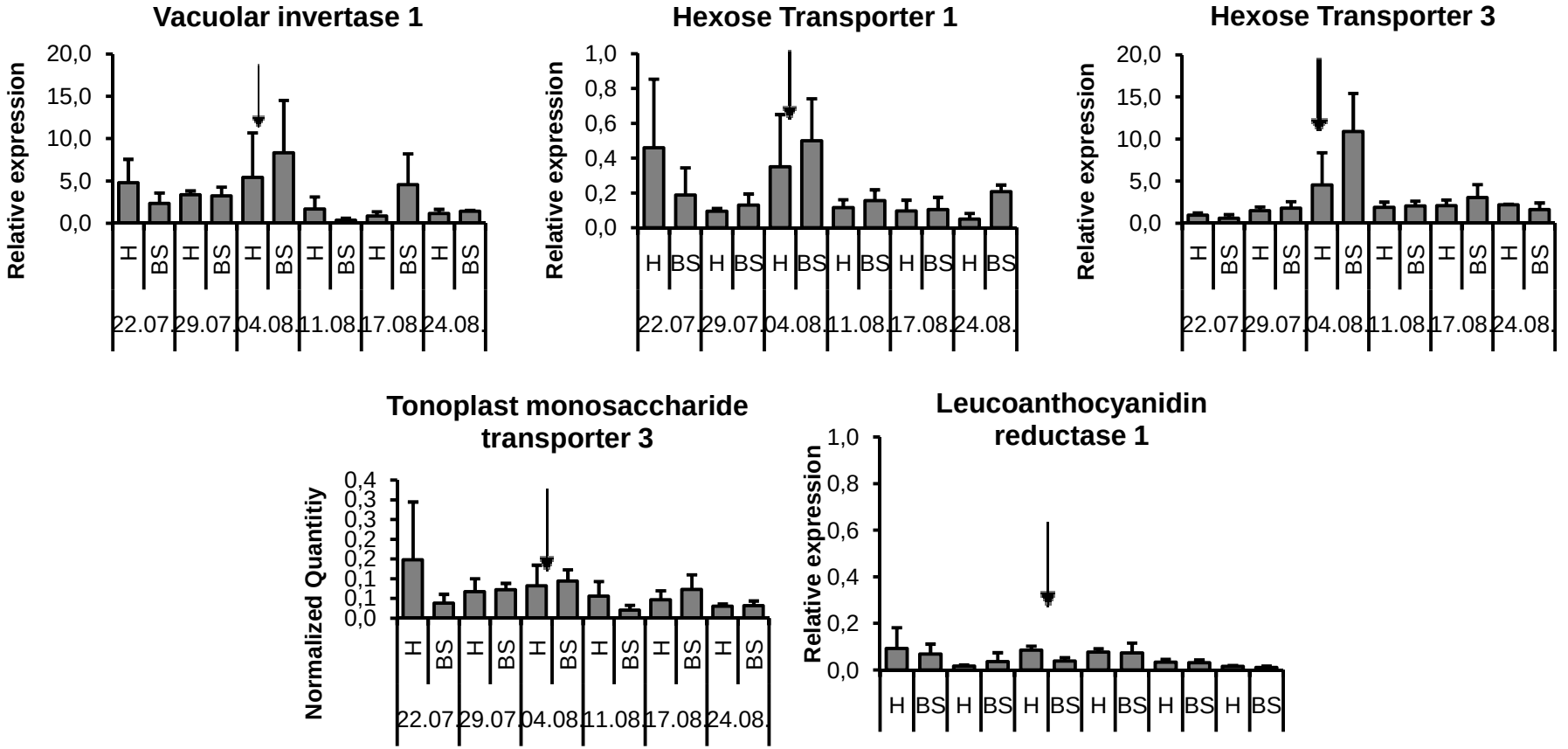

Figure 8: Relative expression of the genes vac INV1(GSVIVT00029907001), HT1 (GSVIVT00004559001), HT3 (GSVIVT00005628001), TMT3 (GSVIVT00019321001) and LAR1 (GSVIVT01011958001) as normalized quantities in healthy and BS affected grape berries. Data represent mean values and standard deviation (N=4 each) calculated according to HELLEMANNS et al. 2007. H= healthy, BS = berry shrivel. Arrow indicates veraison.

IV Summary (English)

"Analyses of Anthocyanin Biosynthesis in Healthy and Berry Shrivel berries of Zweigelt at different developmental stages" M. EITLE, M. GRIESSER, A. FORNECK

Berry shrivel (BS) becomes a serious threat for the current and for the future viticulture in Austria and all over the world. An increasing number of BS cases has been described from South Tyrol and Friuli (Italy; RAIFER cited in RIEDEL 2008), Baden (Germany; BACHTELER and RIEDEL 2011), the US (KELLER 2008) and recently China (FANG et al. 2011). *Vitis vinifera* L. cultivar Zweigelt (Blaufränkisch x St. Laurent) is the most important red wine cultivar in Austria and at the same time very susceptible against BS (KNOLL et al. 2010). Berry shrivel is assumed to be a physiological disorder, since scans for phytoplasms and viruses were negative (KRASNOW et al. 2009). For the winegrowers BS symptoms like lower sugar content of berries (KRASNOW et al. 2009; GRIESSER et al. 2012[1]), a delayed veraison and small flaccid berries (BONDADA and KELLER 2012[2]) are difficult to foresee and thought to be physiologically initiated before they are visible. Compositional changes in the berries caused by BS such as decreased sugar and anthocyanin content, acid and bitter taste (BACHTELER and RIEDEL 2011) combined with low cell viability (BONDADA and KELLER 2012[1]) limits their use for winemaking, so affected clusters have to be considered as yield loss. However the main problem is the lack of information and knowledge of winegrowers and scientists so far. Research is directed to many scientific fields, trying to find explanations, earlier symptoms or management solutions against this physiological disorder.

This thesis focuses on two important physiological processes of berry development affected by BS. The first one is the sugar accumulation in the postveraison. Source organs like mature leaves export photosynthetates (mainly sucrose) via the phloem to sink organs. Once sucrose arrived in the sink organs e.g. berries, apoplastic phloem unloading starts. Firstly sucrose is hydrolyzed to glucose and fructose by invertases in different cell compartments: Cell wall invertases are bound to cell membranes in the apoplast (HAYES et al. 2007), neutral invertases act mainly in the cytosol (NONIS et al. 2008) and vacuolar invertases cleave sucrose inside the vacuoles (ZHANG et al. 2006). Cleaved to monosaccharides, they are translocated by hexose transporters into the cytosol of the sink cells (AFOUFA-BASTIEN et al. 2010). If the monosaccharides are not used for respiration or metabolisation, they cross the tonoplast membrane mediated by tonoplast monosaccharide transporters (ÇAKIR and GIACHINO 2012) to be stored in the vacuoles of mesocarp parenchyma cells.

The first objective of the Thesis is to find changes in the expression levels of genes coding for the mentioned invertases and transporter proteins involved in the sugar transport in BS affected berries. Firstly the decreased sugar accumulation due to BS is confirmed by monitoring the total soluble solids over the berry development period 2011. Secondly the approach to determine changes in the gene expression levels consists in a RNA extraction (REID et al. 2006), cDNA transcription and normalization (PFAFFL 2006), qRT-PCR runs and a statistical evaluation (HELLEMANNS et al. 2007) of the data. The results show that the hexose transporters TMT1 and TMT2 are down regulated by BS. These novel findings are important, because they proof that BS is able to down regulate key transporter proteins involved in the

sugar transport and accumulation in grape berries. However it's important to have in mind that probably much more mechanism are modified negatively by BS and our findings are contributing factors to the overall decreased sugar accumulation.

The second focus is on the anthocyanin biosynthesis. In the beginning of berry development flavonols, then proanthocyanidins and in the end anthocyanins accumulate (CZEMMEL et al. 2012). Anthocyanin accumulation signals veraison, which is expressed by the colour change from green to red or black. The temporal shifts in the flavonoid biosynthesis are regulated and controlled on the one hand by changes in the gene expression levels of specific enzymes. Nine enzymes are directly involved in the biosynthesis of anthocyanins: PAL, C4H, 4CL, CHS, CHI, F3H, DFR, LDOX and UFGT. Two enzymes shift the anthocyanin composition: F3'H and F3'5'H. On the other hand there are enzymes competing for identical substrates: FLS, ANR and LAR (CZEMMEL et al. 2012). Recently several MYB R2R3 transcription factors: MYB5b (DELUC et al. 2008), MYBF1 (CZEMMEL et al. 2009), MYBPA1/2 (CZEMMEL et al. 2012) and MYBA1/2 (JEONG et al. 2004; KOBAYASHI et al. 2013) have been isolated and characterized for grapevine. They enhance gene expression levels and regulate the phenylpropanoid pathway.

The second objective of the thesis is to find changes in the expression levels of genes coding for the mentioned enzymes and their regulatory transcription factors involved in the anthocyanin biosynthesis in BS affected berries. The first step is the confirmation of the inhibited anthocyanin accumulation. This is done by the determination the total anthocyanins during the berry growth period 2011. The results reveal that BS down regulates two key enzymes and their respective transcription factors. The BS modifications of UFGT and MYBA1 and MYBA2 explain the delayed veraison, whereas the down regulation of CHS and MYBPA1 potentially contributes to decreased anthocyanins at harvest. However it has to be mentioned that future research should also consider other parameters like enzyme activity, the role of cofactors and accumulation of other products of the phenylpropanoid pathway e.g. proanthocyanidins and flavonols in order to fully understand the effects of BS on the anthocyanin biosynthesis.

Summarizing the thesis reveals novel findings, adds information and gives hints how berry shrivel influences two major berry development processes. Furthermore it provides general data about gene expression levels of enzymes, transporter proteins and transcription factors in grape berry tissue of the cultivar Zweigelt in Austria.

V Zusammenfassung (Deutsch)

"Analyse der Anthocyaninbiosynthese in Gesunden und von Traubenwelke befallenen Beeren von Zweigelt in verschiedenen Entwicklungsstadien" M. EITLE, M. GRIESSER, A. FORNECK

Die Traubenwelke wird immer mehr zu einer ernsthaften Bedrohung für den aktuellen und für den zukünftigen Weinbau in Österreich und der Welt. Eine wachsende Anzahl von Fällen der Traubenwelke wird aus Südtirol und dem Friaul (Italien; RAIFER zitiert in RIEDEL 2008), Baden (Deutschland; BACHTELER and RIEDEL 2011), den USA (KELLER 2008) und gerade aus China (FANG et al. 2011) gemeldet. *Vitis vinifera* L. cultivar Zweigelt (Blaufränkisch x St. Laurent) ist die bedeutendste Rotweinsorte in Österreich und gleichzeitig sehr anfällig für die Traubenwelke (KNOLL et al. 2010). Es wird angenommen dass von Traubenwelke eine physiologische Störung ist, da Untersuchungen auf Phytoplasmen und Viren negativ waren (KRASNOW et al. 2009). Für die Winzer sind die Symptome der Traubenwelke wie geminderter Endzuckergehalt (KRASNOW et al. 2009; GRIESSER et al. 2012[1]), ein verspäteter Farbumschlag und kleine welke Beeren (BONDADA and KELLER 2012[2]) schwer zu bestimmen. Darüber hinaus wird vermutet, dass die Prozesse physiologisch bereits vor den sichtbaren Symptomen, begonnen haben. Kompositionelle Veränderungen in den Beeren, die von Traubenwelke hervorgerufen werden, wie verminderter Zucker- und verringerter Anthocyangehalt, sowie saurer und bitterer Geschmack (BACHTELER and RIEDEL 2011) kombiniert mit einer geringen Lebensfähigkeit der Zellen, begrenzen die Nutzung der Beeren für die Weinbereitung. Deshalb müssen befallenen Trauben als Ertragsverlust betrachtet werden. Doch das größte Problem ist der momentane Mangel and Informationen und Kenntnis seitens der Winzer und Wissenschaftler. Die Forschung erstreckt sich in viele wissenschaftliche Bereiche und versucht Erklärungen, frühere Symptome und praktische Lösungsansätze gegen die physiologische Störung zu finden.

Diese Thesis beschränkt sich auf zwei wichtige physiologische Prozesse der Beerenentwicklung, die von der Traubenwelke betroffen sind. Der erste Prozess ist die Zuckeranreicherung in den Wachstumsstadien nach dem Farbumschlag der Beeren. Photosynthetisch aktive Organe wie ausgewachsene Blätter exportieren hauptsächlich Saccharose über das Phloem zu den Sinkorganen. Wenn die Saccharose in den Sinkorganen wie beispielsweise Beeren ankommt, beginnt das apoplastische Phloem Entladen. Zuerst wird Saccharose in verschiedenen Zellkompartimenten von Invertasen zu Glucose und Fructose hydrolisiert: Zellwandinvertasen sind an die Membranen im Apoplast gebunden (HAYES et al. 2007), Neutrale Invertasen sind hauptsächlich im Cytosol aktiv (NONIS et al. 2008) und Vakuoläre Invertasen spalten Saccharose in den Zellsafträumen (ZHANG et al. 2006). Einmal zu Monosachariden zerteilt, werden diese von Hexose Transportern in das Cytosol der Sinkzellen transportiert (AFOUFA-BASTIEN et al. 2010). Wenn die Monosaccharide dort nicht für die Zellrespiration oder den Zellstoffwechsel gebraucht werden, werden sie von Tonoplast Monosaccharid Transportern in die Zellsaftvakuolen des Parenchyms des Mesokarps eingelagert (ÇAKIR and GIACHINO 2012).
Das erste Ziel der Thesis ist Unterschiede in den Genexpressionsniveaus, welche für die erwähnten Invertasen und Proteine des Zuckertransports kodieren, in die von Traubenwelke befallenen Beeren zu finden. Als erstes wird die verringerte Zuckeranreicherung,

hervorgerufen durch dir Traubenwelke, bestätigt, indem der Gehalt an löslicher Trockensubstanz über die Beerenentwicklungsperiode 2011 überwacht wird. Als zweites besteht der Ansatz für die Bestimmung der Veränderungen der Genexpression aus einer RNA Extraktion (REID et al. 2006), einer cDNA Transkription und Normalisierung (PFAFFL 2006), mehreren qRT-PCR Durchläufen und letztendlich einer statistischen Bewertung der Daten (HELLEMANNS et al. 2007). Die Ergebnisse zeigen, dass Hexose Transporter TMT1 und TMT2 durch die Traubenwelke unterreguliert werden. Diese neuen Funde sind wichtig, weil sie nachweisen, dass die Traubenwelke Schlüsselproteine des Zuckertransports in Weintrauben beeinflusst. Es muss jedoch auch bedacht werden, dass die Traubenwelke wahrscheinlich vielmehr Mechanismen negativ modifiziert und unsere Funde lediglich beteiligte Faktoren der verringerten Zuckeranreicherung darstellen.

Der zweite fokussierte Prozess ist die Anthocyaninbiosynthese. Am Anfang der Beerenentwicklung werden Flavonole, Proanthocyanidine und am Ende Anthocyane akkumuliert (CZEMMEL et al. 2012). Anthocyananreicherung führt zur Veraison, welche durch den Farbumschlag von grün nach rot oder schwarz gekennzeichnet ist. Die zeitliche Verschiebung innerhalb der Flavonoidbiosynthese und ihre Regulierung sind auf der einen Seite von Veränderungen in der Genexpression spezifischer Enzyme kontrolliert. Neun Enzyme sind an der direkten Anthocyaninbiosynthese beteiligt: PAL, C4H, 4CL, CHS, CHI, F3H, DFR, LDOX und UFGT. Zwei Enzyme verschieben die Anthocyankomposition: F3'H and F3'5'H. Auf der anderen Seite gibt es Enzyme, die für identische Substrate konkurrieren: FLS, ANR und LAR (CZEMMEL et al. 2012). Vor kurzer Zeit wurden einige MYB R2R3 Transkriptionsfaktoren für die Weinrebe isoliert und charakterisiert: MYB5b (DELUC et al. 2008), MYBF1 (CZEMMEL et al. 2009), MYBPA1/2 (CZEMMEL et al. 2012) und MYBA1/2 (JEONG et al. 2004; KOBAYASHI et al. 2013). Diese steigern die Genexpression und regulieren den Phenylpropanoidsyntheseweg.
Das zweite Ziel der Thesis ist von Traubenwelke hervorgerufene Veränderungen der Genexpressionsniveaus zu finden, die für Enzyme und deren regulierende Transkriptionsfaktoren der Anthocyaninbiosynthese kodieren. Der erste Schritt ist die Bestätigung der gehemmten Anthocyananchreicherung. Dies ist durch die Determination der Gesamtanthocyangehalte während der Beerenwachstumsperiode 2011 geschehen. Die Ergebnisse zeigen, dass die Traubenwelke zwei Schlüsselenzyme und ihre dazugehörigen Transkriptionsfaktoren unterreguliert. Die Modifizierung von UFGT, MYBBA1 und MYBA2 erklärt den verspäteten Farbumschlag, wohingegen die Unterregulierung von CHS und MYBPA1 potentiell zum geringeren Endanthocyangehalt in der Lese beiträgt. Nichtsdestotrotz muss erwähnt werden, dass zukünftige Forschung auch andere Parameter wie die Enzymaktivität, die Rolle von enzymatischen Kofaktoren und die Anreicherung anderer Produkte des Phenylpropanoidsyntheseweges berücksichtigen sollte.

Zusammengefasst bringt die Thesis neue Erkenntnisse hervor, fügt Informationen hinzu und gibt Hinweise wie die Traubenwelke bedeutende Beerenentwicklungsprozesse beeinflussen kann. Darüber hinaus enthält sie allgemeine Daten der Genexpressionsniveaus von Enzymen, Transportproteinen und Transkriptionsfaktoren für das Weintraubengewebe der Rebsorte Zweigelt in Österreich.

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I declare under penalty of perjury that I have produced this work independently, without unauthorized assistance of third parties and without the use of any other than the specified resources. Data and concepts, acquired directly or indirectly from other sources are identified by indicating the references. This also applies to figures, graphs, illustrations and the like, as well as, to Internet sources and unpublished sources.

Wien, 03.08.2013 _____

Markus Eitle