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Developmental, molecular and genetic studies on grapevine response to temperature open breeding strategies for adaptation to warming

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Abstract

Aim: In the long term, genetic improvement is one of the major strategies to support sustainable wine production in a changing climate. Over the past 5 years, we have developed an interdisciplinary research program that aimed to: i) characterize the impact of temperature increase sensed by the entire plant or individual bunches on the development and functioning of the plant, ii) identify the physiological and molecular mechanisms regulating the response of vegetative and reproductive development to heat stress and iii) develop tools to map quantitative trait loci (QTLs) of plant and berry development in duly controlled, stable, and contrasting environmental conditions.

Methods and results: Performing high-throughput genomic analyses combined with the use of innovative experimental designs (fruiting cuttings, microvines, single berry sampling) was critical to decipher the ecophysiological and molecular mechanisms involved in the vine response to high temperature.

Conclusion: Warming promotes vegetative growth and hampers plant carbon balance, disturbing flower set and young berry development. High temperatures modify primary and secondary fruit metabolisms, desynchronizing sugar and organic acid metabolisms and delaying sugar and polyphenol accumulation during ripening. The study of day and night transcriptomic and proteomic signatures associated with heat highlighted key players of the response to temperature in the fruit.

Significance and impact of the study: Capitalizing on this knowledge, a new program is being proposed for the selection of cultivars limiting the accumulation of sugars in the berry while maintaining other qualitative compounds.

Keywords: Global warming, temperature, grapevine, biology, adaptation, genetic improvement

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Introduction

Grapevine performance, including productivity and wine quality, is highly dependent on climate. High temperatures (T°) hamper carbon assimilation beyond a 30 °C threshold (Greer and Weedon, 2012) and limit the yield by reducing the number of berries per vine (Rogiers *et al.*, 2011). The composition of the wines also depends on thermal conditions (Spayd *et al.*, 2002 ; Carbonneau *et al.*, 2015) because T° increases sugar concentration in the berry at the expense of malic acid and secondary metabolites, leading to unbalanced wines.

In Europe, wine production relies on close interactions between the variety, the environment and the viticultural practices. Climate change disrupts this balance ; hence varietal adaptation is required to maintain traditional and qualitative viticulture in these regions (Torregrosa *et al.*, 2011 ; Ollat *et al.*, 2014 and 2015). In the long term, genetic improvement is one of the best strategies to support sustainable wine production systems under global warming. Various data confirm that genetic diversity within *Vitis* may be exploited for photosynthesis adaptation to temperature. For example, photosynthesis was not reduced at T°<40 °C in response to heat stress treatments in *V. amurensis* (Luo *et al.*, 2011), and most wild species and hybrids between *V. labrusca* and *V. vinifera* also displayed a better heat tolerance than *V. vinifera* (Xu *et al.*, 2014). Recently, Hochberg *et al.* (2015) observed metabolic adaptations resulting in greater growth inhibition of leaves at 35 °C for cv. Syrah compared to Cabernet Sauvignon. Unfortunately, the lack of physiological and genetic knowledge on the mechanisms of adaptation of grapevine to T° limits the development of non-empirical breeding programs (Torregrosa *et al.*, 2011), insofar as fruit quality, rather than carbon fixation, is considered as the principal selection target. In the last years, much effort has been carried out under vineyard conditions to identify regulatory mechanisms of primary and secondary metabolisms in berries (Deluc *et al.*, 2008 ; Boss and Davies, 2009 ; Hichri *et al.*, 2011 ; Lecourieux *et al.*, 2014) and to identify transcriptomic changes induced by thermal stress applied to the berry (Pillet *et al.*, 2012) or to the whole plant (Carbonell-Bejerano *et al.*, 2013 ; Rienth *et al.*, 2014b ; Rienth *et al.*, 2016).

Despite these recent successes, we are far from a comprehensive picture of the regulatory mechanisms involved in the adaptation of the grape to heat stress. To date, there is no genetic tool or resource suitable to support breeding programs dealing with T° resilience. Over the last 5 years, we have developed a research program aiming to : i) characterize the effects of T° increase, applied at the whole plant or fruit level, on the development and functioning of the plant (organogenesis, biomass partitioning, metabolism of the berry), ii) identify the specific roles of carbon balance and molecular (transcription) mechanisms in regulating yield and quality development under high T° and iii) develop resources to study the genetic structure and stability of developmental and qualitative berry characters against thermal fluctuations.

As with other perennial plants, the biological properties of grapevine cause methodological and experimental difficulties for studying the response to environmental changes and genetic diversity. Indeed, its size, its long juvenile period and its discontinuous reproductive cycle lengthen and complicate the study of such issues. Therefore, this project relied on two alternative models : the microvine (Chaib *et al.*, 2010 ; Rienth *et al.*, 2012 ; Torregrosa *et al.*, 2016), which presents a dwarf stature and continuous fruiting, and fruiting cuttings (Mullins, 1966), which allow to get grapes in the off-season and grow them under strictly controlled conditions (Luchaire *et al.*, 2013 ; Rienth *et al.*, 2013).

This work reveals the central role of carbon balance in the plant response to thermal stress, with critical effects on the distribution of biomass within the plant and also changes in the primary (sugars, organic acids) and secondary (phenolic compounds) berry metabolism. This work also highlights day and night transcriptomic and proteomic signatures associated with heat stresses in grapes. During the program, new tools have been developed for grapevine phenotyping and breeding. The most significant were : i) a spatio-temporal analysis framework of microvine vegetative and reproductive development, ii) stable quantitative trait loci (QTLs) of development *vs* T° and iii) methodologies for RNA purification, proline quantification assays and microvine embryo rescue.

Materials and methods

1. Vine responses to T° applied at whole plant

Temperature effects on biomass partitioning between vegetative and reproductive organs and on carbon balance in microvine

Six experiments (Exp.1 to 6) were performed on two-year-old own-rooted potted microvines (line ML1, Chaïb *et al.*, 2010). Plants (6 to 12 per experiment) were thinned to one main axis and laterals were removed as soon as they appeared. Irrigation was supplied to fit maximal evapotranspiration. In Exp. 1

to 2 (2011) and Exp. 3 to 5 (2013), contrasted temperature treatments were applied over a 1- to 2-month period after the first inflorescences started to ripe. Control day/night air T° was set to 25 °C/15 °C. Contrasted T° were applied both above and below control T°, ranging from 22 °C/12 °C in Exp. 1 to 30 °C/20 °C in Exp. 2, 30 °C/15 °C in Exp. 4 and 30 °C/25 °C in Exp. 5. In Exp. 6, several couples of day/night T° from 20 °C/15 °C to 35 °C/30 °C were applied for 2 days. In all experiments, a 14-h photoperiod was imposed to reach a daily PAR (photosynthetically active radiation) close to 19.0 mol $m^{-2}d^{-1}$. The daily mean VPD (vapor pressure deficit) was maintained close to 1 kPa in Exp. 1 to 5 and close to 2 kPa in Exp. 6.

Spatial changes in biomass partitioning in above-ground organs

The number of unfolded leaves and the rank of the most apical phytomer at flowering (50 % of opened flowers) were recorded for Exp. 1 to 5 at the beginning of the experiment. Three plants were harvested at T0 for Exp. 3 to 5. Berries, leaves and internodes were then harvested and weighed (dry matter) to determine the biomass gain between T0 and final harvest.

Whole plant gas exchange measurements

Exp. 6 was dedicated to whole plant gas exchange measurement. As described in Bédiée *et al.* (2015), net photosynthesis and respiration of whole aerial organs were recorded for 24 h on 3 plants at several day/night T° couples.

Biochemical/transcriptomic berry responses

In order to study the T° effects on microvine fruit development, additional experiments were added to those described above. A first monitoring of microvine fruit development was conducted at 30 °C/20 °C (day/night T°). Biochemical and transcriptomic signatures were studied at day- and nighttime as described in Rienth *et al.* (2014a). Subsequently, short heat stress experiments were conducted at 3 fruit development stages. Plants were acclimatized for 10 days at constant day/night T° (22 °C/12 °C), then heat stressed (37 °C) for 2 h (Rienth *et al.*, 2014b). Long-term T° effects on whole berry development were studied in Exp. 1 and 2. In Exp. 3 and 5, only the *véraison* and ripening stages were studied. The green growth stage of the berry was analyzed in Exp. 4 adding an additional experiment (Exp. 7) using 20 °C/15 °C (day/night T°).

For Exp. 1 and 2, all major biochemical compounds were analyzed during the whole fruit cycle without any transcriptomic characterization. For Exp. 3, 4, 5 and 7, berry RNA was extracted as described in Rienth *et al.* (2014c) and berry transcriptome was studied using a NimbleGen microarray 090818 Vitis exp HX12, which contains 29,549 predicted genes representing 98.6 % of the 12X grapevine gene prediction version V1, and RNA-seq using Illumina paired-end sequencing (Rienth *et al.*, 2016). For stages close to *véraison*, berries were sorted based on their individual sugar and acid composition, in order to homogenize the sampling for transcriptomics.

2. Fruit responses to T° applied at bunch level

To characterize the response of grape to micro-environmental thermal stress, experiments were conducted over 3 years with fruiting cuttings of Cabernet-Sauvignon. Cuttings were grown in small pots, with clusters exposed to an elevated temperature airflow produced by fan heaters (Figure 1). The other



Figure 1 - Experimental system used to investigate the effect of heat at the bunch level.
(a) Cabernet Sauvignon fruiting cuttings were used to expose clusters to an elevated T° airflow produced by fan heaters. (b) The other plant organs were all protected from the warmed airflow by polystyrene foam deflectors.

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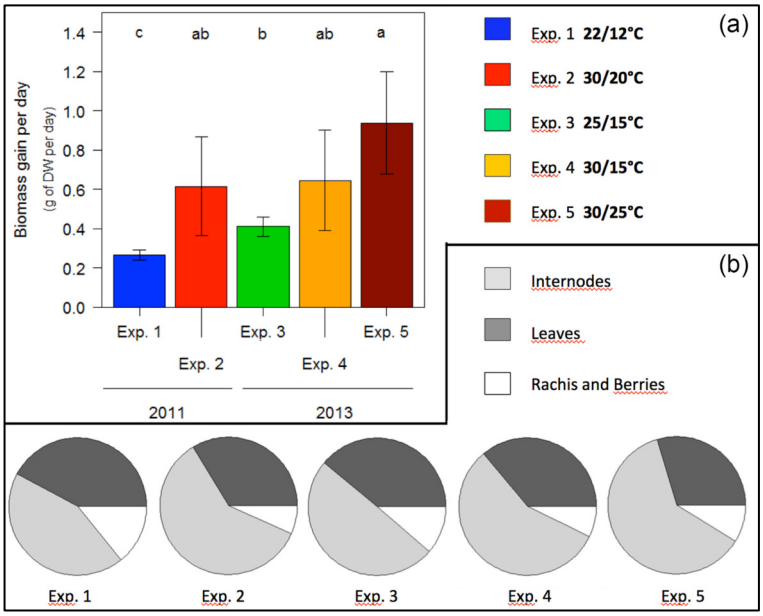


Figure 2 - Biomass gain during thermal experiments along the shoot expressed in g of DW per day (a) and in g per organ type (b). Only the phytomers that expanded during the T° treatment and the ones that had not reached the mid-flowering stage at T0 were considered. Each value is the average of 6-12 plants, depending on the experiment considered (bar corresponds to standard deviation).

plant organs were all protected from the warmed airflow by polystyrene deflectors. High T° was applied at 3 berry developmental stages (middle-green, *véraison*, and mid-ripening) and maintained daily (7:00 am to 7:00 pm) until harvesting. An average daytime fruit T° of 26 °C was set for the control and compared to a 34 °C "hot" treatment. This led to a mean T° difference of about 8 °C between heat stressed and control clusters, i. e. close to the range observed in vineyards between sun-exposed and shaded berries (Pieri and Fermaud, 2004). In order to analyze short- and long-term responses to T°, control and heat stressed berries were collected at 3 different time points (1, 7, and 14 days) and used for biochemical, transcriptomic and proteomic analyses. Transcriptomic analysis was performed using the NimbleGen microarray described above. The relative quantification of proteins was performed using a label-free LC-MS/MS-based shotgun proteomic approach.

3. Genetic mapping of developmental traits

Several crosses were performed between 2 microvine genotypes (ML1 and ML3, heterozygous *VvGAI1*/*Vvgai1* lines) and 4 *V. vinifera* cultivars (Alicante Bouschet, Muscat de Hambourg, Savagin, and Carignan). Crosses were performed to create several segregating progenies, as described in Chaïb *et al.* (2010). A mapping population of 129 microvines from the cross Picovine x Ugni Blanc *flb* (Fernandez *et al.*, 2013) was phenotyped for 43 traits in up to 9 environments (vegetative, reproductive and berry composition at both green lag phase and maturity), including an experiment in growth chambers with 2 contrasted day/night T° (30 °C/25 °C *vs* 20 °C/15 °C). This population was genotyped with a 18K SNP Illumina VeraCode chip (Le Paslier *et al.*, 2013), and 6000 informative SNPs (single-nucleotide polymorphisms) were used to build parental genetic maps with CarthaGene software (De Givry *et al.*, 2005). QTLs were detected in each environment with Rqtl (Broman *et al.*, 2003).

Results and discussion

1. At whole plant level, T° promotes biomass sequestration into the leaves and internodes at the expense of inflorescence rachis and berries

T° elevation significantly increased the above-ground biomass gain per day, specifically in vegetative organs (leaves and internodes; Figure 2a, 2b). In contrast, biomass allocation toward the rachis and the berries was reduced by high T° due to the abortion of inflorescences (data not shown).

As the phyllochron was similar for all experiments (data not shown), plants exposed to high T° developed faster on a calendar day basis. Surprisingly, leaf and internode growths as a function of growing degree days were also higher under warm T°, although they generally follow invariant patterns

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both in annual (Turc and Lecoeur, 1997; Granier and Tardieu, 1998) and perennial plants (Lebon *et al.*, 2004; Dambreville *et al.*, 2013). The inflorescence drop under warm T° likely resulted from the incapacity of the plant to accommodate for the boost in carbon demand. It was previously reported for other species that carbon acquisition is lower and biomass "diluted" when T° increases (Vasseur *et al.*, 2011). Elevated T° were also found to lower the carbon storage in woody reserves in grapevine, with negative impact on fruitfulness and berry composition (Sadras and Moran, 2013; Sadras *et al.*, 2013; Rogiers *et al.*, 2011).

2. At whole plant level, high T° negatively impact microvine daily carbon balance

Net photosynthesis was optimum between 25-30 °C and decreased beyond 30 °C (Figure 3a). Night respiration exponentially increased within the range 15-30 °C (Figure 3b). Such patterns were consistent with those generally reported for grapevine (Zufferey *et al.,* 2000; Huang *et al.*, 2005). Carbon gain thus tended to slightly decrease when T° were higher, specifically at night (Figure 3c). Berry ripening (monitored through sugar accumulation) was much more delayed under warm T° on a thermal time basis than in calendar scale (Figure 4a, 4b). Thus, in contrast with vegetative organogenesis, berry development is not linearly linked to thermal time. The impairment of sugar accumulation by high T° fits with previous observations showing that fleshly fruit berries are very sensitive to heat during ripening (Civello *et al.*, 1997; Greer and Weston, 2010).

Taken together, our results globally fit with some previous findings on grapevine response to elevated T° such as the maintenance of biomass accumulation in vegetative organs, the reduction of carbon storage toward woody reserves and the delay of ripening. Importantly, they showed that with continuous flowering and fruiting, microvine is a suitable model to study the fine tuning of the vegetative and reproductive developmental sequence under T° fluctuations.

3. Effect of T° applied at whole plant on biochemical/transcriptomic berry responses

The microvine made it possible, for the first time, to characterize fleshy fruit development simultaneously during day and night under completely controlled conditions using whole genome microarrays. Up to this point, circadian cycles had only been studied on non-perennial model plants such as Arabidopsis (Schaffer *et al.*, 2001). For 2 green and ripening stages, gene expression changes occurring along development as well as between day/night were

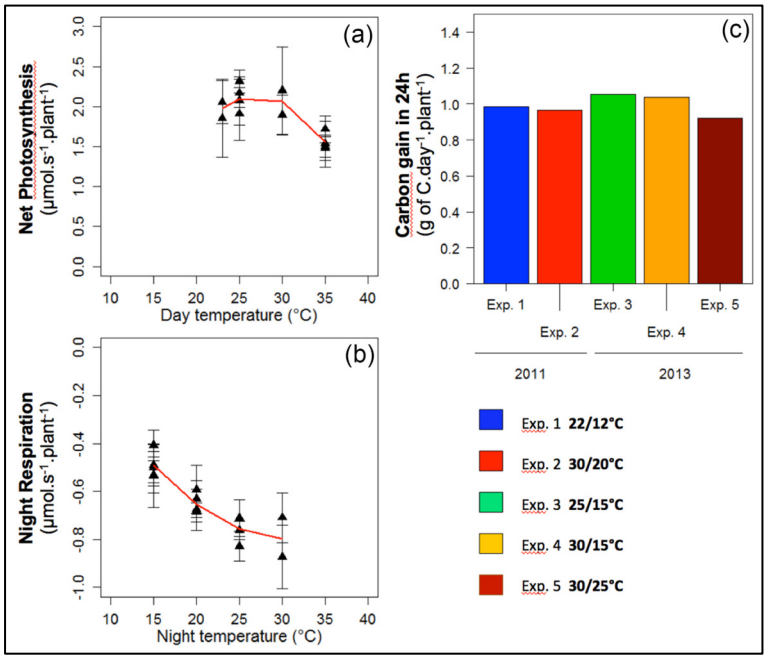


Figure 3 - Microvine whole plant (aerial organs only) net photosynthesis (a) and night respiration (b) responses to day T° and night T°, respectively. Each point is the average of 3 plants during one complete day or night measurement. Data were obtained during Exp. 6. Carbon gain over 24 h was calculated for the Exp. 1 to 5 thermal conditions (c). Carbon gain was estimated from photosynthesis and respiration response to T° fitting curves on (a) and (b).

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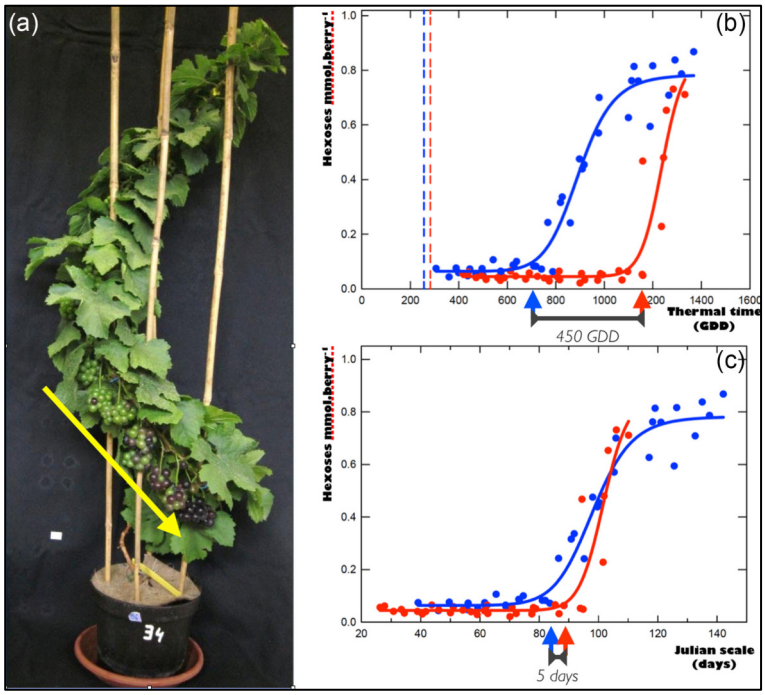


Figure 4 - Effect of +8 °C on the accumulation of sugars in the microvine berry. (a) A ML1 microvine plant during the experiment.
Organs were spatially sampled and spatial positions converted using the phyllochron and the sum of T° above +10 °C. (b) The accumulation of sugars as a GDD (Growing degree days) function. (c). The accumulation of sugars as a calendar function.

studied. All day-detected developmentally regulated transcripts were also modulated at night, whereas, surprisingly, 1,843 supplementary genes displayed night-specific developmental regulation. Hierarchical clustering of those transcripts revealed a similar regulation pattern at day and night with secondary metabolism being more distinctly regulated during night development. A diurnal modulation throughout all stages could only be detected for 9 transcripts, indicating that circadian regulation in the berry has a high stage specificity. Regarding functional categories, mainly cellular organization and photosynthesis were day-upregulated in green berries, whereas secondary metabolism and stress-related genes were night-upregulated in ripening berries.

Short T° stresses confirmed that gene modulation could vary to a large extent according to sampling time. Application of a short stress at 3 different stages at day and night yielded a total of 5,653 modulated transcripts (two-fold change, $p<0.05$). Responsive pathways differed according to time and stage. A clear distinction of ripening stages by single berry selection led to stage-specific detection of malic acid metabolism-related transcripts displaying heat activation, whereas anthocyanin regulatory genes were repressed. Although stress application lasted only 2 h, a heat-induced delay in ripening and sugar accumulation could already be observed at *véraison* at a transcriptomic level. The heat activation of several candidate genes controlling the responses to T° in the berry, *VvGols1* (Galactinol Synthase 1) and *VvHsfA2* (Heat stress factor A2), could be confirmed (Pillet *et al.*, 2012). One complementary Heat Shock transcription Factor (HSF), *VvMbf1c*, previously described in Arabidopsis (Suzuki *et al.*, 2011), could also be identified in the grapevine berry.

In a series of long-term T° treatments using day and night sampling of single berries corresponding to green and ripening stages, a total of 674 millions sequenced reads yielded 10,788 differentially expressed transcripts in response to T°. Green berry development accelerated as evidenced by higher rates of organic acid accumulation and berry growth under higher T°. This could be confirmed when considering the transcriptional signatures of genes related to cell expansion. Transcripts related to tannin synthesis (CHS, PAL) and galloylation were found to be repressed by high T°. Surprisingly, the onset of malate breakdown was delayed to mid-ripening when plants were grown under cold conditions. This observation suggests that malate breakdown is not an

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intrinsic part of the *véraison* program. Whole plant carbon balance could be determinant for the trigger of malate breakdown, which was considered to be non-plastic. Several ATPases and malate transporters displayed development and T° dependent expression patterns, besides less marked but significant regulation of other genes in the malate pathway.

Heat responsive genes (HSPs, HSFs) detected in the short heat stress studies were also found modulated by long-term treatments. However, several of them showed a decreased T° response indicating their role in short-term adaptation to high T°. By contrast, other transcripts maintained a high expression level even during night sampling, when night T° was kept stable besides various T° conditions.

The single berry biochemistry analysis made prior to RNA extraction not only made it possible to reduce biases in transcriptomic results (Carbonell-Bejerano *et al.*, 2016), but also highlighted that the ripening program of single berries can be faster than for whole bunches.

4. At bunch level, T° also dramatically changes the developmental program of the berry

With fruiting cutting experiments, metabolites (sugars, acids, phenolic compounds, amino acids) were quantified. Critical changes were observed for malic acid, anthocyanins, flavonols and some amino acids including GABA. We also noticed that accumulation of sugars and phenolic compounds was postponed by 2 to 3 weeks by heat stress applied at green stage. Transcriptomic analyses identified more than 7,500 transcripts showing differential expression under heat stress. However, most of these responses were found to be stage-specific and only 38 genes exhibited the same deregulation across all conditions.

To understand the biological significance of the differentially expressed genes, a Genome Ontology (GO) category enrichment analysis was performed and revealed both similar and different T° effects according to the developmental stage and the stress kinetics. Several significantly affected functional categories were identified, among which "abiotic-heat stress", "secondary metabolism", "transport", and "signaling". Interestingly, the category "RNA-regulation of transcription" is also highlighted through many heat responsive genes encoding putative transcription factors or epigenetic regulators. Proteomic analyses identified around 2,000 non-redundant proteins. T° led to significant remodeling of the berry proteome with up to 556 deregulated proteins. However, these responses depended on both developmental stage and stress length. The GO category enrichment analyses indicated that the most affected processes belong to stress responses, protein metabolism, and primary and secondary metabolism. Interestingly, less than 20 % of these heat-deregulated proteins were also modulated at the transcriptional level. Taken together, these omics data contribute to explain the dramatic changes in metabolite contents observed in heat stressed berries and highlight the intrinsic capacity of this fleshly fruit to perceive heat stress and to build adaptive responses.

We initiated the functional characterization of some major candidates. Both *VvGols1* and *VvHsfA2* were found upregulated at the transcriptional level in berries under heat stress. *VvGols1* expression profile correlated positively with galactinol accumulation in heat stressed berries. Heterologous expression of *VvGols1* in *E. coli* showed that it encodes a functional galactinol synthase. Transient expression assays showed that the heat stress factor *VvHsfA2* transactivates the promoter of *VvGols1* in a heat stress dependent manner. The results also suggest that galactinol may play a signaling role in these responses (Pillet *et al.*, 2012). To extend this study, several transgenic grapevine material (microvine, hairy roots and embryonic cells) over- or under-expressing *VvHsfA2* and *VvGols1* were produced. The characterization of these transgenic lines is under progress (phenotyping, heat sensitivity, transcriptomic and metabolic modifications). The functional characterization of other heat stress responsive genes that potentially act as transcription factors (TF) or epigenetic regulators was also initiated.

5. Identification and genetic mapping of developmental traits

All traits phenotyped segregated in the Picovigne x Ugni Blanc *flb* population (Figure 5). New correlations were found between malate and total sugars at green lag phase, and between internode length and leaf area (Houel *et al.*, 2015). Broad-sense heritability was unexpectedly lower than 0.40, except for traits related to berries and acids. Dense and reliable genetic maps were built for both parents, with low inter-marker distances. Fourteen QTLs were detected in at least two environments, among which a novel QTL for berry size on chromosome 7, explaining up to 44 % of total variance. This QTL was co-localized with QTLs for the number of berries, clusters and seeds, as well as with QTLs for major berry acids at green lag phase. The dwarf stature of the microvine allowed a fine phenotyping for many traits in a T° range wider than in previously published studies. Quantifying acids at green lag phase allowed us to find the first berry acidity QTLs

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in a *Vitis vinifera* intra-specific cross, and particularly the first QTLs for tartaric acid. These QTLs were found under several environments and then offer opportunities for breeding.

Conclusions

1. On the development of new methods to study the abiotic stress effect

A robust analytical framework was developed to characterize the phenotype of the microvine, especially for inferring temporal data from spatial data (Torregrosa *et al.*, 2016). Thus, it becomes possible to reduce the calendar duration of the experiments to a few days or weeks and to study simultaneously various stages of reproductive development (Rienth *et al.*, 2012; Luchaire *et al.*, 2015).

2. On the central role of carbon in the response to T° fluctuation

Data revealed a relationship between the lower carbon balance within the plant and the abortion of reproductive organs (Luchaire, 2015). In calendar time, while berry development remains stable, T° boosts vegetative organogenesis disturbing source/sink balance. As a consequence, the differential response of reproductive *vs* vegetative system occurs at the expense of berry biomass allocation. Then, T° can change malic acid metabolism in relation to the accumulation of sugar (Rienth *et al.*, 2016, Romieu *et al.*, 2016) by acting on the energy status of the plant. This observation reveals a hitherto unsuspected phenomenon because it was considered a dogma for the vine berry that the decrease in malic acid was triggered at the start of sugar unloading (Carbonneau *et al.*, 2015). These observations provide a new perspective on the capacity of a plant, subjected to limiting carbon assimilation conditions and/or to changes in biomass allocation, to support fruit energy supply. However, these data acquired with the microvine should be validated on other genotypes.

3. On the identification of specific regulatory elements controlling fruit response to heat

Elevated T° applied at the level of the fruit or the entire plant delayed fruit ripening, including sugar and secondary metabolite accumulation. We have found specific regulatory mechanisms that are independent of the carbon status of the plant, showing the existence of a direct impact of T° on fruit behavior. Thus, several regulators, metabolic elements (e.g. *VvGols1*) or transcription factors

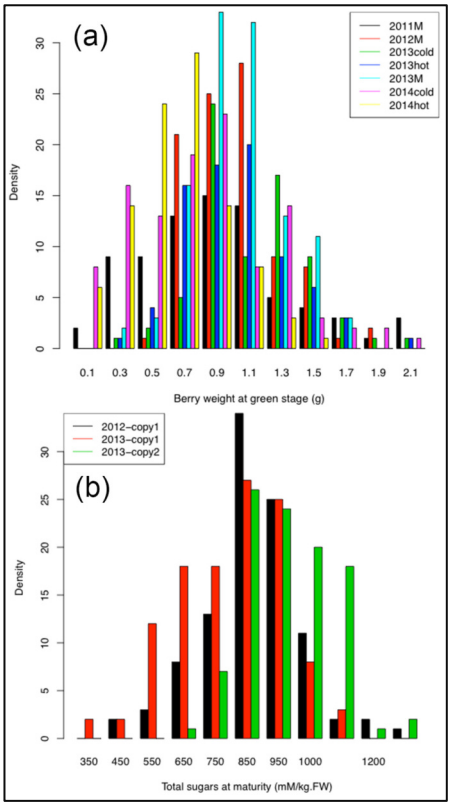


Figure 5 - (a) Berry weight and (b) sugar content individual distributions within the Picovigne x Ugni Blanc *flb* progeny.
M: mean value over two copies. T° in growth room: cool: day/night 20 °C/15 °C; hot: day/night 30 °C/25 °C.

(*VvMbf1* or *VvHsfA2*) were highlighted, opening new avenues for research.

4. On the identification of genetic determinants of adaptation to T°

In this study, 14 QTLs showed a stable behavior with respect to environment fluctuations (Houel *et al.*, 2015). These regions include genetic traits controlling vegetative (e.g. leaf area) and reproductive (e.g. size and acidity of the berry) traits. These resources open new perspectives for future breeding programs. During our program, we have identified interesting phenotypes that could mitigate some negative effects of heat on berry development. Based on these resources, a new program has started to identify QTLs limiting sugar accumulation in the berry, as a major step to select cultivars more suitable for hot conditions (Figure 6).

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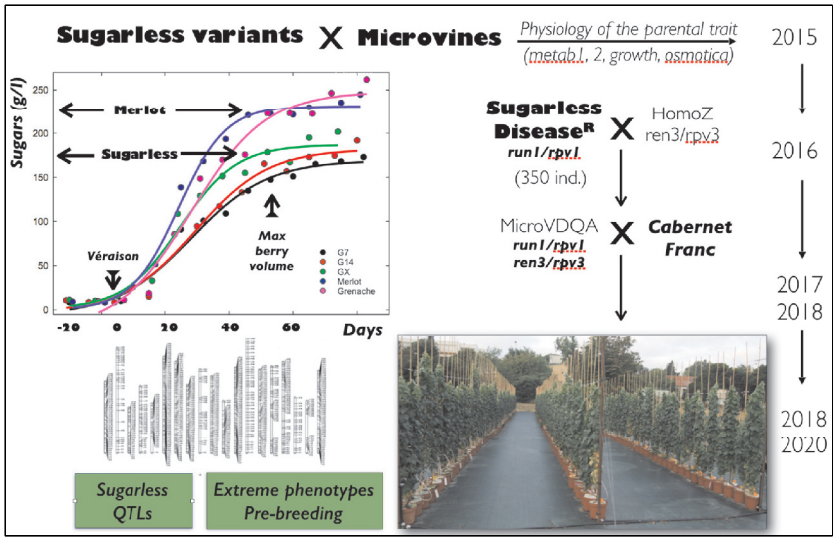


Figure 6 - Scheme of a genetic introgression to combine traits of berry adaptation to warming and plant tolerance to diseases using the microvine system.

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