Changes in Carbohydrate Metabolism in *Plasmopara viticola*-Infected Grapevine Leaves

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The oomycete *Plasmopara viticola* is responsible for downy mildew, a severe grapevine disease. In infected grapevine leaves, we have observed an abnormal starch accumulation at the end of the dark period, suggesting modifications in starch metabolism. Therefore, several complementary approaches, including transcriptomic analyses, measurements of enzyme activities, and sugar quantification, were performed in order to investigate and to understand the effects of *P. viticola* infection on leaf starch and—to a larger extent—carbohydrate metabolism. Our results indicate that starch accumulation is associated with an increase in ADP-glucose pyrophosphorylase (AGPase) activity and modifications in the starch degradation pathway, especially an increased α -amylase activity. Together with these alterations in starch metabolism, we have observed an accumulation of hexoses, an increase in invertase activity, and a reduction of photosynthesis, indicating a source-to-sink transition in infected leaf tissue. Additionally, we have measured an accumulation of the disaccharide trehalose correlated to an increased trehalase gene expression and enzyme activity. Altogether, these results highlight a dramatic alteration of carbohydrate metabolism correlated with later stages of *P. viticola* development in leaves.

Plasmopara viticola is the causal agent for grapevine downy mildew, a severe disease which affects harvest quality and quantity. This disease remains problematic because its control requires frequent fungicide applications in wet weather. *P. viticola* is an obligate biotrophic oomycete which needs living tissue for nutrient uptake, growth, and reproduction. It uses stomata to penetrate into leaves, develops an intercellular mycelium with haustoria for nutrient uptake from mesophyll cells, and subsequently reaches other stomata for sporulation. The disease is characterized by the occurrence of typical “oil-spot symptoms” on the upper side of the leaf. In infected leaves, we

have observed an abnormal starch accumulation at the end of the dark period, suggesting modifications in the carbohydrate metabolism.

Recently, the effects of pathogen infection on plant primary metabolism have aroused new interest (Berger et al. 2007). Plants need photoassimilates to fuel the activation of defense reactions and to mediate physiological adaptations (Bolton 2009) whereas pathogens attempt to manipulate the plant to use carbohydrates for their development. Both compete for sugars, resulting in modifications of photoassimilate production and partitioning within host tissues (Abood and Lösel 2003; Hall and Williams 2000; Scholes et al. 1994).

Plants produce carbohydrates in photosynthetic source organs and export them toward sugar-importing sink organs. During the day, a part of the sugars is retained in leaf chloroplasts and metabolized in transitory starch, which is degraded during the dark period to support leaf metabolism and sucrose export. Starch, the major form of plant carbon storage, is mainly composed of branched amylopectin and also the nonbranched amylose. Both molecules are polymers of α -1,4- and α -1,6-linked glucose units. Starch synthesis involves ADP-glucose pyrophosphorylase (AGPase) (EC 2.7.7.27), starch synthases (EC 2.4.1.21), and branching enzymes (EC 2.4.1.25 3.2.1.33) (Zeeman et al. 2007). The knowledge of starch breakdown mechanisms in plastids remains incomplete. It is proposed that phosphorylation by glucan, water dikinase (EC 2.7.9.4), and phosphoglucan water dikinase (PWD) (EC 2.7.9.4) is required to allow the subsequent degradation by β -amylases (EC 3.2.1.2) (Lloyd et al. 2005; Scheidig et al. 2002; Smith et al. 2005). The role of α -amylases (EC 3.2.1.1) in leaf starch degradation remains uncertain (Lloyd et al. 2005).

Plant sugar concentrations alter in response to diurnal changes and biotic and abiotic stresses (Rolland et al. 2006). In addition to their role as resources for respiration, metabolic intermediates, and storage components, sugars are signaling molecules and can impact gene expression and enzyme activity, especially those related to carbohydrate metabolism and photosynthesis (Koch 2004; Rolland et al. 2002; Smeekens 2000). For example, glucose or sucrose repress the expression of genes encoding the small subunit of ribulose biphosphate carboxylase (EC 4.1.1.39, Rubisco), the chlorophyll a/b binding protein, and α -amylase (Gibson 2005; Krapp et al. 1993), whereas both induce the expression of the gene encoding ex-

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tracellular invertase (EC 3.2.1.26) (Roitsch 1999; Sheen et al. 1999). In *Arabidopsis*, trehalose activates the transcription of AGPase genes (Wingler et al. 2000). Sugars also regulate enzyme activities. For example, extracellular invertase activity is induced by glucose and sucrose in *Chenopodium rubrum* (Roitsch et al. 1995) and by sucrose in tomato (Godt and Roitsch 1997; Sinha et al. 2002).

Invertases are key enzymes for carbohydrate allocation because they catalyze the irreversible cleavage of sucrose, the major form of translocated sugars in plants, into glucose and fructose. Plants contain several isoforms of invertases that differ by their subcellular localization, pH optimum, and isoelectric point (Godt and Roitsch 1997). Soluble intracellular invertases (vacuolar acidic and cytoplasmic alkaline) can be distinguished from acidic apoplastic or cell-wall invertases. The cell-wall invertase activity is an important determinant of local sink strength (Sturm 1999). Invertases are not plant specific and are also present in bacteria and fungi such as *Aspergillus* spp. or *Uromyces fabae* (Voegelé et al. 2006). An increase in invertase activity is a common feature of plant–pathogen interactions (Fotopoulos et al. 2003; Roitsch et al. 2003; Sutton et al. 2007; Voegelé et al. 2006). In compatible plant–biotrophic microorganism interactions, it is associated with a reduction in the rate of net photosynthesis (Chou et al. 2000; Tang et al. 1996) and an accumulation of hexose sugars at the site of infection, resulting in a local shift of source to sink (Scholes et al. 1994). In grapevine leaves infected by powdery and downy

mildew, Brem and associates (1986) have described an increase of acid invertase activity and, more recently, Hayes and associates (2010) have reported changes in the expression of apoplastic invertase and hexose transporter genes.

Whereas grapevine–*P. viticola* interaction has already been described in a context of genetic or induced resistance (Dai 1995; Díez-Navajas et al. 2008; Trouvelot et al. 2008), to our knowledge, neither a deregulation of starch metabolism nor a general view of the effect of the infection on carbohydrate metabolism was reported until now. Therefore, in this study, several complementary approaches, including transcriptomic analyses, measurements of enzyme activities, and sugar quantification, were used in order to understand the mechanisms responsible for starch abnormal accumulation and to investigate the effect of *P. viticola* infection on the leaf carbohydrate metabolism.

RESULTS

P. viticola development induces abnormal starch accumulation.

P. viticola development was monitored by analysis of the ratio of pathogen/leaf DNA content (coefficient of infection) and visualized by microscopy after aniline blue staining (Fig. 1). At 1 day postinoculation (dpi), zoospores have reached stomata, encysted, and initiated germination. The oomycete DNA was detectable from 3 dpi, when hyphae have developed and

Fig. 1. Monitoring of *Plasmopara viticola* development in *Vitis vinifera* 'Marselan' leaves by UV epifluorescence after aniline blue staining and DNA quantification at different times postinoculation. Images present the different stages of development. Infection coefficient representing the ratio between pathogen DNA (*PvACT*) and host DNA (*VvHT5*) quantified by real-time polymerase chain reaction. Leaf samples were collected during the light period (L) and at the end of the dark period (D) over a time course of infection (1 to 7 days postinoculation [dpi]). At 1 dpi, zoospores have reached stomata, encysted, and initiated germination. At 3 dpi, developing hyphae have formed haustoria in contact with the host mesophyll cells. At 5 dpi, the pathogen has highly colonized the host tissues and, at 7 dpi, it is present in the substomatal cavities to prepare sporulation. Scale bars correspond to 10 μ m. Images are representative of three biological replicates. Data correspond to the average of four independent biological replicates $\pm$ standard deviation. Means with different letters (a, b, c) are significantly different ($P < 0.05$).

formed haustoria in mesophyll cells. The coefficient of infection increased significantly from 5 dpi concomitantly to extensive leaf colonization and to the occurrence of the “oil-spot” symptoms. It reached a maximum when symptoms were spread over most of the leaf surface and the oomycete was ready to initiate sporangiophores (7 dpi).

Lugol staining was used to visualize starch in control and *P. viticola*-infected leaves when the coefficient of infection reached the maximum (7 dpi). At the end of the dark period, control leaves showed no starch (Fig. 2A) whereas, in infected leaves, starch was co-localized with the oil-spot symptoms (Fig. 2B). During the light period, starch was evenly distributed in control leaves whereas it seemed preferentially localized in oil spots of infected leaves (data not shown).

Ultrathin cuts of control and inoculated (6 dpi) leaves, collected at the end of the dark period, were observed using transmission electron microscopy. In the spongy parenchyma cells of control leaves, some chloroplasts seemed to contain only small starch amounts, as indicated by the presence of few and relatively small granules (Fig. 2C). In infected leaves, however, chloroplasts contained voluminous starch granules but the integrity of such chloroplasts seemed unaltered (Fig. 2D).

The starch content was determined in leaf extracts by the measure of glucose units released after enzymatic digestion. For leaf extracts sampled during the light period, glucose at approximately 13 mg/g fresh weight (FW) was detected and no significant differences between control and *P. viticola*-inoculated plants were observed (ratio of approximately 1) (Fig. 3). For samples collected at the end of the dark period, glucose

at approximately 0.8 mg/g FW was measured in control plants. It was not significantly different from the values obtained in infected plants at 1 to 5 dpi. At 7 dpi, however, the release of glucose was more than sixfold higher in extracts of infected leaves, indicating a higher starch concentration (Fig. 3).

Fig. 3. Ratio of the starch content of *Plasmopara viticola*-infected versus control grapevine leaves over a time course of infection (1 to 7 days postinoculation [dpi]) in samples collected at the end of the dark period (D) or during the light period (L). Values correspond to the measurement of glucose released after enzymatic degradation of starch. Data correspond to the average of three independent biological replicates $\pm$ standard deviation. Means with different letters (a, b) are significantly different ($P < 0.05$).

Fig. 2. Starch visualization in control and *Plasmopara viticola*-infected grapevine leaves at the end of the dark period. **A** and **B**, Left part of each image corresponds to a photograph of **A**, control and **B**, *P. viticola*-infected leaves at 7 days postinoculation (dpi). Right part corresponds to the same leaves after clarification and lugol staining. The inset image shows a detail of “oil-spot” symptoms. **C** and **D**, Transmission electron micrographs of spongy parenchyma cells of **C**, control and **D**, infected leaves at 7 dpi. H: haustorium, Pv: *P. viticola* hyphae, C: chloroplast, S: starch granule. Scale bar corresponds to 1 μ m.

Modifications of carbohydrate metabolism- and photosynthesis-related gene expression revealed by microarray profiles.

A microarray analysis of control and *P. viticola*-infected grapevine leaves (7 dpi, end of the dark period) revealed that 3,100 of the nearly 30,000 genes studied were affected by infection. Among the regulated genes, 5.4% were annotated as

related to carbohydrate metabolism, including genes involved in starch and soluble sugar metabolism, and 0.6% was connected to photosynthesis (Supplementary Fig. S1, Supplementary Table S1). Gene expression of AGPase, soluble starch synthase, PWD, β -amylase, sucrose phosphate synthase, trehalose phosphate synthase (TPS), Rubisco large subunit, chlorophyll synthase, and chlorophyll a/b binding proteins were repressed,

Table 1. Transcriptional changes for genes involved in carbohydrate metabolism and photosynthesis associated to *Plasmopara viticola* infection

NCBI reference sequence	Annotation	Ratio of gene expression	P value
XM_002277244.1	PSI type II chlorophyll a/b-binding protein	0.20	0.045
XM_002285596.1	α , α -Trehalose-phosphate synthase	0.23	0.015
XM_002271362.1	Sucrose-phosphate synthase	0.25	0.017
AM424156.2	Phosphoglucan, water dikinase	0.25	0.050
XM_002267433.1	Ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit, chloroplastic	0.31	0.027
XM_002281033.1	Glucose-1-phosphate adenylyltransferase (large subunit 1) (AGPase)	0.32	0.032
XM_002277336.1	Soluble starch synthase	0.32	0.032
XM_002263235.1	Chlorophyll synthase, chloroplastic	0.46	0.027
XM_002265662.1	β -Amylase	0.47	0.027
XM_002263662.1	Trehalase	2.89	0.019
XM_002279752.1	β -Bructofuranosidase (invertase)	3.02	0.039
XM_002271938.1	Chlorophyllase-1	9.98	0.009
XM_002282148.1	α -Amylase	13.53	0.044

^a NCBI = National Center for Biotechnology Information.

Fig. 4. Relative expression of genes encoding **A**, ADP-glucose pyrophosphorylase (AGPase); **B**, soluble starch synthase; **C**, β -amylase; **D**, α -amylase; **E**, sucrose phosphate synthase; and **F**, cell wall invertase analyzed by quantitative reverse-transcription polymerase chain reaction. Leaf samples were collected during the light period (L) and at the end of the dark period (D) during a time course of grapevine leaf infection by *Plasmopara viticola* (1 to 7 days postinoculation [dpi]). Expression levels obtained for infected leaves were normalized with respect to *VATP16* and *60SRP* expression and related to the corresponding values of control leaves. Data correspond to the average of three independent biological replicates $\pm$ standard deviation.

while genes encoding α -amylase, invertase, trehalase, and chlorophyllase showed induced expression (Table 1).

Alteration of starch metabolism.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis was performed to validate the variations observed in the microarray profile for a set of genes encoding key enzymes of starch metabolism and to analyze these variations throughout the infection time course. Genes encoding AGPase and soluble starch synthase were both repressed at the end of the infection. The AGPase gene expression was repressed at 7 dpi during both the dark and light periods (Fig. 4A), whereas that of soluble starch synthase was repressed from 5 dpi (Fig. 4B). Whereas the β -amylase gene expression varied slightly over the course of infection and was repressed at 7 dpi during the light period (Fig. 4C), that of α -amylase was clearly induced from 5 dpi, with a maximal activation at 7 dpi at light (Fig. 4D).

AGPase and β - and α -amylase activities were measured in control and infected leaves (7 dpi). The AGPase activity was more than sixfold higher in infected than in control leaves for both sampling periods (Fig. 5A). In control plants, the β -amylase activity was more than three times higher in the dark than during the light period. In infected plants, this activity was simi-

Fig. 5. Activity of **A**, ADP-glucose pyrophosphorylase (AGPase); **B**, β -amylase; and **C**, α -amylase in control and *Plasmopara viticola*-infected grapevine leaves (7 days postinoculation [dpi]). Leaves were collected during the light period (L) and at the end of the dark period (D). Data correspond to the average of three biological replicates $\pm$ standard deviation. Means with different letters (a, b) are significantly different ($P < 0.05$).

lar in the dark and light periods and corresponded to the low value measured for control samples of the light period (Fig. 5B). α -Amylase activity was more than threefold higher in infected than in control leaves for both sampling periods (Fig. 5C).

Alteration of soluble sugar.

To verify whether infection leads to a global deregulation of the sugar metabolism, ^{13}C -spectroscopy was used to draw up and compare the profiles of soluble metabolites in control and *P. viticola*-infected leaves (6 dpi, end of the dark period). ^{13}C -nuclear magnetic resonance (NMR) spectra showed that fructose and glucose seemed more abundant and sucrose less present in infected compared with control leaves (Supplementary Fig. S2). Malic acid was the most abundant Krebs intermediate, and tartaric acid appeared particularly abundant in both control and infected leaves.

Next, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was used to quantify glucose, fructose, and sucrose in samples over the course of infection. At the end of the dark period, glucose, fructose, and sucrose at approximately 9, 6, and 13 mg/g dry weight (DW), respectively, were detected in control leaves. No significant difference could be observed between control and infected leaves before 7 dpi (Fig. 6A). At 7 dpi, however, glucose and fructose concentrations increased significantly in infected leaves, while the sucrose level remained similar to that of control plants (Fig. 6A). For control leaves collected during the light period, the sugar concentrations varied for glucose, fructose, and sucrose at approximately 11, 8.5, and 30

Fig. 6. Ratios of glucose, fructose, and sucrose concentrations of *Plasmopara viticola*-infected versus control grapevine leaves. Samples were collected from 1 to 7 days postinoculation (dpi) **A**, at the end of the dark period or **B**, during the light period and analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection. Data correspond to the average of three independent biological repeats $\pm$ standard deviation. Means with different letters (a, b) are significantly different ($P < 0.05$).

mg/g DW, respectively. The concentrations were quite similar in infected leaves until 7 dpi, whereas sucrose concentration showed a 1.8-fold decrease in infected leaves compared with control leaves (Fig. 6B).

To further investigate the origin of the variations of the sugar content, the expression of genes encoding sucrose phosphate synthase and cell wall invertase, two enzymes implicated in the synthesis and degradation of sucrose, respectively, were followed using qRT-PCR analysis. The gene encoding sucrose phosphate synthase was repressed at 5 dpi at the end of the dark period and at 7 dpi during both the light and dark periods (Fig. 4E). However, that encoding cell wall invertase was clearly induced from 5 dpi during both periods (Fig. 4F). These data fit well with those obtained in the microarray analysis (Table 1).

Finally, invertase activity was assessed in the insoluble fraction of leaf extracts (Fig. 7). At pH 4.5, optimum for cell wall invertases (Avigad 1982), no difference in activity could be measured between control and infected leaves. At pH 7.5, however, a strong increase of invertase activity was observed in infected leaves from 5 dpi, independently of the sampling period. The invertase activities were also assessed in the soluble fraction of the leaf extracts but no significant difference was shown, whatever the pH conditions (data not shown).

Trehalose accumulation.

HPAEC-PAD also allowed trehalose analysis. It was only present in trace amounts in control leaves. In infected leaves, its concentration increased from 5 dpi to reach 2.41 ± 1.1 mg/g DW and 5.16 ± 4.07 mg/g DW at 7 dpi in samples collected in the dark and light periods, respectively.

The microarray analysis showed that the expression of the gene encoding TPS was repressed in infected leaves (Table 1), whereas that encoding trehalose 6-phosphate phosphatase was not differentially regulated (data not shown). The trehalase gene expression was induced from 5 dpi (Table 1; Fig. 8A). Trehalase activity was more than 10 times higher in infected leaves (7 dpi) for both sampling periods (Fig. 8B).

Decrease in the photosynthetic efficiency and in chlorophyll levels.

In infected leaves, Fv/Fm, the maximum efficiency of photosystem II (PSII), was reduced from 5 dpi in the infected area (Fig. 9A). Fv/Fm, the effective PSII quantum yield (Φ_{PSII}), and

Fig. 7. Invertase activity measured at pH 4.5 and 7.5. Ratios of enzyme activity in the insoluble fraction of leaf extracts from control versus *Plasmopara viticola*-infected grapevine samples collected at the end of the dark period (D) and during the light period (L). Data correspond to the average of three independent biological replicates $\pm$ standard deviation. Means with different letters (a, b, c) are significantly different ($P < 0.05$).

photochemical and nonphotochemical quenching (q_P and q_N) were determined in the oil spots and adjacent areas of infected leaves (7 dpi) and in control leaves (Fig. 9B). The Fv/Fm was reduced to 0.63 ± 0.06 in the oil spots, compared with 0.76 ± 0.02 in control leaves. It was accompanied by a decrease of Φ_{PSII} from 0.34 ± 0.03 to 0.19 ± 0.04 and of q_P from 0.63 ± 0.03 to 0.54 ± 0.08 . Meanwhile, the q_N value increased from 0.77 ± 0.04 to 0.84 ± 0.03 . The measured parameters showed no significant difference between noninfected control leaves and green regions showing no symptoms (adjacent to oil spots) in infected leaves (Fig. 9B).

In addition, the chlorophyll content was determined in control and infected leaves at 7 dpi. In control leaves, the total chlorophyll concentration was 24.8 ± 1.8 mg/g FW, and it was reduced to 17.3 ± 0.8 mg/g FW in infected leaves, mainly due to a decrease of the chlorophyll a level (Fig. 9C).

DISCUSSION

In leaves, starch accumulates during the day and is degraded during the dark period. This dynamic is regulated, so that starch catabolism is almost complete at the end of the night. Plants are able to adjust this finely tuned pattern in response to changes in environmental conditions. In *P. viticola*-infected grapevine leaves, we observed an abnormally high starch accumulation at the end of the dark period, suggesting major modifications in starch metabolism regulation. Depending on the pathosystems, a decrease (Horst et al. 2008; Judelson et al. 2009; Storr and Hall 1992) or an increase (Ji et al. 2009; Kim

Fig. 8. A, Trehalase gene expression and B, enzyme activity. A, Expression levels obtained for infected leaves were normalized with respect to *VATP16* and *60SRP* expression and related to the corresponding values of control leaves. Samples were collected during the light period (L) or at the end of the dark period (D) during a time course of grapevine leaf infection by *Plasmopara viticola* (1 to 7 days postinoculation [dpi]). Data correspond to the average of three independent biological replicates $\pm$ standard deviation. B, Quantification of the trehalase activity was performed in control and *P. viticola*-infected grapevine leaves at 7 dpi. Bars represent the average of three biological replicates $\pm$ standard deviation. Means with different letters (a, b) are significantly different ($P < 0.05$).

et al. 2009; Rodríguez et al. 2010) of the starch content was observed in some plant–biotrophic pathogen interactions.

A microarray analysis revealed that the expression of several genes related to the carbohydrate metabolism was modified in infected leaves. The expression of genes encoding starch-degrading enzymes (i.e., a grapevine isoform of a phosphoglucan water, dikinase, and β -amylase) were repressed. PWD plays an essential role in starch degradation because the phosphorylation of semicrystalline starch particles is required for the subsequent degradation by other enzymes (Kötting et al. 2005; Ritte et al. 2002, 2004; Smith and Stitt 2007). β -Amylase is an exo-acting enzyme that degrades the α -1,4-glycosidic bonds from the nonreducing end of the starch molecules, releasing maltose. It plays a major role in the degradation of leaf starch in potato leaves (Scheidig et al. 2002) and probably in other species (Lloyd et al. 2005). These microarray results suggest that starch catabolism is affected in *P. viticola*-infected leaves.

Surprisingly, we have observed a high induction of the expression of a gene encoding α -amylase, an endo-acting enzyme that degrades 1,4-glycosidic bonds to release malto-oligosaccharides. This enzyme is mostly described in the context of sink tissues, as potato tubers or cereal endosperm, but con-

flicting reports exist about its function in foliar tissues (Lloyd et al. 2005). The rice isoform AMY1 was shown to have a significant function in leaf starch degradation (Asatsuma et al. 2005). It seems to play no essential role in *Arabidopsis* (Yu et al. 2005), except in leaves during senescence or infection with *Pseudomonas syringae* pv. *tomato*, where the gene encoding α -amylase A1 is induced (Doyle et al. 2007). Up to now, to our knowledge, no data on the function and the importance of the amylase isoforms in grapevine leaf starch metabolism were available. In our study, qRT-PCR confirmed that the expression of the β -amylase gene was repressed in infected leaves, whereas it was clearly induced for the α -amylase gene. Concerning enzyme activities, the β -amylase activity was reduced in infected leaves at the end of the dark period whereas it remained unchanged for the light period. The activity of α -amylase was induced in infected leaves for both sampling periods. These results suggest that, in *Plasmopara viticola*-infected leaves, α -amylase replaces β -amylase for starch degradation. The observed starch accumulation might result from a lower efficiency of α -amylase to degrade starch.

We have also studied enzymes involved in starch synthesis, including the key enzyme AGPase. The microarray analysis,

Fig. 9. Photosynthetic parameters in control and *Plasmopara viticola*-infected leaves. **A**, Imaging of the maximum efficiency of PSII (Fv/Fm); **B**, values for Fv/Fm, effective PSII quantum yield (Φ_{PSII}), and photochemical (q_p) and nonphotochemical (q_{NP}) quenching parameters; and **C**, chlorophyll concentrations in control and *P. viticola*-infected leaves (7 days postinoculation [dpi]). **A**, Representative false-color images of control and *P. viticola*-infected leaves during the course of infection (1 to 7 dpi). Scale represents 0.5 cm. **B**, Measurements were performed in infected (oil spots and adjacent area without symptoms) and control leaves. Data correspond to the average of 35 measurements $\pm$ standard deviation. Means with different letters (a, b) are significantly different ($P < 0.05$). **C**, Data correspond to the average of three biological replicates $\pm$ standard deviation. Means with different letters (a, b) are significantly different ($P < 0.05$).

confirmed by qRT-PCR, revealed that the expression of the gene encoding the large subunit 1 of AGPase was repressed in infected leaves. However, it was not correlated to a reduction but an increase of the enzyme activity. In *Arabidopsis*, changes in the transcription of this gene do not systematically lead to a change in protein abundance or enzyme activity because a strong post-translational regulation is important to control starch metabolism (Gibon et al. 2004; Lu et al. 2005; Smith et al. 2004). Therefore, in *P. viticola*-infected leaves, starch accumulation could result from the increase of AGPase activity and alteration of starch degradation.

At 7 dpi, both glucose and fructose levels were higher in infected than in control leaves at the end of the dark period. Microorganisms can induce changes in soluble sugar concentrations (Horst et al. 2008; Ji et al. 2009; Jobic et al. 2007; Rodriguez et al. 2010). For example, an increase in soluble sugars was observed in *Arabidopsis*, wheat, or tobacco infected by *Albugo candida*, *Puccinia graminis*, or *Phytophthora nicotianae*, respectively (Chou et al. 2000; Scharte et al. 2005; Tang et al. 1996; Wright et al. 1995). In plant-pathogen interactions, sugars play several roles: energy source to fuel the activation of defense reactions, regulators of gene expression (sugar signaling), and nutrients. They can induce pathogenesis-related (PR) proteins (Roitsch 1999) and sink-specific genes (such as invertase) and repress source-specific genes, such as the chlorophyll subunit a/b binding protein and Rubisco (Rolland et al. 2002). In our case, because sugars accumulate just before sporulation, a role in plant defense seems uncertain but they certainly serve as nutrients for the pathogen.

Sucrose is the main form of sugar transported in plants and invertase catalyzes its cleavage into glucose and fructose. At 7 dpi, infected leaves showed a low level of sucrose, but only during the light period. However, we have shown an increase in invertase activity in the insoluble fraction after 3 dpi, independently of the sampling period. Therefore, one could expect a lower sucrose level at the end of the dark period and a higher level of glucose and fructose during the light period compared with the obtained results. Such discrepancies might reflect distinct day and night carbohydrate metabolism of *Plasmopara viticola*. It is consistent with developmental events such as sporulation, which preferentially occurs in the dark. An increase in cell-wall invertase activity seems to be a common feature in plants infected by viruses (Herbers et al. 2000), bacteria (Sturm and Chrispeels 1990), necrotrophic fungi (Benhamou et al. 1991), and biotrophic fungi (Chou et al. 2000; Fotopoulos et al. 2003; Hall and Williams 2000; Roitsch et al. 2003; Scholes et al. 1994; Sutton et al. 2007; Voegelé et al. 2006). In grapevine, Hayes and associates (2010) have reported the induction of a cell-wall invertase gene expression in downy and powdery mildew-infected leaves. Because host and pathogen possess invertases, it is difficult to establish which one is activated during the interaction. Few works stated the plant or pathogen origin of invertases during interactions. An immunological approach was used to show the involvement of a fungal invertase in sunflower-*Sclerotinia sclerotiorum* and grapevine-*Botrytis cinerea* interactions (Jobic et al. 2007; Ruiz and Ruffner 2002). In the *Vicia faba*-*U. fabae* interaction, Voegelé and associates (2006) have observed an increase of both plant and pathogen invertase gene expression. In our pathosystem, because *P. viticola* is an obligate intra-tissular biotroph pathogen, we cannot state whether the invertase activity determinant for the carbohydrate changes observed is of plant or pathogen origin. The increase in the invertase activity might be linked to the starch accumulation observed. Indeed, Fernie and associates (2001) have reported a correlation between an increase in invertase activity and starch synthesis in potato discs treated by palatinose.

The invertase detected in the insoluble fraction had an alkaline optimum pH, whereas major plant invertases activated during a compatible interaction with biotrophic fungi are extracellular acid enzymes (Roitsch et al. 2003). To our knowledge, only one study has reported an alkaline cell-wall invertase in rice cell suspensions but not in a context of plant-pathogen interaction (Hsiao et al. 2002). Thus, further experiments would be required in order to characterize the alkaline invertase found in *P. viticola*-infected grapevine leaves.

An increasing level of the disaccharide trehalose was observed in *P. viticola*-infected leaves. Brem and associates (1986) have reported a depletion of sucrose and a diversion of glucose to trehalose in *P. viticola*-infected leaves but it was after the beginning of sporulation. In most plants, trehalose is found only in trace amounts (Goddijn et al. 1997; Paul et al. 2008); however, there is growing interest in its metabolic pathway because it has effects on growth, development, metabolism, and stress resistance and it may play an important regulatory role in carbohydrate metabolism or signaling (Goddijn and Smeekens 1998; Paul 2007). In *Arabidopsis*, it activates *ApL3* gene expression, AGPase activity, and starch accumulation (Fritzzius et al. 2001; Kolbe et al. 2005; Ramon et al. 2007; Wingler et al. 2000). In soybean, it affects sucrose synthase and invertase activities (Müller et al. 1998). The trehalose precursor, trehalose 6-phosphate (T6P), also regulates starch biosynthesis via post-translational redox modification of AGPase (Kolbe et al. 2005; Lunn et al. 2006; Schluepmann et al. 2003); therefore, the increase in trehalose concentration—or T6P—during leaf infection might be involved in the starch accumulation observed. Few studies have reported a trehalose accumulation in plant organs infected by pathogens (Fernandez et al. 2010). In *Arabidopsis*, trehalose activates the expression of genes encoding a defense-related transcription factor and a PR protein (Schluepmann et al. 2004). In wheat, it confers a protection against the necrotrophic agent *Blumeria graminis*, associated with papilla deposition, H₂O₂ production, and an increase in phenylalanine ammonia lyase and peroxidase activities (Reignault et al. 2001; Renard-Merlier et al. 2007). It could also act as a virulence factor in some interactions such as *Arabidopsis thaliana*-*Plasmodiophora brassicae* (Brodmann et al. 2002). In this case, a trehalase activity was also detected and supposed to be produced by the plant to counteract trehalose accumulation. In our pathosystem, it is unclear whether trehalose is produced by the plant or by the oomycete. Its accumulation was correlated to increasing trehalase gene expression and enzyme activity. The accumulated trehalose might be degraded by the plant as a regulation process or by the pathogen for nutrition.

Starch and sugar metabolic pathways are known to be regulated by the circadian rhythm (Graf et al. 2010; Lu et al. 2005; Smith and Stitt 2007; Smith et al. 2004; Zeeman et al. 2007); therefore, we have studied the gene expression and enzyme activities in both darkness and light. In our conditions, the day and night regulation of gene expression was visible in control samples and was maintained in infected leaves. However, it is not visible on the graphs because data of infected leaves were related to the corresponding values of control leaves. For enzymatic activities, only β -amylase showed a circadian regulation in control leaves. As previously described, the diurnal regulation of gene expression does not always directly impact the protein level, underlining the importance of post-translational regulation (Lu et al. 2005; Smith et al. 2004).

We have observed a repression of the expression of genes encoding chlorophyll synthase, chlorophyll a/b binding protein, and Rubisco and an induction of chlorophyllase gene expression in infected leaves. This was associated with a decrease of the leaf chlorophyll content. Moreover, measurement

of chlorophyll fluorescence indicated a reduction of the Fv/Fm associated with an increase in the q_N values in the oil spots, whereas both parameters were similar in control leaves and the green area surrounding oil spots. Moriondo and associates (2005) also described a similar Fv/Fm in uninfected leaves and the green area of downy mildew-infected leaves. However, they did not determine it in oil spots. Several studies have shown that rates of photosynthesis are reduced near areas infected with pathogens (Berger et al. 2007; Chou et al. 2000; Scharte et al. 2005; Swarbrick et al. 2006; Zou et al. 2005) and can be associated with a loss of chlorophyll, as observed in leaves infected by rusts and downy and powdery mildew (Ahmad et al. 1983; Scholes et al. 1994; Wright et al. 1995). In oat leaves infected with crown rust, Scholes and Rolfe (1996) also observed reduced photosynthesis efficiency in infected area and an increase of q_N values that they associated with a high demand for ATP. In stress situations, increases in q_N values are often accompanied by photoinactivation of PSII reaction centers leading to a dissipation of excitation energy as heat rather than as photochemistry (Melis 1999). In our conditions, the repression of genes encoding the Rubisco large subunit and chlorophyll a/b binding protein may also explain the reduction of the photosynthetic efficiency. A lower rate in photosynthesis is associated with an increase in invertase activity, a hexose accumulation, and a downregulation of photosynthetic gene expression. These events reflect a source to sink transition in carbohydrate production and partitioning within host tissues as described before for compatible interactions (Abood and Lösel 2003; Chou et al. 2000; Hall and Williams 2000; Scholes et al. 1994; Swarbrick et al. 2006; Tang et al. 1996).

In conclusion, in *Plasmopara viticola*-infected leaves, we have described, for the first time, an abnormal starch accumulation associated with an increase of starch synthesis (increase in AGPase activity) and modifications in the starch-degradation pathway (increase in α -amylase activity and decrease in β -amylase activity). These events occur together with a source-to-sink transition marked by an increase in hexose levels and invertase activity and a reduction of photosynthetic capacity. Altogether, these results highlight a dramatic alteration of carbohydrate metabolism. All these modifications occurred at the end of infection when the leaves were highly colonized and may be associated with the pre-sporulation stage. The originality of this study remains in the identification of key events from gene expression toward the sugar dynamic.

MATERIALS AND METHODS

Biological material.

Grapevine (*Vitis vinifera* L. 'Marselan') herbaceous cuttings were grown as previously described (Allègre et al. 2007) in a greenhouse at 25 ± 4 and $18 \pm 7^\circ\text{C}$ (day and night, respectively).

The *P. viticola* strain was maintained on Marselan plants and inoculation was performed as previously described (Allègre et al. 2009). Briefly, the lower side of leaves was sprayed with a suspension of 10^4 sporangia/ml in distilled water, incubated overnight at a relative humidity of 100%, and placed in the conditions described above. For mock inoculation used as a control, the leaf lower surface was sprayed with water and the plants placed in the conditions described above.

For all experiments, the third and fourth (from the shoot base) leaves of control and inoculated plants were harvested at the end of the dark period or after 10 h of light. For mRNA, DNA, soluble sugar, total protein and starch extractions, and aniline blue staining, leaves were collected at 1, 3, 5, and 7 dpi

and immediately frozen in liquid nitrogen. For the other experiments, samples were collected at 6 or 7 dpi. Six plants were used per treatment and time point and three biological repetitions were performed.

Visualization of pathogen development.

Leaf discs (7 mm) from infected and control plants were harvested at 1, 3, 5, and 7 dpi and *P. viticola* was visualized using blue aniline staining as previously described (Trouvelot et al. 2008).

Transmission electron microscopy.

Samples were prepared and examined as previously described (Trouvelot et al. 2008). For each leaf, sections were cut in three different zones along the leaf fragment. At least 60 sections were observed per zone.

Leaf starch staining by lugol.

Leaves were clarified in absolute ethanol (10 min at 90°C), rinsed in water, incubated for 10 min in diluted lugol solution (1:16 [vol/vol]) (Sigma-Aldrich, St Louis), rinsed twice, and photographed immediately.

Starch extraction and quantification.

Starch was extracted and quantified from 500 mg of leaves according to Smith and Zeeman (2006).

NMR spectrometry.

Perchloric acid extracts were prepared using 5 g of leaves according to the method described by Aubert and associates (1996) and NMR spectrometry was performed as described by Jobic and associates (2007).

HPAEC-PAD analysis of soluble sugars.

Samples were ground in liquid nitrogen and lyophilized. The powder (50 mg) was treated by 1 ml of 80% ethanol (30 min, 80°C) in an ultrasonic bath. After centrifugation (10 min, $15,000 \times g$), 10 μl of supernatant was injected on an ICS 3000 Dionex instrument (Dionex Corp., Sunnyvale, CA, U.S.A.). The sugar contents were determined by HPAEC-PAD using a guard and analytical column CarboPac PA100 (Dionex P/N043055), according to Dionex instructions (Dionex Application Note 122 [2004]).

Total protein extraction and enzyme activities.

Proteins were extracted as described by Dancer and associates (1990). The protein concentration of the extracts was estimated according to the method of Bradford (1976). Invertase activities of soluble and insoluble fractions were assayed as described by Dancer and associates (1990). Trehalase activity of the soluble fraction was quantified according to Brodmann and associates (2002).

Chloroplast protein extraction.

Chloroplast proteins were extracted from enriched chloroplast fractions according to a protocol modified from Elias and Givan (1978) and Triboush and associates (1998). Leaves (10 g) were ground with sand, added by 100 ml of extraction buffer (50 mM HEPES-KOH [pH 8.0], 330 mM sorbitol, 5 mM ascorbic acid, 5 mM cysteine, 2 mM EDTA, 1 mM PMSF, and 2.5% [wt/vol] polyvinyl-polypyrrolidone), filtered through two layers of cloth (mesh size 35 μm), and centrifuged ($200 \times g$, 20 min, 4°C). The supernatant was transferred to a new tube and centrifuged ($6,000 \times g$, 25 min, 4°C). The pellet was washed with 1 vol of washing buffer (50 mM HEPES-KOH [pH 8.0], 330 mM sorbitol, 2 mM EDTA, and 1 mM PMSF), resuspended in 1 vol of protein buffer (10 mM HEPES-KOH

[pH 8.0], 5 mM MgCl₂, and 1 mM PMSF), incubated on ice for 10 min, and centrifuged (10,000 × g, 25 min, 4°C). Proteins of the supernatant were concentrated using Microcon spin columns (cut-off 30 kDa; Millipore, Billerica, MA, U.S.A.). The protein concentration of the samples was estimated according to the method of Bradford (1976).

Ceralpha and Betamyl-3 kits (Megazyme, Wicklow, Ireland) were used to determine α- and β-amylase activities in chloroplast protein extracts diluted in the kit's extraction buffer (1:10 [vol/vol]). The release of *p*-nitrophenol from the specific substrates was quantified according to the manufacturer's instructions. The AGPase activity in the pyrophosphorolysis direction was assessed as previously described (Tiessen et al. 2002).

RNA extraction.

Total RNA was extracted from 500 mg of ground leaves as described by Reid and associates (2006). DNA contaminations were removed with the DNase 1-Kit (Sigma-Aldrich) according to the manufacturer's instructions. The concentration and purity of RNA extracts were determined by spectrophotometry. For microarray analysis, the quality was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.A.).

Total RNA (4 µg) was used to synthesize the first-strand cDNA using the SuperScript III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, U.S.A.) with a 1:1 mixture of Oligo-dT and random hexamer primers following the kit's technical notice.

Microarray analysis.

The microarray analysis was performed at the University of Verona (Italy) on a NimbleGen *V. vinifera* chip (Roche NimbleGen, Madison, WI, U.S.A.). This array was based on the 12X grapevine genome assembly produced by the French-Italian consortium. In all, 29,582 transcripts were analyzed with four probes per transcript (one replicate per probe). RNA samples for hybridization were extracted from control or infected leaves (7 dpi, end of the dark period), as described above, including three independent biological replicates. cDNA labeled with Cyanine 3 (Cy3) was produced from total RNA (1 µg) and hybridized as indicated in the NimbleGen array user guide. The array was scanned using the Axon GenePix 4400A Microarray scanner (Molecular Devices, Sunnyvale, CA, U.S.A.) and images were analyzed using the NimbleScan software (Roche NimbleGen). Fluorescence data were extracted using the robust multi-array algorithm with quantile normalization (Supplementary Table S2).

Student's *t* test and statistical analysis of microarrays method (two-class unpaired test) (Tusher et al. 2001) were used to identify differentially expressed genes (fold change < 0.5 [repressed] or > 2 [induced]; *P* < 0.05; false discovery rate < 5%).

Primer design and validation.

Primer pairs were designed (Supplementary Table S3) using PerlPrimer software (Marshall 2004) and the sequence data of *V. vinifera* 'Pinot noir' (Jaillon et al. 2007) provided by the Genoscope Grape Genome Browser. All primers were examined for their target specificity by end-point PCR and the products sequenced by the Sanger method (Genoscreen, Lille, France).

qRT-PCR.

The ABsolute QPCR SYBR Green ROX Mix (Thermo Fisher Scientific, Waltham, MA, U.S.A.) was used with a final primer concentration of 500 nM and 5 ng of total cDNA. The thermal cycling profile was: 95°C for 15 min and 45 cycles at

95°C for 20 s, 60°C for 30 s, and 72°C for 30 s (LightCycler480; Roche Applied Science, Penzberg, Germany). Each real-time assay was tested in a dissociation protocol to ensure that the amplicon was a single product. Amplification efficiency for each sample was calculated from the fluorescence raw data with the LinRegPCR program (Ruijter et al. 2009).

Samples were treated in technical triplicates and the mean values of cycle threshold (Ct) and efficiency were used for further analysis. *VATP16* and *60SRP* (*L18e*) were used as internal control genes (Gamm et al. 2011). Fold change in RNA expression was estimated using Ct by the ΔΔCt method (Livak and Schmittgen 2001).

Pathogen quantification.

Pathogen quantification was performed as described before (Silvar et al. 2005; Valsesia et al. 2005). DNA was extracted from control and infected leaves and *P. viticola* sporangia according to Porebski and associates (1997). DNA (100 ng) was used in a qRT-PCR mix with the ABsolute QPCR SYBR Green ROX Mix (Thermo Scientific), applying a final primer concentration of 500 nM. Pathogen and plant DNA contents were quantified using a *P. viticola* actin primer and a *V. vinifera* *HT5* primer pair (Hayes et al. 2010; Schmidlin et al. 2008), respectively. The thermal cycling profile using a LightCycler480 (Roche Applied Science) was as described above.

Chlorophyll analysis.

The leaf chlorophyll a fluorescence was quantified on attached leaves using an IMAGING-PAM Chlorophyll Fluorometer (Walz, Effeltrich, Germany). Measurements were performed on the central parts of the leaf adaxial side after preconditioning in the dark. From these measurements, several fluorescence parameters were calculated according to Schreiber and associates (1986) and Genty and associates (1989): Φ_{PSII} and Fv/Fm. Φ_{PSII} represents the number of electrons transported by a PSII reaction center per mole of quanta absorbed by PSII and Fv/Fm is the ratio of variable to maximal fluorescence. In addition, both *q_p* and *q_N* were calculated according to van Kooten and Snel (1990). On each image, the values of the selected fluorescence parameters were averaged. Images of Φ_{PSII} were displayed with the help of a false color code ranging from 0.000 (black) to 1.000 (pink).

For chlorophyll quantification, 3 g of leaves was ground in a mortar with sand and calcium carbonate and extracted with 20 ml of 95% methanol. The sample was filtered and analyzed by spectrometry at 645 and 663 nm (Hitachi U-2001 spectrophotometer, Hitachi Scientific Instruments, Mountain View, CA, U.S.A.), and the levels of chlorophylls were determined according to Wellburn and Lichtenthaler (1984).

Statistical analysis.

Data obtained for pathogen, carbohydrate, and chlorophyll quantification; enzyme activities; and chlorophyll fluorescence measurement were analyzed by one-way analysis of variance and means were compared using Fisher's least significant difference test to detect significant differences (*P* < 0.05) between treatments. All analyses were performed with the statistical program STATGRAPHICS (release 5.1; Manugistic, Inc., Rockville, MD, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Genoscope Grape Genome browser:
www.genoscope.cns.fr/externe/GenomeBrowser/Vitis
 Genoscope Vitis vinifera webpage: www.cns.fr/spip/Vitis-vinifera-e.html
 NimbleGen website :
www.nimblegen.com/products/exp/custom.html#vitus
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