

Chapter 3

Interactions Between Grapevines and Grapevine Yellows Phytoplasmas BN and FD

Abstract Many aspects of interactions between grapevine yellows phytoplasmas (GYP) and their grapevine hosts remain unclear. However, based on the available data, it appears that damage caused by GYP to their hosts is greater than might be expected from their relatively low titre in the phloem and their uneven distribution throughout the plant. Moreover, it is hard to define the limits between the common plant responses to an infection and the real GYP activities towards obtaining the necessary compounds for their life within the host. Collective evidence suggests that the accumulation of soluble carbohydrates in GYP-infected plants results in feedback inhibition of photosynthesis, which causes a source–sink transition. In many microbe–plant interactions, cell-wall invertase, which hydrolyses sucrose to glucose and fructose, plays an important role in disease expression. However, it appears that in the grapevine–GYP interaction, another enzyme has a leading function, sucrose synthase, on the basis of providing both fructose for the GYP and UDP-glucose for the plant responses to the infection. In parallel to the responses of the genes involved in primary carbohydrate metabolism, sink-specific secondary metabolism pathways that involve genes and metabolites of the shikimic acid and oxidative pentose phosphate pathway are induced, along with genes involved in direct defence responses. The observed changes in metabolism of GYP-infected plants suggest that infected grapevines respond to this pathogen through induced salicylic-acid-dependent systemic acquired resistance.

3.1 Introduction

Like other biotrophic pathogens, phytoplasmas that thrive in the plant sap or in insect haemolymph have evolved intimate and sophisticated modes of parasitism that trigger various responses on the host cells. As with other known bacterium–plant interactions, the plant responses to phytoplasmas is highly diversified. Moreover, considering the very low titres of phytoplasmas in host cells, which vary according to plant organ and season (Prezelj et al. 2013), as for most plant diseases (Agrios 2005), the amount of damage caused to the host by these pathogens is much greater than it would be expected from their mere removal of nutrients. However, our knowledge of this specific interaction is very rudimentary, due to difficulties to routinely grow phytoplasmas in axenic cultures (Contaldo et al. 2016; see also the

first chapter of this book) or to maintain them in their original host plants under greenhouse conditions, where there can be very high rates of recovery or decline. Phytoplasma studies are thus highly dependent on the unpredictable conditions of their natural environments, which in the case of grapevine yellows phytoplasmas (GYP) are the vineyards. Similar studies have already shown the importance of field experiments to evaluate complex correlations and to recognize inter-relationships among the biotic and abiotic factors that structure the ecosystems (Schmidt et al. 2004; Izawa 2015). Nevertheless, an understanding of the specific interactions between GYP and their grapevine host will not only lead to better understanding of the basic aspects of how phytoplasmas infect plants and how plant hosts respond to such infection, but will also result in new treatment approaches for the elimination of these pathogens from plants or to produce plants that are resistant to phytoplasmas.

A substantial part of the knowledge of how phytoplasmas interact with their hosts has been predicted from the definition of their draft and complete genome sequences (Kube et al. 2012). Little data are available on phytoplasma interactions with their insect hosts (Bai et al. 2006; Sugio et al. 2011a, b). On the other hand, there is more information available on plant responses to phytoplasma infection. Several metabolic changes of GYP-infected plants have been revealed using classical biochemical techniques (Bertamini et al. 2002; Rusjan et al. 2012a, b; Rusjan and Mikulic-Petkovsek 2015), and these have suggested that phytoplasmas affect both the primary and secondary metabolism of the host plant. The introduction of “omics” approaches to phytoplasma research has boosted the knowledge of plant responses to phytoplasma presence, and has confirmed previous ideas of the presence of integrated plant responses to this infection. However, most of these studies on GYP-infected plants have been carried out at the transcriptome level (Albertazzi et al. 2009; Hren et al. 2009a, b), so rarely at the proteome level (Margaria and Palmano 2011; Margaria et al. 2013), and with only two studies at the metabolome level (Prezelj et al. 2016a, b). This appears strange, as the metabolites are the end products of the cellular regulatory processes, and their levels can be regarded as the ultimate response of biological systems to genetic or environmental changes (Fiehn 2002; Weckwerth 2011).

In this chapter, the plant responses to infection with ‘*Candidatus* Phytoplasma solani’ strains (BN) that are associated to “bois noir” (BN) and phytoplasma FD that is associated to “flavescence dorée” (FD) will be reviewed. Although these phytoplasmas are not closely related phylogenetically, they are associated with similar symptoms on grapevine, which would appear to be related to a more general plant response to their presence. However, due to the quarantine status of FD, more information is available for grapevines infected with BN. Therefore, this interaction is commonly used as a model for studying grapevine host strategies to cope with GYP.

3.2 How Phytoplasmas Interact with Their Hosts, Based on Analysis of Their Genomes

The determination of five complete genome sequences (Andersen et al. 2013; Kube et al. 2008, 2012; Oshima et al. 2004; Tran-Nguyen et al. 2008), and additionally of 12 draft genome sequences, including the draft sequence of BN (Mitrović et al. 2014), has opened new ways for the analysis of these pathogenic bacteria. As a result of their evolutionary adaptation to intracellular life in their hosts, phytoplasmas have small genomes that encode minimal metabolism. Phytoplasma genomes have a high proportion of complex transposons (known as potential mobile units; Bai et al. 2006), and phage-derived sequences, which result in the different genome sizes seen for phytoplasmas (Kube et al. 2012). Analysis of the genomic core of the BN strains has revealed a complete set of proteins that encode for their replication, for DNA structure and modification, and for DNA repair and transcription (Mitrović et al. 2014). The BN genome sequence contains genes that encode the membrane proteins Vmp1 and Stamp, which are believed to be involved in phytoplasma–host interactions (Cimerman et al. 2009; Fabre et al. 2011), and also gene sets that are necessary to build the general Sec-dependent pathway. In addition, other genes that have been identified in the BN genome sequence enclose those that encode ABC transporters, and proteins involved in the first stages of glycolysis and in energy-yielding pathways, including alternative energy-yielding pathways. The use of the alternative energy-yielding pathways, can provide an important carbon source for the phytoplasmas (Kube et al. 2012). As for some other known phytoplasma genome sequences, the gene that encodes the membrane-bound phosphoenolpyruvate-dependent phosphotransferase system is missing from the BN genome, although a gene that encodes a truncated sucrose phosphorylase is present.

It is believed that phytoplasmas can produce effectors that regulate characteristic targets in their hosts, to direct variations in the development of plants and insects. However, at present, it is largely unclear which phytoplasma effectors are involved in host manipulation (Sugio et al. 2011a, b, Chen et al. 2012; Rümpler et al. 2015), and such effectors have not been characterised in the genomes of GYP.

3.3 Pre-symptomatic Grapevine Responses to Grapevine Yellows Phytoplasma Infection

Studies on the distribution and persistence of BN in grapevine cultivars Ancellotta, Lambrusco Salamino, Sangiovese, Trebbiano Romagnolo and Chardonnay (Terlizzi and Credi 2007; Hren et al. 2009a) have shown that a very low proportion of asymptomatic leaf samples of otherwise infected plants are BN positive. Again, a low proportion of phloem samples from dormant cane, cordon and roots from otherwise BN-infected grapevines were BN-positive when checked during winter (Terlizzi and Credi 2007). Low titres of phytoplasmas were also shown at the beginning of

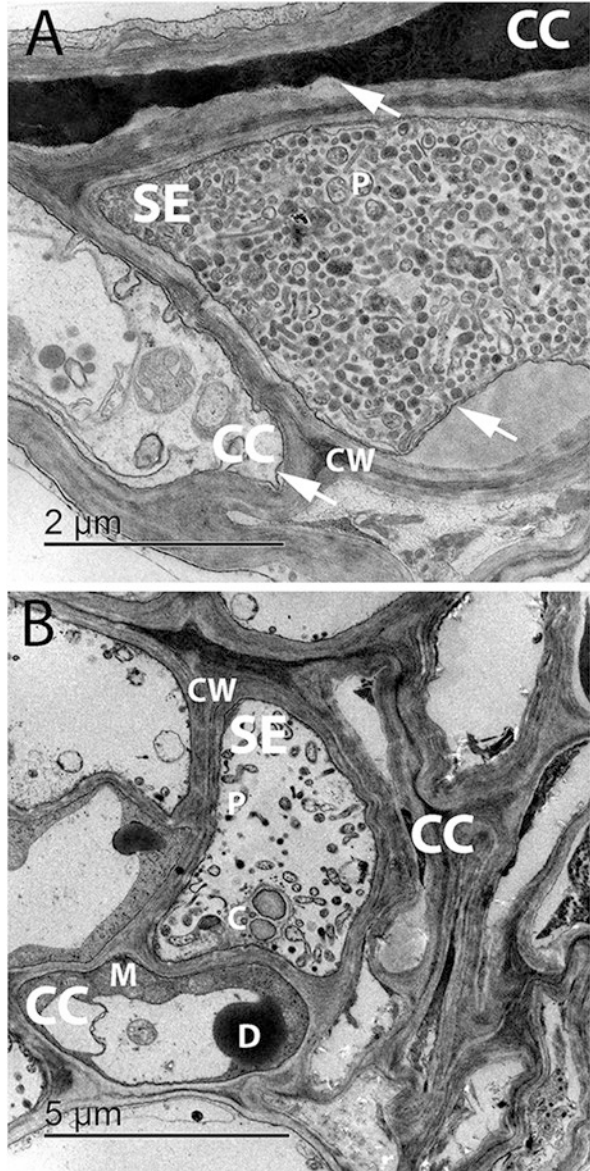
the growing season for FD in grapevine cultivars Blaufränkisch and Refosco d'Istria, although these increased in close association with the later expression of symptoms. In plants with very high concentrations of FD in tissues with symptoms, phytoplasmas have also been detected in the symptomless tissues (Prezelj et al. 2013). FD has been detected in flowers, petioles, berry tissues and tendrils, with the highest titre in berries in the late growing season (Prezelj et al. 2013). Collectively, these data indicate an uneven distribution of phytoplasmas inside plants, and their sporadic systemic spread throughout grapevines, thus having a more indirect influence on the host metabolism. However, for efficient management strategies, it would be beneficial to have a plant-host marker for accurate detection of phytoplasmas even when their titres are below the present limits of detection.

A thorough transcriptional analysis of genes shown to be differentially expressed in BN- and FD-infected grapevines revealed that only a few genes were significantly different before symptoms development. While the enzyme products of most of these genes are more general components of host-plant defence responses, the up-regulation of the *DMR6* gene detected in grapevines infected with BN and FD is of particular interest (Dermastia et al. 2015; Prezelj et al. 2016a, b), *DMR6* encodes a 2-oxoglutarate and Fe(II)-dependent oxygenase, although its biological role is uncertain at the moment. However, it has been shown that in *Arabidopsis* lacking a functional *DMR6* protein, susceptibility to downy mildew was reduced (Van Damme et al. 2008), and it has been suggested that *DMR6* acts as a suppressor of plant immunity (Zeilmaker et al. 2015). Although it remains to be determined how specific the pre-symptom expression of the *DMR6* gene is in terms of phytoplasma diseases, this might represent a potential early marker of GY diseases.

3.4 Ultrastructure of Phloem Infected with Grapevine Yellows Phytoplasmas

Phloem is the main plant tissue where phytoplasmas can thrive (Christensen et al. 2004). Its ultrastructure has been investigated in grapevine cv. Chardonnay and tomato infected with '*Ca. P. solani*' strains (Santi et al. 2013a, b; Buxa et al. 2015), and in the broadbean (*Vicia faba*) infected with FD (Musetti et al. 2010). In infected phloem, many companion and phloem parenchyma cells show plasmolysis, with the consequent cytoplasm condensation, or undergo necrosis (Fig. 3.1) (Santi et al. 2013a). Infected leaves are characterised by Ca^{2+} influx into sieve tubes, which leads to sieve-plate occlusion through callose deposition or protein plugging (Musetti et al. 2013a). Phytoplasma infection is additionally associated with changes in the plasma membrane surface and distortion of the sieve-element reticulum (Fig. 3.2). It has been suggested that actin is displaced from the sieve-element microplasm and aggregates on the phytoplasma surface. Therefore, these aggregates might represent a connection between phytoplasmas and the sieve-element cytoskeleton (Buxa et al. 2015).

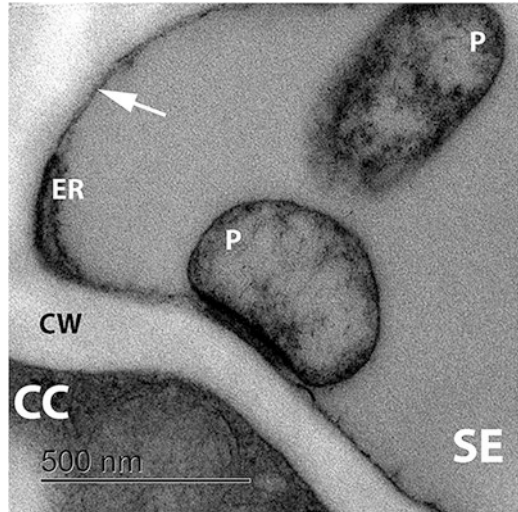
Fig. 3.1 Transmission electron micrograph of the main vein cross-section of symptomatic *C. roseus* infected with ‘Ca. P. solani’ (genotype CPM4_At1). (a) In sieve elements (SE) associated with companion cells (CE), which has not been lethally damaged (e.g., CC on the left of the picture) phytoplasmas (P) are numerous. However, plasmolysis (marked by an arrow) has already occurred. CC on the top of the picture has collapsed; its cell wall is bent and the cytoplasm is condensed. (b) In the SE associated with completely collapsed CC, phytoplasmas decline; a deteriorated chloroplast (C) is visible. In damaged CC electronically dense deposits (D) are common (Photo: M. Tušek Žnidarič)



3.5 Infection of Grapevine with Grapevine Yellows Phytoplasmas Affects Photosynthesis

There is growing evidence that several steps in photosynthesis of GYP-infected grapevines are repressed during an infection (Albertazzi et al. 2009; Bertamini and Nedunchezhian 2001a, b; Bertamini et al. 2002; Hren et al. 2009a; Margaria et al.

Fig. 3.2 Transmission electron micrograph of the main vein cross-section of asymptomatic tissue culture of *C. roseus* infected with 'Ca. P. ulmi'. Phytoplasma (P) in the sieve element (SE) with a distorted reticulum (ER) is attached to the cell membrane (indicated by an arrow). CW, cell wall; CC, companion cell (Photo: M. Tušek Žnidarič)



2013). However, it is not clear if the observed alterations are directly linked to the presence of the phytoplasmas or are general, non-specific, responses to the biotic stress (Bertamini et al. 2002; Hren et al. 2009a). Data support the theory that chloroplasts are key players in symptom development (Zou et al. 2005).

In leaves of cv. Chardonnay infected with BN, three and 11 genes that encode chlorophyll a/b binding proteins in photosystem I and photosystem II, respectively, are significantly down-regulated (Hren et al. 2009a). Significant repression of the *RbcL* gene that encodes rubisco large subunit (Hren et al. 2009a) is consistent with the loss of this protein in infected grapevine (Bertamini et al. 2002). In addition, the gene that encodes rubisco activase is also significantly down-regulated in infected plants. In infected leaves, the expression of genes involved in the cytochrome b6f complex in the electron-transport pathway decreases (Hren et al. 2009a), which correlates directly with the observed down-regulation of genes encoding ATP synthase. This is also in agreement with the biochemical model of photosynthesis, in which the regeneration of ribulose biphosphate is dependent on the rate of electron transport required for the generation of energy and reducing equivalents of ATP and NADPH. In leaves of grapevine infected with BN, decreases were seen for the chlorophyll, carotenoid and soluble protein contents and the activities of rubisco, nitrate and nitrite reductase (Bertamini and Nedunchezian 2001a; Endeshaw et al. 2012; Rusjan et al. 2012a).

In grapevines infected with FD, similar decreases in photosynthesis have been detected at the protein level (Margaria and Palmano 2011; Margaria et al. 2013). Several proteins are down-regulated: two related to the dark reactions (i.e., rubisco, rubisco activase), and four related to the light-dependent reactions of photosystem II (i.e., chloroplastic ATP synthase CF1 α subunit, ATP synthase CF1 beta subunit, oxygen-evolving enhancer protein 2, Mn-stabilising protein). A gradual decrease in net photosynthesis in FD-infected cv. Barbera was more severe during no drought than during drought years (Vitali et al. 2013).

3.6 Grapevine Yellows Phytoplasma Infection is Associated with Prominent Changes in Carbohydrate Metabolism

The mechanisms of carbon partitioning and its accumulation in grapevine are poorly understood. However, several plant pathogens, including BN and FD, can manipulate host metabolism to turn infected tissues into a carbohydrate sink that provides them with hexoses (Berger et al. 2007; Hren et al. 2009a; Santi et al. 2013a). This source-to-sink switch is regulated by cytokinins (Roitsch and Ehness 2000). However, at the moment it is not known whether a significant transcript increase of the gene *HP* from the cytokinin signalling pathway in BN-infected and FD-infected vein-enriched samples (Hren et al. 2009a; Prezelj et al. 2016a) is related to this transition.

It is generally believed that the feedback inhibition of photosynthesis that causes chlorosis, and is described above, is the result of accumulation of carbohydrates in source leaves (Christensen et al. 2005). Accumulation of glucose, fructose, sucrose and starch in infected source leaves is indeed a common effect of phytoplasma infections (Lepka et al. 1999; Guthrie et al. 2001; Maust et al. 2003; Junqueira et al. 2004; Gai et al. 2014). In whole leaves of cv. Chardonnay infected with BN, the levels of glucose, fructose and sucrose do not significantly differ before the development of symptoms; instead, they significantly increase after the appearance of symptoms (Prezelj 2014). On the other hand, in symptomatic leaves of cv. Blaufränkisch infected with FD, the concentrations of fructose and glucose are only slightly higher, while those of sucrose and starch significantly increase upon infection (Prezelj et al. 2016a).

Sucrose is the major form of carbohydrate loaded into the phloem of photosynthetic source leaves, and with a concentration of up to 1 M in the phloem sieve tubes it might represent a food supply for phytoplasmas. However, the sucrose concentration is not significantly different in BN-infected (Prezelj et al. 2016b) and FD-infected (Prezelj et al. 2016a) vein-enriched leaf samples of cv. Chardonnay and cv. Blaufränkisch, respectively. Sucrose produced in mesophyll cells can load into the phloem either following a symplastic route through plasmodesmata or through apoplastic mechanisms, which requires the movement of assimilates across membranes that is conducted by specialised transport proteins located within the membranes. Although there is very little information available about the sugar transporters that are involved in phytoplasma pathogenicity, there is evidence that some genes that encode such transporters are expressed in the phloem of GYP-infected grapevine leaves (Santi et al. 2013a, b; Prezelj et al. 2016a). In cv. Chardonnay leaves infected with BN, a transcript of the gene that encodes sucrose transporter *SUC27* is down-regulated in comparison with uninfected leaves. On the other hand, genes that encode a novel family of hexose and sucrose transporters, known as *SWEET*s (Eom et al. 2015), show differential expression in leaf-vein-enriched samples of cv. Blaufränkisch infected with FD (Prezelj et al. 2016a). There are, however, fluctuations in the expression throughout the growing season of the *SWEET1* gene from clade I, which is involved mainly in transport of monosaccharides, the *SWEET10* gene from clade III, which is involved mainly in sucrose transport (Chen et al. 2010, 2012), and the *SWEET17a* gene from clade IV, which is involved in vacuolar trans-

port of fructose (Guo et al. 2014) and suggested to function predominately in sink organs (Chardon et al. 2013), irrespective of infection (Prezelj et al. 2016a). Despite this, the transcript level of *SWEET17a* is affected by FD infection, as it is significantly higher in infected grapevines (Prezelj et al. 2016a). Expression patterns of SWEET orthologues of clade III in *Arabidopsis* and rice show induction by biotrophic bacteria and by fungi (Chen 2014), which suggests a certain level of pathogen dependence of the SWEETs. However, prior to the study of Prezelj et al. (2016a), there were no reports of pathogens association with SWEETs from clade IV. Further studies are now needed to confirm the role of *SWEET17a* in phytoplasma infection.

The only known enzymes in plants that produce hexoses by sucrose cleavage are the invertases (Roitsch and González 2004) and sucrose synthase (Koch 2004) (Fig. 3.3). Source-to-sink transition is usually characterised by increased activity of invertases, which irreversibly hydrolyse sucrose to glucose and fructose (Roitsch and González 2004). Three groups of invertases can be distinguished: neutral (nINV), acid insoluble bound to the cell wall (cwINV), and acid soluble localised in the vacuole (vacINV). In grapevines two vac INV genes (i.e., *INV1*, *INV2*) encode translation products that are 62% identical, but have different expression patterns (Davies and Robinson 1996). *INV1* is expressed predominately in berry skin and flesh. For *INV2*, in addition to its elevated expression levels in young leaves, its expression is greatest in young flowers, with lower expression levels in berries and no expression in fully expanded leaves (Davies and Robinson 1996). Accordingly, in vein-enriched samples of phytoplasma-free expanded leaves, there are no detected transcripts of *INV1*, and the level of the transcript of *INV2* is very low (Hren et al. 2009a). The transcript corresponding to *INV2* is then significantly increased in vein-enriched samples of leaves infected with BN (Hren et al. 2009a). A slight increase in the transcription of the gene that encodes cwINV has been reported for grapevines infected with BN (Santi et al. 2013a), but not in another study about the same interaction (Hren et al. 2009a). Similarly, the expression of *INV2* significantly changes throughout the growing season in both healthy and FD-infected vein-enriched samples, and is up-regulated in FD-infected samples in August (Prezelj et al. 2016a). In leaf samples infected with BN (Covington et al. 2016) and FD (Prezelj et al. 2016a), the activities of the different invertase isozymes are not significantly higher than in healthy controls. Of particular interest, in the Madagascar periwinkle (*Catharanthus roseus*) and tomato infected with stolbur phytoplasmas, no differential expression of the *vacINV* gene was observed in leaf tissue, although its enzymatic activity was increased (Machenaud et al. 2007).

Sucrose synthase reversibly catalyses sucrose breakdown to UDP-glucose and fructose, and this enzyme is localised in both companion cells and sieve elements of the phloem (Koch 2004). The genes that encode sucrose synthase are up-regulated in grapevine infected with BN (Hren et al. 2009a, b; Santi et al. 2013a, b) and FD (Prezelj et al. 2016a, b) (Fig. 3.3). It has been suggested that in grapevine infected with BN, up-regulation of *SUSY4* is co-regulated with down-regulation of *SUC27*,

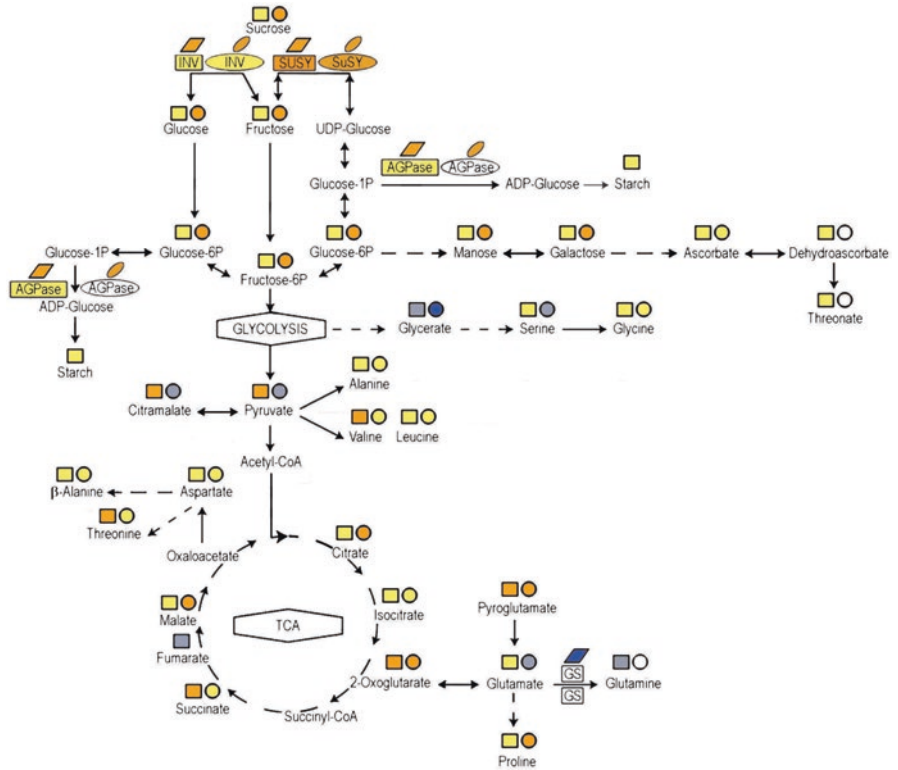


Fig. 3.3 Schematic representation of the metabolites in the primary metabolism pathways affected during FD (squares) and BN (circles) infection of grapevines, together with selected studied enzymes. Metabolite levels: *light blue*, uninfected sample > infected sample (not significant); *dark blue*, uninfected sample > infected sample ($p < 0.05$); *yellow*, infected sample > uninfected sample (not significant); *orange*, infected sample > uninfected sample ($p < 0.05$). Enzyme activities: *open rectangle/large ellipse*, infected sample not significantly different from uninfected sample; *yellow rectangle/large ellipse*, infected sample > uninfected sample ($p < 0.05$); *orange rectangle/large ellipse*, infected samples > uninfected sample ($p < 0.05$). Gene expression: *orange rhomb/small ellipse*, enzyme in infected sample > uninfected sample ($p < 0.05$); *dark blue rhomb/small ellipse*, enzyme in infected sample > uninfected sample ($p < 0.05$); *dark blue rhomb/small ellipse*, enzyme in infected sample < uninfected sample ($p < 0.05$); *white*, not determined

which induces the establishment of the source-to-sink transition in the phloem of the leaf, and thus the access to sugars for the phytoplasmas (Santi et al. 2013a, b). In cv. Blaufränkisch, expression of *SUSY4* in uninfected samples decreases through the season. On the other hand, the transcript levels in plants infected with FD only decreases until July, and then increases in August, when the symptoms are fully developed (Prezelj et al. 2016a). A significantly higher abundance of the *SUSY4* transcript in FD-infected vein-enriched tissues in August agreed with the substantial increase in the activity of sucrose synthase at the same point in the growing season. Under phloem conditions of high sucrose and low fructose, sucrose synthase probably operates as a sucrose-degrading enzyme. However, continuous mobilisation of

sucrose via sucrose synthase depends upon the removal of fructose (Geigenberger et al. 1993). Fructose can be used directly by FD, as it was proposed for *Spiroplasma citri*, another member of the Mollicutes class of bacteria (Gaurivaud et al. 2000). Fructose use such as that proposed for these spiroplasmas might impair sucrose loading into sieve tubes by the companion cells, which would result in accumulation of carbohydrates in the source leaves, as seen in FD-infected grapevines. However, a model reported for *S. citri* operation involved a putative role for acid invertase, although this enzyme was not studied in this system (André et al. 2005).

Several lines of evidence support additional associations of sucrose synthase with phytoplasma pathogenicity, specifically with carbohydrate accumulation in the mesophyll of infected leaves. This appears to be related to physically obstructed phloem loading and transport due to callose depositions in sieve tubes, as observed by transmission electron microscopy in *V. faba* infected with FD (Musetti et al. 2013a, b), as well as in cv. Chardonnay heavily infected with BN (Hren et al. 2009a, b; Santi et al. 2013a; Dermastia et al. 2015). The sieve-tube localisation of sucrose synthase (Koch 2004) might facilitate its role in directly supplying UDP-glucose for rapid biosynthesis of callose plugs in the sieve pores. Callose deposition is a dynamic process that is coordinated through the activities of callose synthase and the callose hydrolysing enzyme β -1,3-glucanase. Among seven genes that encode callose synthase in the grapevine genome, only *CAS2* (Santi et al. 2013a, b; Hren et al. 2009a, b; Dermastia et al. 2015) and *CAS7* (Santi et al. 2013a, b) are up-regulated in BN-infected leaves. Based on observations that glucanase activities are enhanced under conditions that promote callose accumulation, a short life span of callose molecules shifted towards catabolism has been suggested (Zabotin et al. 2002). In this regard, transcriptional analyses of cv. Chardonnay showed significant increases in transcription of β -1,3-glucanase genes upon infection with BN (Hren et al. 2009a; Landi and Romanazzi 2011). The level of the *Glc* transcript is also increased in different grapevine cultivars that are infected with FD (Margaria 2013; Prezelj 2014).

The starch that can accumulate in the mesophyll of GYP-infected leaves (Prezelj 2014; Prezelj et al. 2016a, b) might be synthesised through glucose 1-phosphate metabolised from UDP-glucose as a product of sucrose synthase, or alternatively from glucose as a product of the invertase activity (Fig. 3.3). However, the significantly higher sucrose synthase activity in GYP-infected grapevines and unchanged invertase activity upon infection (Prezelj et al. 2016a, b; Covington et al. 2016) imply the involvement of the sucrose synthase pathway. Up-regulation of the *AGPL* gene, which encodes the large subunit of ADP-glucose pyrophosphorylase that is a rate-limiting enzyme in starch biosynthesis (Ballicora et al. 2004), is a key feature of grapevine infections with GYP (Hren et al. 2009a, b; Prezelj 2014; Dermastia et al. 2015; Prezelj et al. 2016a, b). In agreement with the abundant *AGPL* transcript in infected leaves, there is also a trend towards higher ADP-glucose pyrophosphorylase activity in FD-infected leaves of cv. Blaufränkisch, and significantly higher starch concentrations (Prezelj et al. 2016a, b). The high starch concentrations in phytoplasma-infected mulberry leaves have been explained by lower expression of genes and/or lower activity of enzymes for the degradation of starch (Gai et al.

2014). In contrast with this observation, the gene encoding α -amylase in grapevines infected with BN is up-regulated (Hren et al. 2009a). Although transient starch degradation has not been followed in GYP-infected grapevines, the possibility of its phosphorolytic degradation leading to the increase in hexose-6-phosphates observed in GYP-infected vein-enriched leaf samples (Prezelj et al. 2016a, b) cannot be excluded. However, the expression of genes that encode the enzymes involved in starch degradation, glucan, water dikinase and β -amylase (Smith et al. 2005) are not differentially expressed in grapevines infected with BN (Hren et al. 2009a).

3.7 Grapevine Yellows Phytoplasma Infection Affects the Plant-Energy-Associated Network

It has been shown in other plant–pathogen interactions that the increased activities of carbohydrate transporters and sucrolytic enzymes, and the increase in respiratory metabolism, are coupled with the promotion of a favourable energy balance for plant defence (Rojas et al. 2014). Induction of the energy-associated network is indicated by increases in fructose 6-phosphate (Prezelj et al. 2016a, b), multiple isoforms of the enolase involved in glycolysis (Margaria et al. 2013), and several metabolites of the tricarboxylic acid cycle, including malate, citrate (Prezelj et al. 2016a, b) (as the predominant organic acids in phloem and xylem sap; Ziegler 1975) (Fig. 3.3) and aconitase (Margaria et al. 2013), which catalyses the reversible isomerisation of citrate to isocitrate in grapevine tissues infected with GYP. It has been suggested that the content of these metabolites is regulated as a consequence of phytoplasma infection (Kube et al. 2012). As shown by the comparison of known phytoplasma genomes, including that of BN (Kube et al. 2012; Mitrović et al. 2014), phytoplasmas lack all enzymes from the membrane-bound phosphoenolpyruvate-dependent phosphotransferase system, which most bacteria use as an energy efficient way of simultaneously importing and phosphorylating sugars such as sucrose, glucose and fructose. In contrast, the genes needed to perform the energy-investing initial part of the glycolytic pathway are present in all known phytoplasma genomes, as also in the genome sequences of BN and FD (Carle et al. 2011; Kube et al. 2012; Mitrović et al. 2014). This discrepancy would be overcome by an uptake system allowing the use of phosphorylated hexoses from the phytoplasma host. It has been suggested that phytoplasma can use sucrose and trehalose compounds from phloem sap or insect haemolymph, respectively, using the phosphoglucose isomerase encoded in all known phytoplasma genomes. However, this step would not be necessary if fructose 6-phosphate is available (Kube et al. 2012). On this basis, significantly increased amounts of fructose 6-phosphate in BN-infected and FD-infected grapevines (Prezelj et al. 2016a, b) (Fig. 3.3) might also be used by phytoplasmas. Fructose 6-phosphate can enter the phytoplasma glycolysis pathway if it is converted to fructose 1,6-bisphosphate by plant phosphofruktokinase, the gene for which is in fact up-regulated in BN-infected grapevines (Hren et al. 2009a). In addition, malate concentrations

increase upon GYP infection (Prezelj et al. 2016a, b), which might enter the suggested alternative energy-yielding pathway of glycolysis (Kube et al. 2012) that includes the step of malate uptake through the MleP symporter that is encoded in the BN genome (Mitrović et al. 2014).

3.8 Changed Concentrations of Amino Acids in Infected Vein-Enriched Grapevine Samples

When exposed to stress conditions, plants accumulate an array of metabolites, and particularly amino acids (Hayat et al. 2012). In grapevine vein-enriched samples, the concentrations of several amino acids (i.e., serine, glycine, valine, leucine, alanine, β -alanine, threonine, aspartate, pyroglutamate, proline) increase upon infection with BN and FD (Prezelj et al. 2016a, b) (Fig. 3.3). A large body of evidence suggests a positive correlation between proline accumulation and plant stress. Proline protects plants from stress and helps the plants to recover from it, through its actions as a metal chelator, an antioxidative defence molecule, and a signalling molecule (Hayat et al. 2012; Anil Kumar et al. 2015). The sixfold and threefold increases in proline in BN-infected and FD-infected plants, respectively (Prezelj et al. 2016a, b), is in line with this suggestion. In addition, a gene that encodes cysteine synthase is up-regulated in cv. Chardonnay infected with BN (Albertazzi et al. 2009; Hren et al. 2009a), as well as its protein product in FD-infected cv. Nebbiolo (Margaria and Palmano 2011).

At the same time, the level of chloroplast glutamine synthetase (Margaria et al. 2013), as well as its product glutamine (Prezelj et al. 2016a, b), are decreased in FD-infected plants (Fig. 3.3). It has been shown that *Pseudomonas syringae* pv. *tabaci* in tobacco provokes chlorosis through inhibition of glutamine synthetase, which results in accumulation of toxic levels of ammonia (Anzai et al. 1989). This ammonia could uncouple photosynthesis and photorespiration, and destroy the thylakoid membrane of the chloroplast, thereby causing chlorosis as a prominent symptom of GYP infection. The final effect is the reduced ability of the plant to respond actively to phytoplasmas as has been suggested for *P. syringae* pv. *tabaci* (Agrios 2005).

3.9 Changes in the Flavonoid Pathway in Grapevine Yellows Phytoplasma Infected Grapevines

Flavonoids comprise a large class of plant secondary metabolites of >10,000 compounds with very diverse roles, including grapevine responses to biotic stress (Gutha et al. 2010; Vega et al. 2011). After cv. Chardonnay infection with BN, the amounts of transcripts of several genes that encode enzymes involved in biosynthesis of phenolic compounds are increased, as well as the activities of their enzyme products

(Fig. 3.4). Specifically, genes that encode phenylalanine ammonia lyase, chalcone synthase, flavanone 3-hydroxylase, and leucoanthocyanidin dioxygenase are up-regulated in leaves upon BN infection (Hren et al. 2009a; Landi and Romanazzi 2011; Dermastia et al. 2015). In addition, the enzyme activities of phenylalanine ammonia lyase, chalcone synthase/ chalcone isomerase, flavanone 3-hydroxylase, and polyphenoloxidase (Rusjan et al. 2012a) are also increased in infected leaves. As a consequence, BN infection leads to increases in hydroxycinnamic acids (e.g. caftaric acid, sinapic acid glucose derivate, coumaric acid), flavanols (e.g., procyanidin B1, procyanidin dimer 3, catechin, epicatechin) and flavonols (e.g., quercetin 3-O- glucuronide, quercetin 3-O-glucoside) in leaves, especially in the period up to véraison. At the véraison stage in infected berries, the amounts of caftaric and coumaric acids, p-coumaroyl hexose, procyanidin B1, procyanidin trimer, quercetin-3-O-glucoside, quercetin-3-O-glucuronide and quercetin-3-O-xyloside are diminished. At berry softening, BN infection significantly increases the contents of total phenolics, hydroxycinnamic acids and flavanols, but decreases the flavonol contents, especially in symptomatic berry skins. At harvest, BN infection is associated with an additional significant decrease in coumaric acid and p-coumaroyl pentose; moreover, increases are also seen for procyanidin B1, procyanidin dimers and trimers, kaempferol-3-O-glucoside, and for most of the quercetins identified, except for quercetin-3-O-xyloside. During this period, non-symptomatic berries from infected plants show similar dynamics in their total phenolics contents, compared to berry skins from uninfected plants. On the other hand, the total flavanols and flavonols contents are similar to those in symptomatic berries (Rusjan et al. 2012b). Incomplete lignification of the shoot and the one-year-old canes of grapevines is a frequent symptom of infection with GYP. Lignification is a complex process that involves the phenylpropanoid and cinnamate/monolignol pathways (Boerjan et al. 2003). Infected canes have been demonstrated to have 4.6-fold higher induction of flavonols, 1.8-fold higher of flavanols, and 1.3-fold higher of stilbenoids, in comparison with BN-free canes. Thus incomplete cane lignification in phytoplasma-infected grapevines is associated with changes to many phenolic substances, especially individual flavonoids and stilbenoids, in the earlier phenological stages of cane lignification. Moreover, the significantly higher concentrations of hydroxycinnamic acid and monolignol derivatives, and of flavanone, in canes from BN-infected grapevines suggest alterations to the monolignol pathway, which might be responsible for lack of cane maturation (Rusjan and Mikulic-Petkovsek 2015).

The flavonoid pathway is also affected in FD-infected leaves of grapevine cultivars Barbera, Nebbiolo and Blaufränkisch (Fig. 3.4). Activation of anthocyanin accumulation in infected leaves has been indicated through transcript analysis of the genes of several enzymes involved in this pathway (e.g., chalcone synthase, flavanone-3-hydroxylase, leucoanthocyanidin dioxygenase, UGT-glucose:anthocyanin 3-O-glucosyltransferase, UAGT-transcription factor, anthocyanidine reductase, leucoanthocyanidine reductase, flavonol synthase, FLS-transcription factor) (Margaria et al. 2014; Prezelj et al. 2016a), and quantifica-

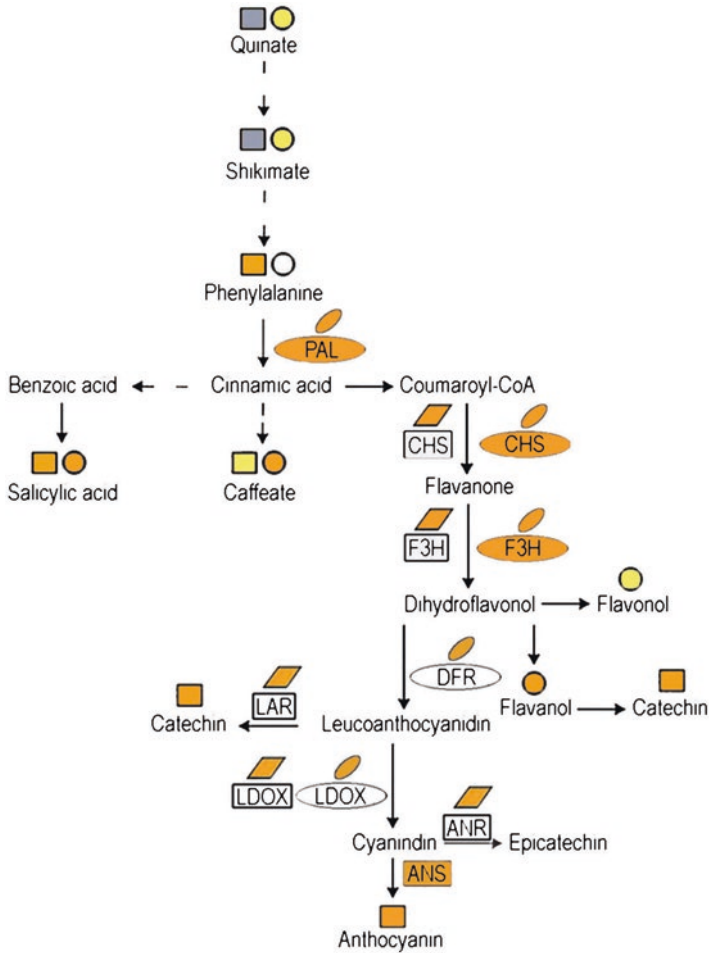


Fig. 3.4 Schematic representation of the metabolites in the phenylpropanoid metabolism pathways affected by FD (squares) and BN (circles) infections of grapevines, together with some of the enzymes that have been studied. Metabolite levels: *light blue*, uninfected sample > infected sample (not significant); *dark blue*, uninfected sample > infected sample ($p < 0.05$); *yellow*, infected sample > uninfected sample (not significant); *orange*, infected sample > uninfected sample ($p < 0.05$). Enzyme activities: *open rectangle/large ellipse*, infected sample not significantly different to uninfected sample; *yellow rectangle/large ellipse*, infected sample > uninfected sample; *orange rectangle/large ellipse*, infected samples > uninfected sample ($p < 0.05$). Gene expression: *orange rhombs/small ellipse*, enzyme in infected sample > uninfected sample ($p < 0.05$)

tion of anthocyanins (e.g., flavonols, proanthocyanidins). This is less pronounced in infected leaves of cv. Nebbiolo, which is known to be less susceptible to FD infection (Margarita et al. 2014).

3.10 Induced Salicylic-Acid-Dependent Systemic Acquired Resistance in Grapevines Infected with Grapevine Yellows Phytoplasmas

The source–sink transition upon pathogen infection is typically linked to coordinated defence responses, which enhances the expression of defence-related genes and the production of secondary metabolites (Ehness et al. 1997; Roitsch 1999; Rojas et al. 2014). Studies on GYP-infected grapevines are in agreement with these suggestions. Among the several classes of pathogenesis-related (PR) proteins, the significant up-regulation of genes from group 5 (PR-5; which encode thaumatin-like and osmotin-PR proteins) and group 2 (PR-2; which encodes β -1,3-glucanase) has been shown in BN-infected leaves (Albertazzi et al. 2009; Hren et al. 2009a, b; Landi and Romanazzi 2011; Santi et al. 2013b; Dermastia et al. 2015). As well as catalysing the hydrolysis of β -1,3-linkages of the cell-wall polymers found in plants, β -1,3-glucanases can hydrolyse fungal cell-wall β -1,3/1,6-glucans, and are induced when plants are stressed by microbial pathogens and/or herbivores (Iglesias & Meins 2000; Renault et al. 2000; Hao et al. 2008). At the moment, the possibility that increased gene transcription of β -1,3-glucanase in grapevine is due to phytoplasma infection cannot be ruled out. Increased gene transcription of β -1,3-glucanase potentially makes callose degradation products usable for phytoplasmas and/or to facilitate the phytoplasma spread through the plant, as has been suggested for planthopper attacks (Hao et al. 2008) and the spread of plant viruses (Iglesias and Meins 2000). On the other hand, under stress conditions, osmotin helps in the accumulation of the osmolyte proline, which quenches reactive oxygen species (ROS) and free radicals (Anil Kumar et al. 2015), and has been shown to be greatly increased in grapevines infected with GYP (Prezelj et al. 2016a, b).

The *PR-2* and *PR-5* genes are commonly used as molecular markers for salicylic-acid-dependent systemic acquired resistance signalling, and their expression is coordinately regulated by salicylic acid (Frías et al. 2013). It has been suggested that BN induces salicylic-acid-dependent systemic acquired resistance in leaves of infected tomatoes, which delays phytoplasma multiplication, in contrast to the jasmonic-acid-dependent and ethylene-dependent defence pathways (Ahmad et al. 2015). Significant up-regulation of the *PR-2* and *PR-5* genes in BN-infected samples, together with significant up-regulation of the gene encoding the enzyme responsible for biosynthesis of methyl salicylate, S-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase (Hren et al. 2009a; Dermastia et al. 2015), and the 26-fold increase in salicylic acid-glucopyranoside (Prezelj et al. 2016a, b), support this idea.

In cv. Blaufränkisch infected with FD, up-regulation of the *PR-2* and *PR-5* genes is not significant, and also the increase in salicylic acid and salicylic-acid-glucopyranoside (i.e., 7.52-fold, 13.13-fold, respectively) is not as pronounced as after BN infection. In contrast, the *PR-5* gene product increases 77-fold in FD-infected cv. Nebbiolo, which is known to be less susceptible to FD than other cultivars (Margaria and Palmano 2011). This might indicate the more aggressive

nature of FD and the stronger susceptibility of cv. Blaufränkisch. It has been suggested that although FD infection triggers a defence response, this response is not sufficient to block the FD infection (Gambino et al. 2013; Prezelj et al. 2016a).

3.11 The Recovery and Oxidative-Stress Phenomena

The progress of GY diseases appears to depend on antagonistic influences of the spontaneous disappearance of symptoms from the crown of the affected plants and of plant reinfection. The phenomenon of spontaneous disappearance of symptoms is known as recovery (Bertaccini and Duduk 2009), and although interesting, this is still a very unclear process. This can occur as either latent infection with temporal remission of symptoms, or complete recovery from the pathogen (Maixner 2006). Growing evidence has indicated that recovery is accompanied with ultrastructural and biochemical changes in the phloem, where phytoplasmas are located. Specifically, in phloem of grapevine cv. Chardonnay recovered from infection with BN the tissue is well preserved, in contrast to the similar one in infected plants. Callose deposits at sieve plates of recovered plants resemble more those of healthy than infected plants (although they sometimes occlude the lumen of the sieve pores), and P-protein occurs as condensed plugs or as filaments (Santi et al. 2013a). In grapevine cv. Prosecco (now cv. Glera), it was shown that recovery from FD infection coincides with accumulation of H_2O_2 in the sieve elements (Musetti et al. 2007). Additional work on cv. Barbera recovered from FD confirmed up-regulation of several genes that encode enzymes involved in H_2O_2 metabolism (i.e., germin-like protein, glycolate oxidase), as well as the presence of large amount of H_2O_2 (Gambino et al. 2013). H_2O_2 is one of the ROS that are commonly present in all aerobic cells. ROS can act up and/or downstream of various signalling cascades. The extent of ROS accumulation determinates their role in the cell, where at low levels, ROS can act as important signal-transduction molecules, or at high levels as toxic molecules with strong oxidant power. Their production and quantity is constantly equilibrated with the ROS scavenging systems (Camejo et al. 2016). It has been suggested that recovered plants can accumulate H_2O_2 because of the stable down-regulation of two genes that encode the main enzymatic H_2O_2 scavengers: catalase and ascorbate peroxidase (Musetti et al. 2007). Proteomic analysis of recovered cv. Barbera grapevines confirmed down-regulation of ascorbate peroxidase (Margarita et al. 2013), and catalase activity was lower in recovered grapevines of cv. Prosecco in comparison with FD-uninfected and infected plants (Musetti et al. 2007). However, in BN-uninfected and BN-infected plants of cv. Chardonnay, the changes in catalase activity appear insignificant (Landi and Romanazzi 2011).

In cv. Chardonnay recovered from BN, the transcript levels of sucrose synthase and vacuolar invertase were similar to those of healthy plants, although sucrose transporters and cell-wall invertase were expressed to greater degrees in recovered leaves than in healthy leaves (Santi et al. 2013a, b; Dermastia et al. 2015). It has been suggested that recovered plants acquire structural and molecular changes that lead to increases in sucrose transport and defence signalling (Santi et al. 2013a, b).

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