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Rootstock influences the effect of grapevine leafroll-associated viruses on berry development and metabolism via abscisic acid signalling

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Abstract

Grapevine leafroll-associated virus (GLRaV) infections are accompanied by symptoms influenced by host genotype, rootstock, environment, and which individual or combination of GLRaVs is present. Using a dedicated experimental vineyard, we studied the responses to GLRaVs in ripening berries from Cabernet Franc grapevines grafted to different rootstocks and with zero, one, or pairs of leafroll infection(s). RNA sequencing data were mapped to a high-quality Cabernet Franc genome reference assembled to carry out this study and integrated with hormone and metabolite abundance data. This study characterized conserved and condition-dependent responses to GLRaV infection(s). Common responses to GLRaVs were reproduced in two consecutive years and occurred in plants grafted to different rootstocks in more than one infection condition. Though different infections were inconsistently distinguishable from one another, the effects of infections in plants grafted to different rootstocks were distinct at each developmental stage. Conserved responses included the modulation of genes related to pathogen detection, abscisic acid (ABA) signalling, phenylpropanoid biosynthesis, and cytoskeleton remodelling. ABA, ABA glucose ester, ABA and hormone signalling-related gene expression, and the expression of genes in several transcription factor families differentiated the effects of GLRaVs in berries from Cabernet Franc grapevines grafted to different rootstocks. These results support that ABA participates in the shared responses to GLRaV infection and differentiates the responses observed in grapevines grafted to different rootstocks.

KEYWORDS

Closteroviridae, leafroll disease, plant–virus interaction, rootstock–scion interaction, *Vitis vinifera*

1 | INTRODUCTION

Grapevine leafroll-associated viruses (GLRaVs) are among the most consequential pathogens affecting grapevine and have considerable economic impact (Atallah et al., 2012; Fuchs et al., 2021; Ricketts et al., 2015). GLRaVs are diverse and belong to the family Closteroviridae, with six species and numerous strains in three genera (Fuchs, 2020; Naidu et al., 2015). Grapevines are often infected with several of these viruses simultaneously (Prosser et al., 2007; Rwahnih et al., 2009). Given their impact and global distribution, efforts to manage the spread of GLRaVs, characterize their effects, and understand the interaction between the vine and GLRaVs have been undertaken.

Generally, plant responses to viruses include numerous changes in gene expression, gene regulation, and metabolism (Alazem & Lin, 2015; Bester et al., 2017; Blanco-Ulate et al., 2017; Moon & Park, 2016). Pathogens and stresses elicit conserved responses from their hosts (Amrine et al., 2015; Jiang et al., 2015; Postnikova & Nemchinov, 2012; Rodrigo et al., 2012; Shaik & Ramakrishna, 2013). Infections with GLRaVs have been associated with poorer fruit quality, lower yield, and leaves that curl, redden, and become brittle. Gene expression studies that implicate regulatory systems in the leafroll disease phenotype are few in number and have focused on the impact of GLRaV-3, highlighting changes in the expression of senescence-associated and flavonoid biosynthetic pathway genes (Espinoza, Medina, et al., 2007, Espinoza, Vega, et al., 2007; Gutha et al., 2010; Vega et al., 2011). Additional transcriptomic study could help generate novel hypotheses concerning the controls that are fundamental to GLRaV responses (Gaiteri et al., 2014; Mandadi & Scholthof, 2013; Moon & Park, 2016).

Though common responses might be expected in infected plants given the relatedness of GLRaVs, there is considerable variability in the severity of GLRaV infections. Some GLRaV infections appear without symptoms or are mild (Kovacs et al., 2001; Montero et al., 2016; Poojari et al., 2013), but others cause significant changes in photosynthesis, metabolism, and gas exchange in leaves (Bertamini et al., 2004; Endeshaw et al., 2014; Guidoni et al., 1997, 2000; Pereira et al., 2012). Changes in fruit yield, organic and amino acids, titratable acidity, potassium, sugars, and flavonoids are also observed (Alabi et al., 2016; Cabaleiro et al., 1999; Kliewer & Lider, 1976; Lee et al., 2009; Lee & Martin, 2009). These are influenced by host genotype (Kovacs et al., 2001; Montero et al., 2016), which virus or combination of viruses is present (Credi, 1997; Guidoni et al., 2000; Komar et al., 2010), and environmental conditions (Cui et al., 2016). Leaf reddening, for example, is only observed in red-fruited grapevines (Naidu et al., 2015).

Evidence relating GLRaV responses to rootstock is mixed (Golino, 1993; Komar et al., 2010). In a study of Cabernet Franc vines grafted to different rootstocks, the effect of GLRaV infection on pruning weight depended on rootstock and the largest effects were observed in Kober 5BB-grafted vines (Rowhani et al., 2015). Similarly, fruit yield was influenced by both infection type and rootstock, with Kober 5BB-grafted vines most severely affected by a mixed infection with GLRaV-2, GLRaV-3, and grapevine fleck virus (Golino, Wolpert, et al., 2008). In another report, Red Globe scion buds infected with a strain of GLRaV-2 were used to inoculate Cabernet Sauvignon plants grafted to 18 different rootstocks; the infection was lethal in plants grafted to several rootstock genotypes, including Kober 5BB (Alkowni et al., 2011; Uyemoto et al., 2001).

This study used Cabernet Franc grapevines infected with zero, one, or two GLRaVs and grafted to two different rootstocks to (a) identify leafroll effects in ripening berries that were conserved across experimental conditions, and (b) determine whether or not GLRaV responses could be distinguished in berries from plants grafted to different rootstocks. Grapevines were grown in a single experimental vineyard and evaluated in four consecutive years. Vine growth and several measures of fruit composition were taken in the first two years. Total soluble solids (TSS) were measured in all four years. RNA sequencing (RNA-Seq), hormone, and metabolite data were collected from Cabernet Franc berries at four stages during ripening in the third and fourth years. RNA-Seq reads were mapped to the Cabernet Franc genome, which was sequenced in long PacBio reads, assembled using the FALCON-Unzip pipeline, and scaffolded using Hi-C data. The same samples were used to measure the levels of stress and ripening-associated hormones and metabolites. Among these were abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA). Though many of the GLRaV effects occurred in individual years, a subset of reproducible conserved responses and rootstock-differentiating responses were discovered.

2 | RESULTS

2.1 | GLRaV species and rootstock influence canopy density, cluster weight, and fruit composition

Cabernet Franc grapevines infected with individual and pairs of GLRaVs (GLRaV-1, GLRaV-1 [+]; GLRaV-3, GLRaV-3 [+]; GLRaV-4 strain 5, GLRaV-4 [+]; GLRaV-1 and GLRaV-2, GLRaV-1,2 [+]; GLRaV-1 and GLRaV-3, GLRaV-1,3 [+]) and grafted to different rootstocks (Millardet et de Grasset [MGT] 101-14 and Kober 5BB) were studied during grape berry ripening in a dedicated experimental vineyard at the University of California, Davis.

Typical grapevine leafroll disease symptoms (i.e., leaf reddening and curling) were observed by mid-ripening (Figure 1a). In addition, there was a visible, stark reduction in canopy density and cluster size in GLRaV-1,2 (+) versus GLRaV (−) in vines grafted to Kober 5BB that was not readily apparent in vines grafted to MGT 101-14 with the same infection status (Figure 1b).

Vine growth, cluster weight, and other measures were collected in 2015 and 2016 (Figure 2, Figure S1). The effect of GLRaV infection on dormant pruning weight, berry weight, pH, and tartaric acid content in 2015 and on moisture content, total anthocyanin content, and titratable acidity in 2016 differed significantly based on the rootstock present (analysis of variance [ANOVA], $p < .05$). This interaction was significant for malic acid in 2015 and 2016. Significant

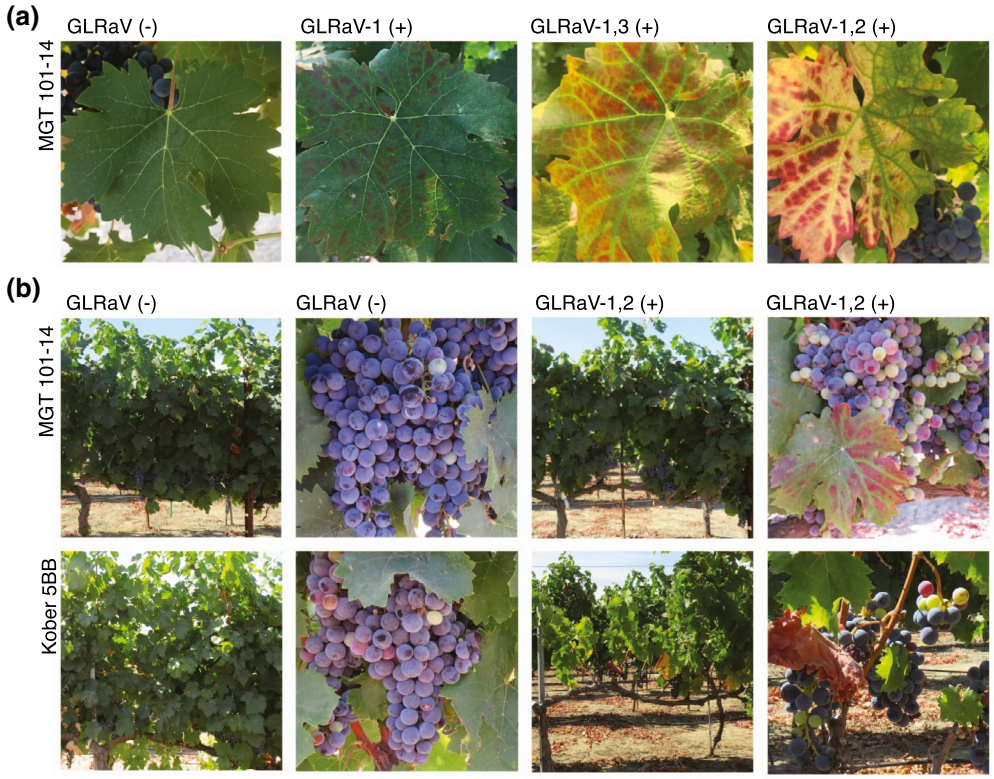

FIGURE 1 Examples of the effects of GLRaVs on Cabernet Franc leaves, canopy density, and cluster size. (a) Photographs depicting leaves from Cabernet Franc grapevines grafted to MGT 101-14. The photographed leaves were from (left to right) GLRaV (−), GLRaV-1 (+), GLRaV-1,3 (+), and GLRaV-1,2 (+). The photograph of the GLRaV (−) leaf was taken on 2018-08-13. The photographs of the leaves from GLRaV (+) were taken on 2018-08-08. The purpose of these photographs is to depict the range of leafroll disease symptoms in leaves observed in the study. The symptoms should not be construed as specific to certain infections. (b) Canopy and berry clusters from GLRaV (−) and GLRaV-1,2 (+) in different rootstock conditions on 2017-08-14

differences in dormant pruning weight, total cluster weight, and tartaric acid were observed in plants with different GLRaV infection status and rootstock (Tukey honestly significant difference [HSD] test, $p < .05$). In contrast, few or no significant differences between GLRaV (+) given the same rootstock were observed for total anthocyanins, moisture content, malic acid content, pH, titratable acidity, weight per berry, or yeast assimilable nitrogen and overwhelmingly in a single year if at all (Figure S1).

Overall, GLRaV infection tended to reduce dormant pruning weight and cluster weight. The dormant pruning weights and cluster weights of GLRaV-1,2 (+) was significantly lower than those of GLRaV (−) and other GLRaV (+) (Figure 2a,b); this was observed for both rootstock genotypes. Significant differences in fruit tartaric acid levels were observed only in 2015 and were between GLRaV-1,3 (+) grafted to different rootstocks and between plants with different GLRaV infection status (Figure 2c). In each year except 2015, there was a significant interaction between rootstock and GLRaV infection status in terms of TSS at harvest (ANOVA, $p < .05$, Figure 2d). This interaction was significant at each other developmental stage in 2017 and at prevéraison in 2018 (Figure S1). Significant differences in TSS at harvest were found between GLRaV (−) and GLRaV (+) in each year except 2017 (Figure 2d). Overall, significant reductions in TSS relative to GLRaV (−) were limited to the dual infections and GLRaV-3 (+). Significant differences were observed between rootstocks in GLRaV 1,2 (+) at every developmental stage, albeit only in 2017 (Figure 2d, Figure S1).

These data provide limited evidence that (a) different GLRaV infections may or may not affect various aspects of vine growth and fruit composition, (b) some of these differences are rootstock-specific, and (c) although some of these effects are observed across years, year-to-year differences may impact whether or not effects occur.

2.2 | GLRaVs elicit reproducible changes in gene expression across infection types and rootstocks

We used RNA-Seq to sequence the transcriptome of 384 Cabernet Franc berry samples collected from plants grafted to different rootstocks (Kober 5BB or MGT 101-14), with different GLRaV infection status, at four developmental stages (prevéraison, véraison, mid-ripening, and harvest), and in two consecutive years (2017 and 2018).

Because of the remarkable structural and gene content variability among grape cultivars (Da Silva et al., 2013; Minio et al., 2019; Venturini et al., 2013), we built a genome reference specifically

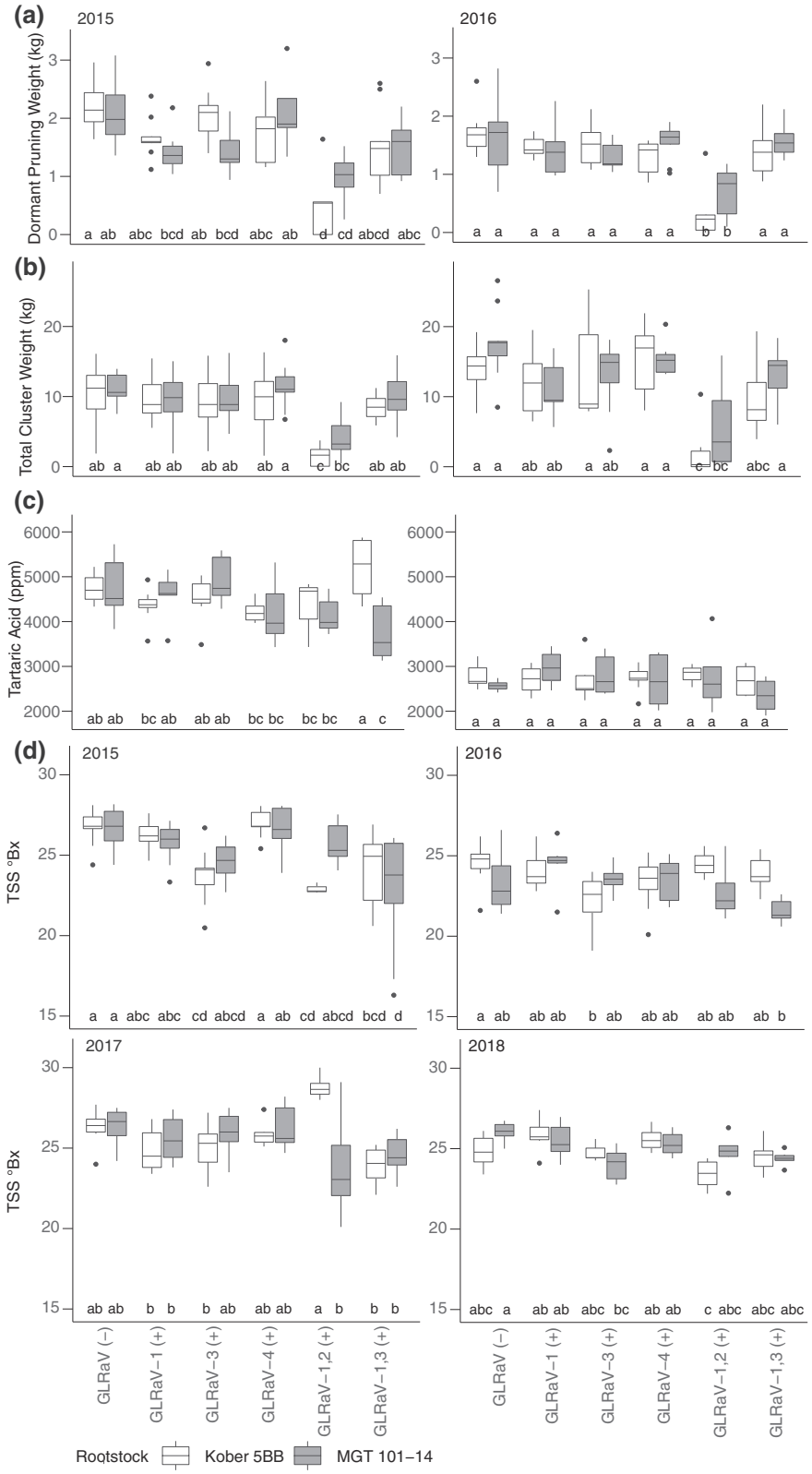

FIGURE 2 Effects of GLRaV infection on (a) dormant pruning weight, $5 \le n \le 9$, (b) total cluster weight, $5 \le n \le 9$, and (c) tartaric acid content, $3 \le n \le 9$, in 2015 and 2016. (d) Total soluble solids(TSS) at harvest in four consecutive years. 2015–2016, $5 \le n \le 9$; 2017–2018, $n = 6$. Group differences are indicated with nonoverlapping letters (Tukey HSD, $p < .05$)

for the analysis of these RNA-Seq data. The Cabernet Franc genome was assembled into 504 primary contigs (N50 = 5.74 Mb) for a total assembly size of 570 Mb. This is comparable to the size of the Zinfandel (591 Mb; Vondras et al., 2019), Cabernet Sauvignon (590 Mb; Chin et al., 2016), Chardonnay (490 Mb; Roach et al., 2018), and Pinot Noir PN40024 (487 Mb; Jaillon et al., 2007) genomes. In total, 3,085 additional haplotigs were assembled with an N50 of 184 kb (Table S3). The primary assembly and haplotigs were annotated with 33,563 and 19,146 protein-coding genes, respectively (Table S3).

Ripening was associated with transcriptomically distinct developmental stages. Samples clustered primarily by developmental

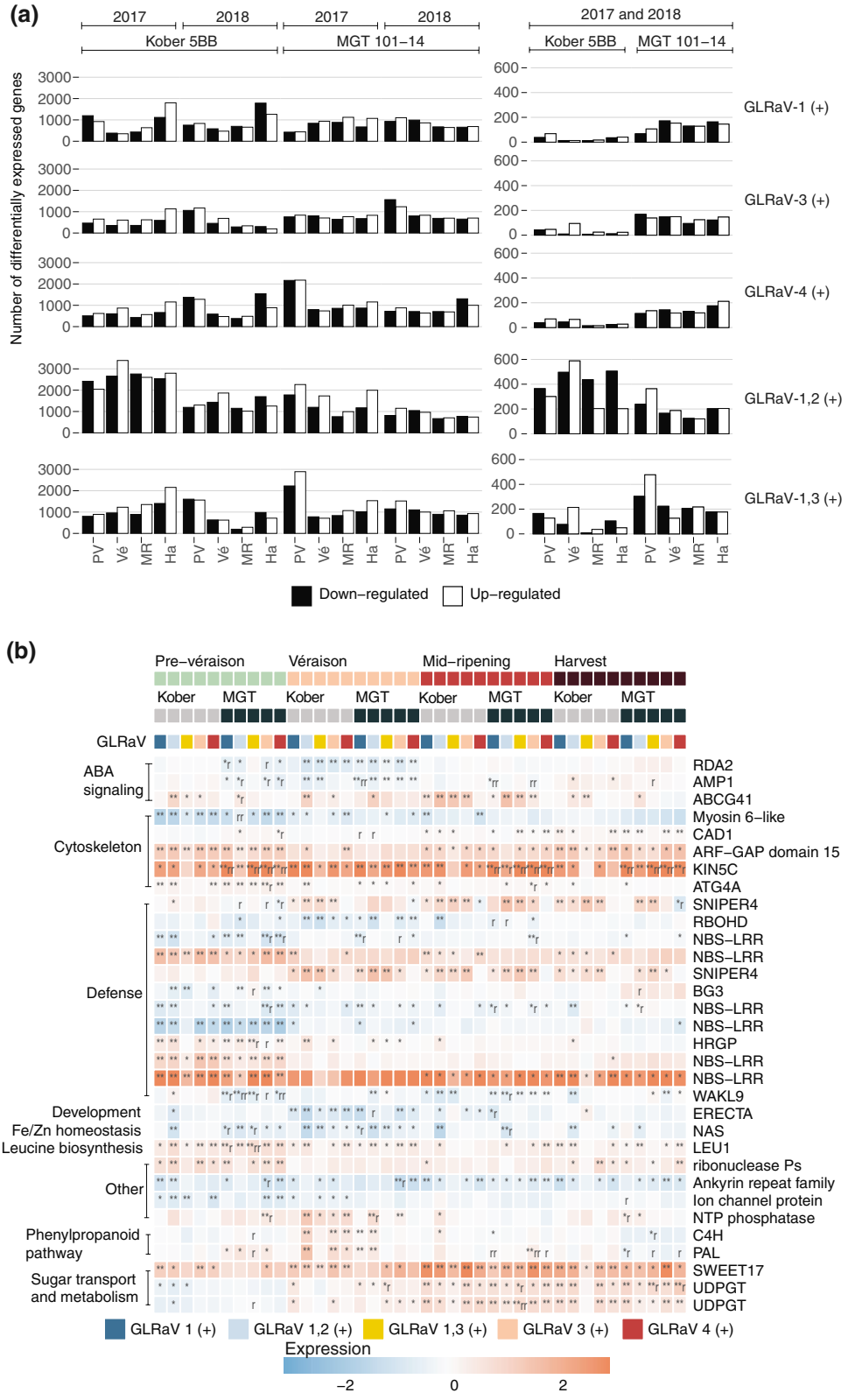

FIGURE 3 The conserved responses of ripening Cabernet Franc berries to GLRaV infection(s). (a) Barplots showing the number of differentially expressed ($p < .05$) genes up- and downregulated in GLRaV (+) versus GLRaV (−) in berries from Cabernet Franc grapevines grafted to different rootstocks (Kober 5BB [Kober] and MGT 101-14 [MGT]) at each developmental stage (prevéraison [PV], véraison [Vé], mid-ripening [MR], and harvest [Ha]) in 2017, in 2018, and in both years. (b) Heatmap showing the responses to GLRaVs ($p < .05$) in both rootstock conditions and in more than one GLRaV infection condition. *Differentially expressed in 1 year; **differentially expressed in both years. One or two letters "r" indicate that the effect of a particular GLRaV infection differs between rootstocks at the same developmental stage in 1 or 2 years. Any notation requires the direction (up/downregulation) of the effect to be consistent in both years, even if a significant change occurred in only 1 year

stage and secondarily by year, though samples at harvest clustered separately (Figure S2). Genes with comparable, significant responses (consistently upregulated or downregulated, $p < .05$) in both years of the study were selected to identify reproducible responses to GLRaVs during ripening. Gene expression in GLRaV (+) was compared to gene expression in GLRaV (−) grafted to the same rootstock at the same developmental stage (Figure 3). In addition, the effects of each GLRaV infection on gene expression at each developmental stage were compared in plants grafted to different rootstocks (Figure S3).

On average, 7.1% of the genes differentially expressed between GLRaV (−) and GLRaV (+) were reproduced in both years (Figure 3a). This percentage was slightly above average for plants with dual, relatively more severe, infections (8.6%, GLRaV-1,3 [+]; 8.9%, GLRaV-1,2 [+]) and below average for individual infections (5.8%, GLRaV-1 [+]; 6% GLRaV-4 [+]; 6.2% GLRaV-3 [+]). A subset of 32 genes significantly changed their expression level in two or more GLRaV (+) infection conditions, in both rootstock conditions, and at least one developmental stage (Figure 3b). These genes constitute the "conserved" responses to GLRaVs in Cabernet Franc berries during ripening.

The majority of these differentially expressed genes are associated with defence, ABA signalling, and cytoskeleton organization and biogenesis (Figure 3b; Table S4). Six of these were genes encoding nucleotide-binding site and leucine-rich repeat-containing (NBS-LRR) proteins; half of these were upregulated. Two F-box genes encoding SNIPER4 were upregulated (Huang et al., 2018), as was a gene encoding a hydroxyproline-rich glycoprotein (HRGP). HRGP and NBS-LRR proteins are associated with pathogen detection (DeYoung & Innes, 2006). Genes encoding a respiratory burst oxidase protein D (RBOHD), a wall-associated kinase-like protein (WAKL), and a β-glucosidase 3 (BG3) were downregulated. RBOHD participates in the production of reactive oxygen species (ROS) and hypersensitive responses (HRs) to pathogens (Otulak-Kozieł et al., 2019). RBOH family proteins are targeted by Snf1-related kinase 2 (SnRK2) phosphorylation, a key component of the ABA signalling pathway. Likewise, a *WAKL* gene in citrus participates in JA and ROS signalling (Li et al., 2020). Among the functions of β-glucosidases are the activation of ABA and SA by freeing them from the conjugates that render them inactive (Jia et al., 2016; Morant et al., 2008; Seo et al., 1995; Sun et al., 2015; Zhang et al., 2013). Several ABA-related genes were among the conserved GLRaV responses, including an upregulated ABC transporter and two downregulated genes, *AMP1* and *RDA2*. *AMP1* negatively regulates ABA sensitivity (Shi et al., 2013). *RDA2* participates in the inhibition of ABA signalling and the promotion of MAPK signalling (Park et al., 2019).

Five genes related to cytoskeleton organization were sensitive to GLRaV infection. Only one of these, a myosin VI motor protein-coding gene, was downregulated. The four others were an autophagy gene (*ATG4A*) and *constitutively activated cell death 1* (*CAD1*), which function in autophagy, lytic pore formation, and HRs (Haxim et al., 2017; Morita-Yamamuro et al., 2005; Yoshimoto et al., 2004), *Kinesin-like 5C* (*KIN5C*), which encodes a microtubule motor protein (Reddy & Day, 2001), and an *ARF-GAP* encoding ADP-ribosylation factor GTPase-activating protein domain 15, which helps efficiently load vesicles and remodel the actin cytoskeleton (Inoue & Randazzo, 2007).

Several additional general functional categories were present among the 32 genes that exhibited conserved responses to GLRaVs (Figure 3b). Genes encoding phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H), which catalyse the first two steps of the phenylpropanoid pathway, two genes encoding UDP glucosyltransferases (UDPGTs), which conjugate sugars, and *SWEET17*, encoding a sugar transporter, were upregulated, as was a gene encoding 3-isopropylmalate dehydratase, an enzyme in the leucine biosynthetic pathway. Two genes, encoding an LRR receptor-like kinase (LRR-RLK) called ERECTA and nicotianamine synthase (NAS), were downregulated. ERECTA participates in organ development and resistance to bacterial and fungal pathogens (Goff & Ramonell, 2007). *NAS* expression increases Fe and Zn abundance in rice (Moreno-Moyano et al., 2016; Nozoye, 2018).

Generally, these genes and their changes in expression suggest that a common response to GLRaVs in Cabernet Franc berries during ripening includes the modulation of pathogen-detecting genes, an increase in ABA transport and signalling, a decrease in ROS-related signalling, and an enhancement of cytoskeleton remodelling, vesicle trafficking, phenylpropanoid metabolism, sugar transport and conjugation, and leucine biosynthesis.

2.3 | Rootstock influences the impact of GLRaV on ABA metabolism

The same berry samples used for RNA-Seq were used to measure the levels of three hormones associated with ripening and/or stress, including SA, JA, and ABA, and additional metabolites, including xanthoxin, a precursor to ABA, and ABA glucose ester (ABA-GE), a conjugate of ABA implicated in its long-distance transport (Jiang & Hartung, 2008). The mean levels of SA and JA were significantly influenced by year and/or by interactions between year, rootstock, and GLRaV at prevéraison (ANOVA, $p < .05$), but no significant differences were observed between individual groups (Tukey HSD, $p > .05$) (Figure S4). In contrast, year alone had a significant impact on the levels of ABA and related metabolites measured at each developmental stage, but largely did not interact with rootstock or GLRaV infection type to affect the abundance of ABA and related metabolites (Figure S4). In addition, the effect of GLRaV infection on ABA and ABA-GE content significantly differed based on rootstock (Figure S4). Significant differences in ABA and ABA-GE content were observed between rootstocks in plants with identical infection status and between plants with different GLRaV status grafted to the same rootstock (Tukey HSD, $p < .05$). Such differences were scarcely observed for xanthoxin, a precursor to ABA (Figure 4a, Figure S4).

Significant differences between rootstock genotypes in the abundance of these metabolites were observed most at prevéraison and in GLRaV (−), GLRaV-3 (+), and dual infections (Figure 4a,

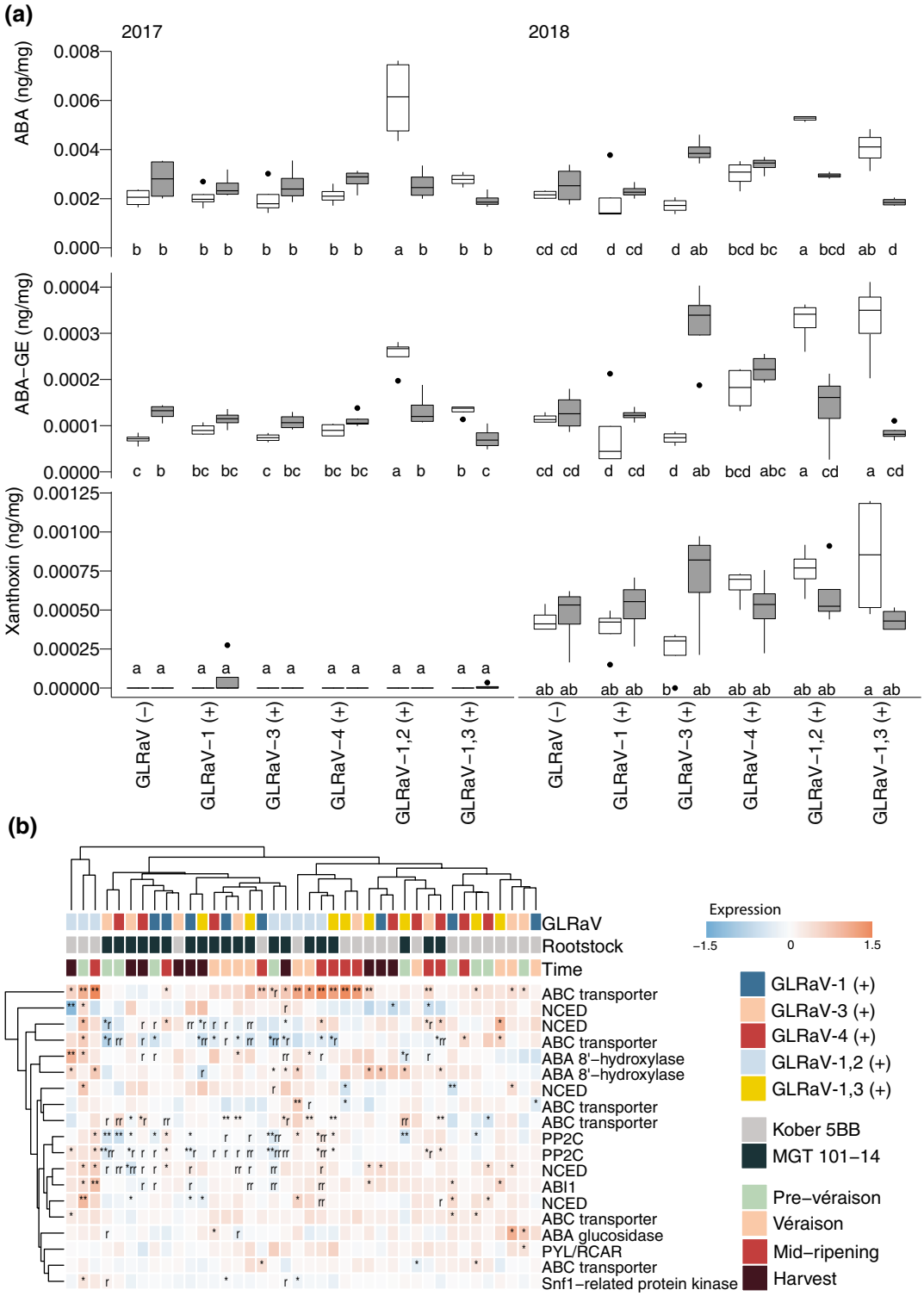

FIGURE 4 ABA metabolism and signalling pathways are sensitive to GLRaV infection. (a) Boxplots showing the abundance of abscisic acid (ABA), ABA glucose ester (ABA-GE), and xanthoxin in 2017 and 2018 at prevéraison. Groups with nonoverlapping letters are significantly different (Tukey HSD, $p < .05$). (b) The effect of GLRaV infection(s) and rootstock on ABA biosynthesis and signalling genes. *Differentially expressed in 1 year; **differentially expressed in both years. One or two letters "r" indicate that the effect of a particular GLRaV infection differs between rootstocks at the same developmental stage in 1 or 2 years. Any notation requires the direction (up/downregulation) of the effect to be consistent in both years, even if a significant change occurred in only 1 year

Figure S4). In GLRaV (–) and most single infection conditions, the levels of all three metabolites tended to be higher in berries from plants grafted to MGT 101-14 than in berries from plants grafted to Kober 5BB. The opposite tended to be true when two GLRaVs were present. With one exception, significant changes in the abundance of ABA and ABA-GE in GLRaV (+) versus GLRaV (–) were typically

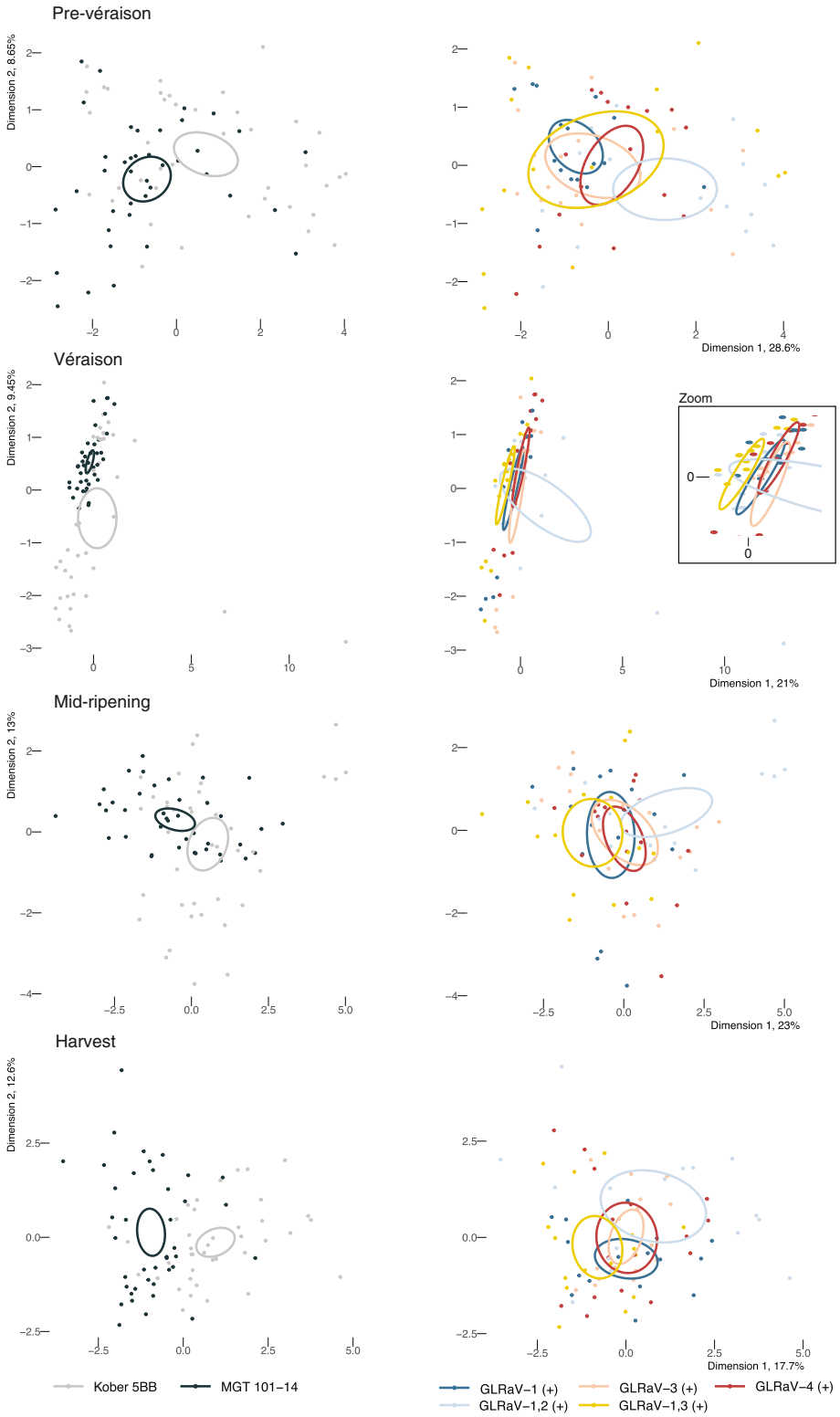

FIGURE 5 Multiple factor analysis (MFA) of the effects of GLRaV infection. This scatterplot shows the distribution of samples along the first two MFA dimensions at each developmental stage. For each rootstock (left) and each GLRaV infection condition (right), 95% confidence ellipses are drawn

increases and were most often observed in berries from Kober 5BB-grafted plants (Tukey HSD, $p < .05$). Though slight reductions in these metabolites were observed versus GLRaV (−), these were almost always nonsignificant.

We further investigated ABA biosynthesis and signalling using a previously published, curated set of genes (Pilati et al., 2017). Their expression largely clustered according to rootstock, with some exceptions; Kober 5BB GLRaV-1,2 (+), for example, tended

to cluster separately from other Kober 5BB-grafted plants. Nonetheless, the effects of GLRaVs differed between rootstocks for many of these genes (Figure 4b). One of these, encoding an ABC transporter (VITVvi_vCabFran04_v1_P438.ver1.0.g381930), is also included in Figure 3; significant increases in its expression were observed in both years, in both rootstock conditions, and for several GLRaV infections. All other significant changes in GLRaV (+) versus GLRaV (−) that were reproduced in both years occurred in only one rootstock or the other. These changes were sparse. However, significant differences between rootstocks in identical GLRaV (+) were reproduced in both years for 9 out of these 19 genes. Significant differences between rootstocks in at least one year were observed for 16 out of these 19 genes. On average, three genes encoding 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), both ABA 8′-hydroxylase genes, three ABC transporter genes, one gene encoding PP2C, and PYL/RCAR were upregulated in berries from plants infected with GLRaV in both rootstock conditions. One ABC transporter gene was downregulated in both rootstock conditions. Of the remaining eight genes, most were downregulated across development only in berries from MGT 101-14-grafted plants.

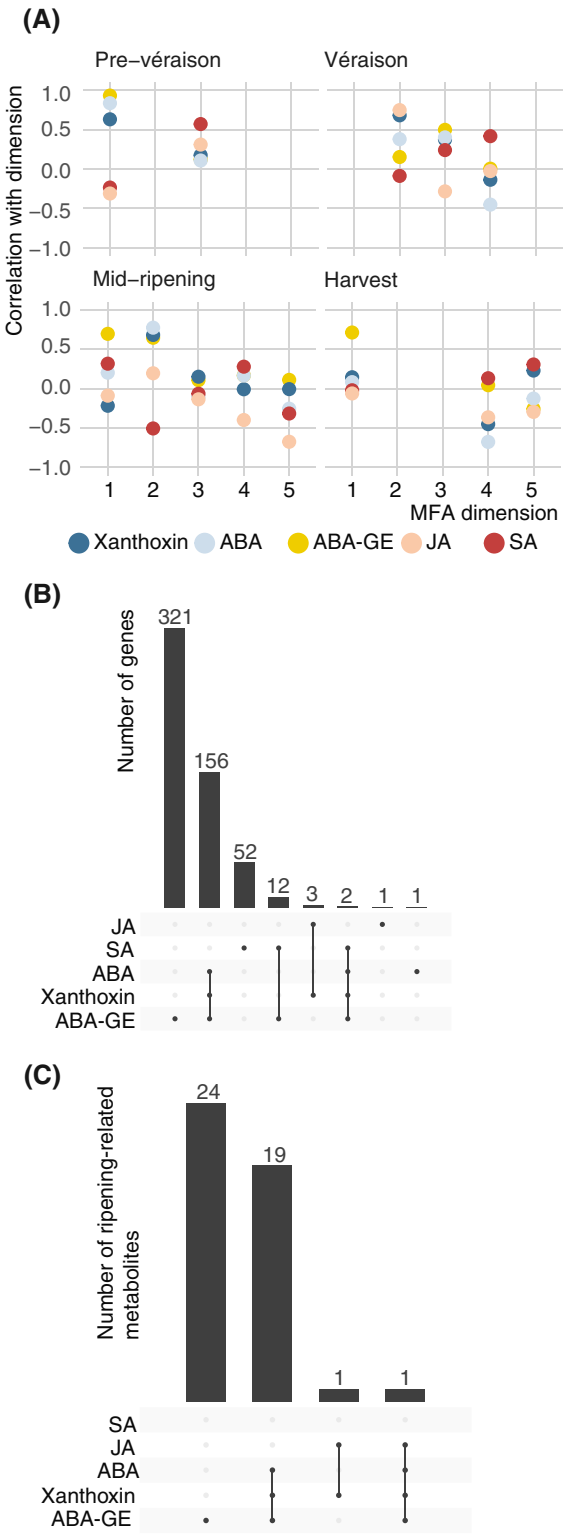

FIGURE 6 The roles of genes, hormones, and metabolites in a multiple factor analysis (MFA) of GLRaV effects. (a) Correlation between hormones and hormone-related metabolites to rootstock-differentiating MFA dimensions. If two variables both have either a strong positive or a strong negative correlation (|corr| > 0.5) to the same rootstock-differentiating MFA dimension, their relationship is counted in the UpSet plots in(b) and (c), which are analogous to Venn diagrams. (b and c) The numbers of (b) genes and (c) ripening-related metabolites with similar relationships to rootstock-differentiating MFA dimensions as the hormone(s) and/or hormone-related metabolites indicated below each bar. ABA, abscisic acid; ABA-GE, ABA glucose ester; JA, jasmonic acid; SA, salicylic acid

2.4 | Gene expression, hormone, and other metabolite data distinguish the effects of GLRaVs in different rootstocks

Differential expression analysis identified 1,809 genes (a) that were differentially expressed ($p < .05$) in at least 1 year, (b) that were differentially expressed in only one rootstock condition and more than one GLRaV infection type versus GLRaV (−), and/or (c) for which the effects of more than one GLRaV infection significantly differed between rootstocks (Figure S5). RNA-Seq, hormone, and metabolite data from ripening Cabernet Franc berries were integrated in a multiple factor analysis (MFA) to relate these variables and distinguish the effects of GLRaVs given different rootstocks (Figure 5, Figure S6, Table S5).

As input for the MFA, all genes differentially expressed between GLRaV (−) and GLRaV (+) or between rootstocks were used, plus all hormones and metabolites measured. Overall, the rootstocks were distinct at each developmental stage (Figure 5). Some of the GLRaV (+) conditions could be distinguished from others at prevéraison, véraison, and harvest. At prevéraison, GLRaV-1,2 (+) differed overall from GLRaV-1 (+). At véraison, GLRaV-1,3 (+) differed from every other GLRaV (+) condition except GLRaV-1,2 (+). At harvest, the two dual infections were different than one another and GLRaV-1,2 (+) differed from GLRaV-1 (+) (Figure 5).

Next, we identified which variables were best correlated with each rootstock-differentiating MFA dimension. At each

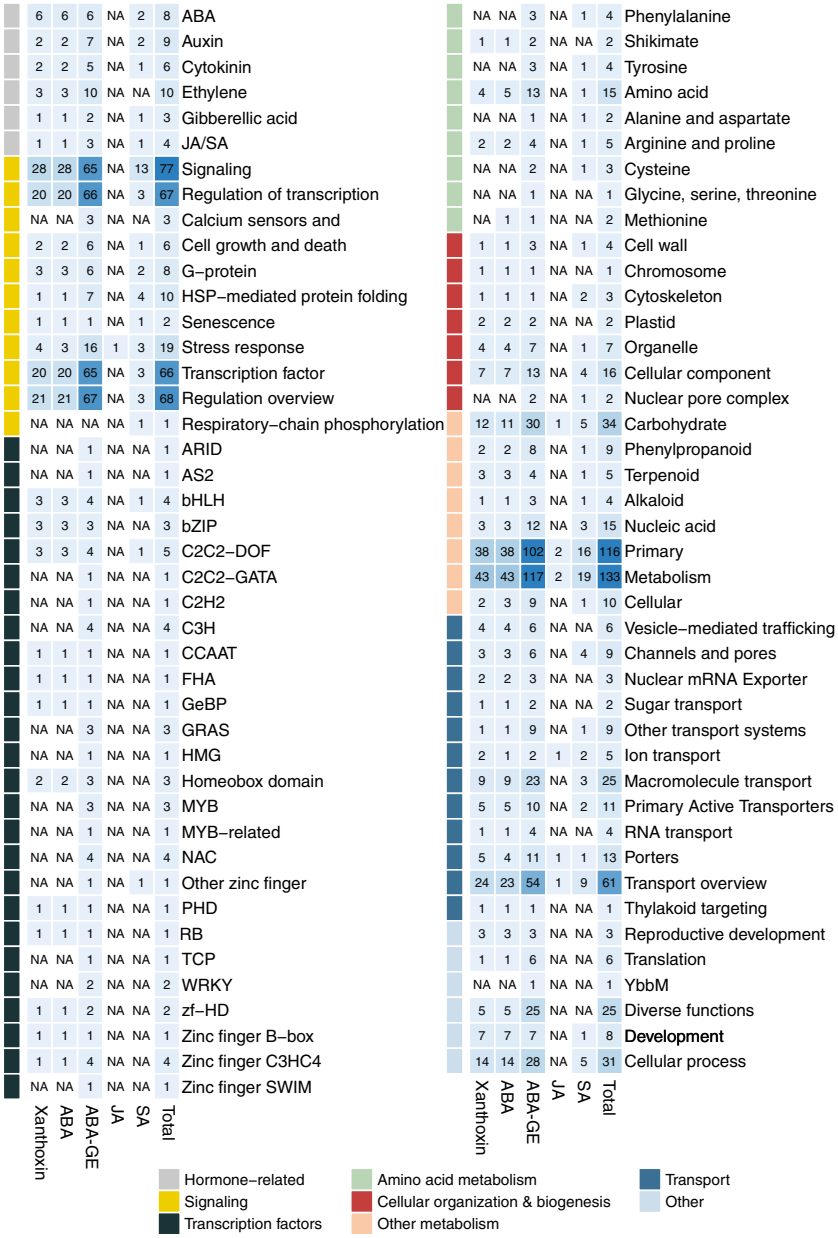

FIGURE 7 Functional categories of genes correlated (|corr| > 0.5) to the same rootstock-differentiating multiple factor analysis dimensions as hormones and/or hormone-related metabolites. The counts of genes, per category, related to each hormone and metabolite are shown. ABA, abscisic acid; ABA-GE, ABA glucose ester; JA, jasmonic acid; SA, salicylic acid

developmental stage, ABA and/or ABA-related metabolites were correlated with at least one of the first two MFA dimensions (Figure 6a). The rootstock-dependent disparity in ABA levels and ABA-related gene expression (Figure 4) is consistent with the observation that ABA and related metabolites tended to be highly correlated with rootstock-differentiating MFA dimensions over time and that ripening initiates earliest in Kober 5BB plants with dual infections in terms of TSS (Figure S1).

There were 548 genes that shared high correlation (|corr| > .5) to rootstock-differentiating MFA dimensions with hormones or hormone-related metabolites. Most of these genes had shared positive or negative correlations to the same dimensions as ABA, xanthoxin, and/or ABA-GE (Figure 6b). Categories of genes with functionally relevant relationships to each hormone or hormone-related metabolite were over-represented (hypergeometric test, $p < .05$ and $n > 1$ gene) among the genes that shared high correlation to rootstock-differentiating MFA dimensions with each hormone. ABA signalling, starch biosynthesis and catabolism, and C2C2-DOF transcription factor-encoding genes were over-represented among the genes correlated to the same dimensions as ABA and xanthoxin. The latter two categories were significantly over-represented among the genes correlated with the same dimensions as ABA-GE. Genes related to heat shock protein (HSP)-mediated protein folding, chaperone-mediated protein folding, the cation channel-forming HSP-70, channels and pores, the reductive carboxylate cycle, and carbon fixation were over-represented among those correlated with the same MFA dimensions as SA. Similarly, most ripening-related metabolites measured were correlated with the same MFA dimensions as ABA, xanthoxin, and/or ABA-GE (Figure 6c).

Overall, the effects of GLRaVs differ between rootstocks primarily in terms of ABA and related metabolites. This finding is especially salient because of the role that ABA plays as a ripening promoter

near véraison, in root–scion communication, and in plant stress. ABA, metabolites, and genes that were well correlated to rootstock-differentiating MFA dimensions and were differentially expressed were scrutinized more closely.

2.5 | Rootstock influences the impact of GLRaV on hormone signalling genes and transcriptional controls

There were 548 genes in 85 functional categories that were well correlated with rootstock-differentiating MFA dimensions and differentially expressed between GLRaV (+) grafted to different rootstocks or in GLRaV (+) versus GLRaV (−) in only one rootstock condition (Figure 7). These functional categories were generally related to hormone and other types of signalling, amino acid and other metabolic pathways, transcription factors, transport, and cellular organization and biogenesis (Figure 7). Most of these genes coincided with ABA and related metabolites along rootstock-differentiating MFA dimensions (Figure 7).

The distribution of expression for four transcription factor families differed significantly between rootstocks at all four developmental stages (Figure S7, Kolmogorov–Smirnov test, $p < .05$). This included bHLH, C2C2-DOF, FHA, and homeobox domain transcription factors. The distribution of expression of 39 hormone signalling-related genes differed significantly between rootstocks (Figure 7, Figure S7, File S1, Kolmogorov–Smirnov test, $p < .05$). This was true at each developmental stage for ABA, gibberellin (GA), and auxin signalling genes and at three developmental stages for JA/SA, cytokinin (CK), and ethylene signalling genes (Figure S7). Genes related to all of these hormone families had similar roles in MFA and were associated with ABA, including the JA/SA signalling genes (Figure 7). This may reflect interactions between hormone signalling pathways. In addition, the effects of GLRaV infections (dual infections and GLRaV-4 [+]) on histone H1 expression were not equal in plants grafted to both rootstocks (Figure S8). Linker histone H1 contributes to higher-order chromatin structure (Hill, 2001).

There were seven ABA signalling pathway genes (excluding VITVvi_vCabFran04_v1_P495.ver1.0.g468110, which was also associated with GA signalling) that differentiated GLRaV effects in plants grafted to different rootstocks (Figure 8). The effect of GLRaV on expression for all of these differed between rootstocks (Figure S7). This included *SOS2*, *KEG*, three *PP2C* genes (*HAB1*, *AHG3*, *DBP*), and two genes encoding ABA-responsive element (ABRE)-binding proteins (*AREB2*, *ABI5*). SOS2 is a kinase appreciated for its role in the salt stress response, seed germination, GA signalling (Trupkin et al., 2017), and ABA signal transduction via its interaction with ABI2 and ABI5 (Ji et al., 2013; Zhou et al., 2015). SOS2 was upregulated in both rootstock conditions before and at véraison and downregulated after véraison. KEG is a negative regulator of ABA signalling; it maintains low levels of ABI5 in the absence of stress by ubiquitination and degradation (Lyzenga et al., 2013; Stone et al., 2006) and helps regulate endocytic trafficking and the formation of signalling complexes on vesicles during stress (Gu & Innes, 2011). KEG was downregulated in Kober 5BB and downregulated in MGT 101-14 at véraison and mid-ripening.

In the presence of ABA, ABA receptors (PYR/PYL/RCAR family proteins) bind PP2Cs like HAB1 and AHG3 to inhibit their phosphatase activity. As a result, ABA signal transduction is permitted via SnRK2 phosphorylation of ABRE-binding proteins (Hirayama & Shinozaki, 2010). ABI5 and AREB2 are bZIP transcription factors that bind to ABREs to drive ABA signalling and ABI5 can integrate signals across hormone signalling pathways (Skubacz et al., 2016). The effects of GLRaVs on these genes in Kober 5BB were consistent with an enhancement of ABA signalling during ripening. In Kober 5BB, HAB1, AHG3, AREB2, and ABI5 were upregulated. In MGT 101-14, the PP2Cs were downregulated at two or more developmental stages; AREB2 and ABI5 were upregulated at and after véraison.

2.6 | Rootstock influences the impact of GLRaV on the flavonoid biosynthetic pathway

In addition to analysing hormones and hormone-related metabolites, we analysed metabolites associated with the shikimate, phenylpropanoid, and flavonoid pathways (Figure 9a,b) and their biosynthetic and regulatory genes (Figure 9c) in Cabernet Franc berries during ripening (Figure S9). Significant differences in expression versus GLRaV (−) were detected, as well as significant differences in the effects of GLRaV infection between different rootstock conditions (Figure 9c). The effects of GLRaV infection on the genes associated with this pathway were generally consistent with the change in abundance of corresponding metabolites (Figure 9b). Overall, these genes tended to be upregulated in GLRaV (+) at véraison (Figure 9c). After véraison, the amount of upregulation tended to decrease, or genes were downregulated (Figure 9c). The three amino acids examined (phenylalanine, tryptophan, and tyrosine) tended to be less abundant in GLRaV (+) across the developmental stages and the largest decreases were observed at harvest (Figure 9b). Mixed effects of GLRaVs were observed on the abundance of hydroxycinnamic acids (caftaric and coutaric acid), *t*-resveratrol, and anthocyanins. Significant changes versus GLRaV (−) tended to occur in only 1 year. During ripening, these were significantly more abundant in Kober 5BB GLRaV-1,2 (+), Kober 5BB GLRaV-4 (+), and/or MGT 101-13 GLRaV-3 (+) (Figure 9b). Significant decreases were observed for GLRaV-1 (+) and GLRaV-1,3 (+). Though nonsignificant, the size of the downward effect of some GLRaV infections on these metabolites tended to increase towards harvest. Finally, flavanols (epigallocatechin and catechin) and flavonol (quercetin) glycosides tended to be elevated in GLRaV (+) (Figure 9b). The size of this effect tended to be greatest before and at véraison and decreased towards harvest. Significant differences between rootstocks were observed for GLRaV-1,2 (+) in both years and for GLRaV-1 (+), GLRaV-1,3 (+), and GLRaV-3 (+) in individual years;

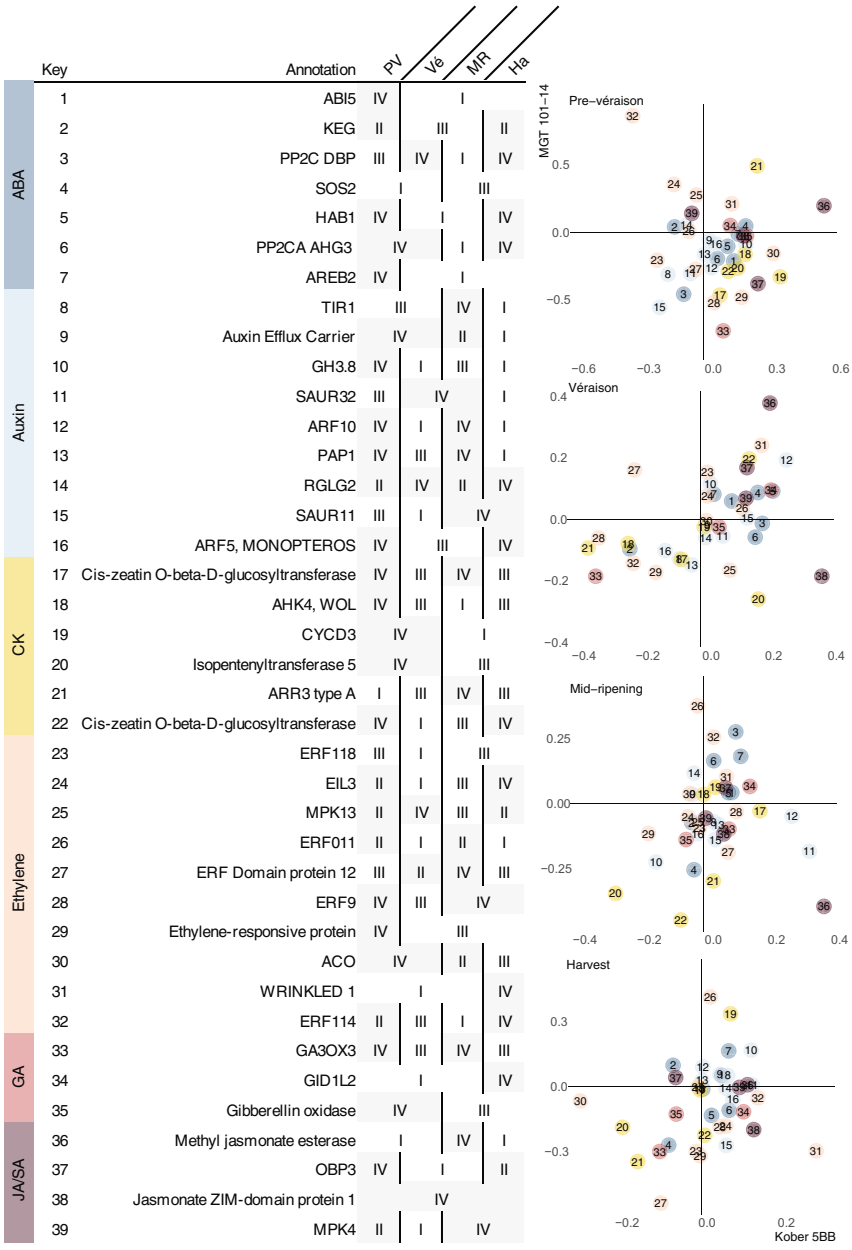

FIGURE 8 The effect of GLRaVs on hormone signalling gene expression in grape berries from plants grafted to different rootstocks. Quadrants are numbered counterclockwise from top right (I) to bottom right (IV). Individual genes are numbered 1–39. The key (left) indicates in which quadrant each gene can be found at each developmental stage. Developmental stages are abbreviated. PV, prevéraison; Vé, véraison; MR, mid-ripening; Ha, harvest. ABA, abscisic acid; CK, cytokinin; GA, gibberellin; JA, jasmonic acid; SA, salicylic acid

the increase in flavonols and flavanols tended to be greater in berries from Kober 5BB GLRaV (+) than in those from MGT 101-14 GLRaV (+).

3 | DISCUSSION

GLRaVs affect viticulture on nearly every continent (Akbaş et al., 2007; Charles et al., 2009; Fiore et al., 2008; Golino, Weber, et al., 2008; Habili & Nutter, 1997; Jooste et al., 2015; Mahfoudhi et al., 2008) and can a have considerable economic impact on a major crop. The presence and severity of symptoms in GLRaV-infected grapevines is influenced by host genotype, rootstock, which GLRaV is present, and environmental conditions. In addition to the assembly and annotation of the Cabernet Franc genome, a valuable resource that might be applied for the larger purpose of understanding grapevine genomic diversity and evolution, the dedicated experimental vineyard used in this study is a tremendous asset for the study of GLRaV infections over time in a common environment. This work identified responses to GLRaVs in grape berries during ripening, including those that are conserved across experimental conditions and responses that differ based on the rootstock present. We propose which hormones and signalling pathways at least partially govern the responses observed and likely influence leafroll disease symptoms.

The effects of dual infections, particularly GLRaV-1,2 (+), were most distinctive. All of the leafroll viruses selected for this study belong to the *Closteroviridae* family and all but one belong to the *Ampelovirus* genus; GLRaV-2 belongs to the genus *Closterovirus*. All of the GLRaVs used in this study contain a conserved replication gene block (RGB) but are diverse outside of the RGB (Naidu et al., 2015). In addition to host genotype and environment, the sequences

downstream of the RGB may account for the disparities in responses observed between infection types. These sequences encode a quintuple gene block and/or viral suppressors of host RNA silencing (VSRs; Naidu et al., 2015). Some VSRs are characterized (Gouveia & Nolasco, 2012), but most are not. Further research might determine their specific effects and relationship to host cellular machinery (Chapman et al., 2004; Wu et al., 2010).

Changes in the expression of NBS-LRR genes were among the conserved responses to GLRaVs and were the single largest category of genes among them. NBS-LRR genes confer resistance to

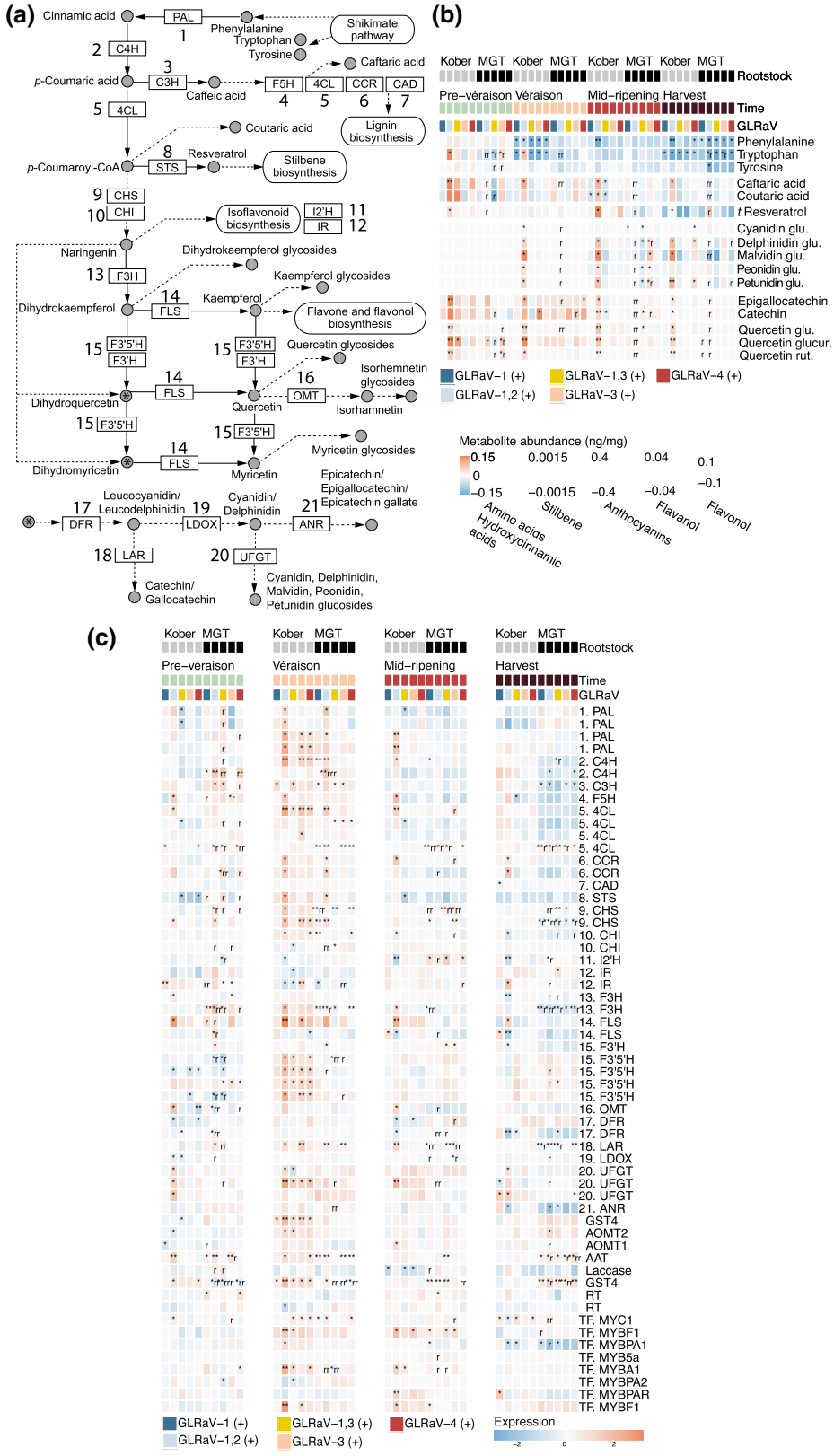

FIGURE 9 Differentially expressed genes and selected metabolites produced in the shikimate, phenylpropanoid, and flavonoid pathways. (a) Pathway diagram. (b) Metabolite abundances and (c) related biosynthetic and regulatory gene expression relative to GLRaV (−) in identical rootstock and at the same developmental stage. Notation requires the direction (up/downregulation) of the effect to be consistent in both years, even if a significant change occurred in only a single year. Glycosides are abbreviated: 3-*O*-glucoside [glu], 3-*O*-glucuronide [glucur], and 3-*O*-rutinoside [rut]. *Differentially expressed/abundant in 1 year; **differentially expressed/abundant in both years. One or two letters "r" indicate that the effect of a particular GLRaV infection differs between rootstocks at the same developmental stage. Kober, Kober 5BB rootstock; MGT, MGT 101-14 rootstock. PAL, phenylalanine ammonia-lyase; C4H, trans-cinnamate 4-monooxygenase; C3H, *p*-coumarate 3-hydroxylase; F5H, ferulate-5-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; STS, stilbene synthase; CHS, chalcone synthase; CHI, chalcone isomerase; I2′H, isoflavone 2′-hydroxylase; IR, isoflavone reductase; F3H, flavonone 3-hydroxylase; FLS, flavonol synthase; F3′H, flavonoid 3′-monooxygenase; F3′5′H, flavonoid 3′,5′-hydroxylase; OMT, *O*-methyltransferase; DFR, dihydroflavanol 4-reductase; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxgenase; UFGT, UDP-glucose:anthocyanidin/flavonoid 3-*O*-glucosyltransferase; ANR, anthocyanidin reductase; GST4, glutathione *S*-transferase 4; AOMT, anthocyanin *O*-methyltransferase; AAT, anthocyanin acyl-transferase; RT, UDP-rhamnose:rhamnosyltransferase. The pathway annotation is based on KEGG pathways (www.genome.jp/kegg/pathway.html, accessed 13 February 2021) and Blanco-Ulate et al. (2017)

powdery and downy mildew in grapevine (Riaz et al., 2011; Zini et al., 2019). The abundance of these genes varies among *Vitis* species and are particularly dense at resistance loci (Cochetel et al., 2021). The HR to viruses is mediated by resistance (R) genes. SA level and pathogenesis-related gene expression increase for systemic acquired resistance. HR is a means of prohibiting pathogen spread and can confer resistance when a corresponding dominant avirulence protein is produced by the pathogen (Balint-Kurti, 2019; Moffett et al., 2002). However, GLRaV infections are systemic and persist over time, and SA does not seem to play a preeminent role in the response to GLRaV infections. In a previous study of GLRaV-3 infections in Cabernet Sauvignon and Carmenère, the authors also remarked on the induction of expression of defence genes but their inability to impede systemic infection (Espinoza, Vega, et al., 2007). Both SA and ABA can participate in the response to viruses, though considerably less is understood about the role of ABA (Alazem & Lin, 2015; Koornneef & Pieterse, 2008; Kunkel & Brooks, 2002) and its relationship to NBS-LRRs. Notably, however, ABA deficiency is associated with an increase in R gene efficacy in incompatible interactions with *Pseudomonas syringae* and in a manner independent of SA (Mang et al., 2012).

Hormones have been implicated in mediating defence- and development-related networks and are over-represented at network hubs (Amrine et al., 2015; Jiang et al., 2015; Müller & Munné-Bosch, 2015; Vandereyken et al., 2018). Hormones like ABA, SA, and JA act as important signalling molecules during ripening and defence. The pathways engaged under stress are often tailored to particular pathogens. This entails coordination between hormone pathways (Gao et al., 2011; Vos et al., 2015). Interestingly, the effects of GLRaVs on gene expression in several hormone signalling pathways differed between rootstocks. A subsequent effort could be made to measure the abundances of hormones not quantified here, like cytokinins, GAs, and ethylene. Of the hormones considered in this study, however, the abundance of ABA and ABA-GE tended to increase in GLRaV (+) and this was influenced by rootstock. ABA can antagonize SA and JA signalling pathways and suppress ROS signalling (Alazem & Lin, 2015). WRKY transcription factors regulate and/or are regulated by ABA, SA, and JA (Gao et al., 2011; Jiang & Deyholos, 2009; Li, 2004; Liu et al., 2015; Xin et al., 2016). SA and ABA both can interact with RNAi, which is a fundamental component of antiviral defence. *AGO1* expression is positively correlated with ABA levels and the expression of *miR168a*, which regulates AGO1, and contains ABREs in its promoter (Alazem & Lin, 2015). Levels of ABA and ABA-GE increase in tobacco mosaic virus-infected leaves. One way in which ABA might aid plant defence is by increasing callose deposition to impair virus movement (Alazem & Lin, 2015); a gene encoding callose synthase is upregulated in the leaves of grapevine virus B-infected plants (Chitarra et al., 2018).

Relatively more is known about ABA's function as a ripening promoter (Koyama et al., 2010; Wheeler et al., 2009), in response to drought stress (Cochetel et al., ; Deluc et al., 2009), and in transmitting long-distance signals from roots to aerial organs and vice versa (Ferrandino & Lovisolo, 2014; Manzi et al., 2015). In a study of the impact of GLRaV-3 infection, drought stress, and a combination of both on grapevine plantlets in vitro, individual stresses both induced increases in ABA levels (Cui et al., 2016). Drought stress increases ABA levels and induces the flavonoid pathway in both tea plants (Gai et al., 2020) and grapes (Deluc et al., 2009). Our findings, in which ABA abundance tends to increase in GLRaV (+), are different than that observed for red blotch virus-infected berries, in which ABA abundance and *NCED* expression decrease in infected fruits (Blanco-Ulate et al., 2017).

The results of our analysis of metabolites associated with the phenylpropanoid and flavonoid pathways are mixed in their consistency with previous work. Though nonsignificant decreases in anthocyanin levels were observed, anthocyanin levels significantly increased in several GLRaV (+) conditions, albeit usually in individual years. These findings differed from others; some observed significant decreases in anthocyanin levels in fruits from GLRaV-infected plants (Lee & Martin, 2009; Vega et al., 2011) and others observed no significant changes in anthocyanin at harvest (Alabi et al., 2016; Endeshaw et al., 2014). In agreement with the results by Vega et al. (2011), flavonol levels were elevated in GLRaV (+) and the largest differences versus GLRaV (−) occurred at the first two stages, *FLS* expression was downregulated in GLRaV (+) at harvest, and *CHS* and *MYBPA1* were upregulated at véraison and generally downregulated at harvest.

In the present study, changes in the abundance of ABA and related metabolites distinguished the effects of GLRaVs between rootstocks. The parentage of the two rootstocks used in this study,

Kober 5BB and MGT 101-14, includes *Vitis riparia*. The other parents of Kober 5BB and MGT 101-14 are *Vitis berlandieri* and *Vitis rupestris*, respectively. These rootstocks were developed at different times. MGT 101-14 originated in France in 1882 and Kober 5BB originated in Austria in 1930 (https://fps.ucdavis.edu/). Rootstocks are chosen for the advantages they confer to the scion given a particular set of circumstances, often having to do with resistance to *Phylloxera*, nematodes, scion vigour, soil type, and abiotic stress tolerance (Corso & Bonghi, 2014; Tramontini et al., 2013; Warschefsky et al., 2016). *V. riparia*, *V. rupestris*, and *V. berlandieri* are asymptomatic hosts of GLRaVs (Naidu et al., 2014). Yet, the particularly severe response to GLRaV-1,2 (+) was not entirely unexpected in Kober 5BB-grafted vines (Alkowni et al., 2011; Golino, Wolpert, et al., 2008; Uyemoto et al., 2001). It would be interesting to determine (a) whether differences in viral titre exist between Kober 5BB, MGT 101-14, and Cabernet Franc and between different infection conditions and (b) whether such differences, if they exist, influence the severity of leafroll disease. Differences in wood abnormalities given different rootstocks (including Kober 5BB) have been observed for particular isolates causing grapevine rugose wood disease (Credi, 1997).

Notably, nutritional deficiencies in phosphorus, magnesium, and potassium produce symptoms that resemble those typically observed in GLRaV-infected plants (Gohil et al., 2016). Magnesium deficiency tolerance (Livigni et al., 2019), the impact of phosphorus deficiency on canopy growth (Grant & Matthews, 1996), and potassium uptake and channels are influenced by rootstock (Wolpert et al., 2005). Potassium uptake, channel activity, and related gene expression are also regulated by ABA (Blatt, 2000; Köhler et al., 2003; Rogiers et al., 2017; Song et al., 2016), and the application of ABA to tomato roots by drip irrigation affects fruit mineral composition (Barickman et al., 2019). Furthermore, elevated levels of potassium are observed in leafroll virus-infected Burger and Sultana fruits (Hale & Woodham, 1979; Kliewer & Lider, 1976) and in leaf petioles but potassium levels are lower in leaf blades (Cook & Goheen, 1961). Perhaps potassium deficient and GLRaV (+) phenotypes are similarly governed by ABA and fine-tuned by rootstocks. If some portion of scion ABA originates in roots and/or if rootstock can influence scion ABA levels and signalling genes, as observed here and by others (Chitarra et al., 2017), then perhaps this partially accounts for the variation in response observed between rootstocks. This experiment did not include a comprehensive survey of phytohormones, which would be beneficial, but ABA's function in root–shoot communication, its role in ripening, and the results here make it a good candidate around which to study the basis of leafroll disease symptom variability going forward. In addition, the transport of RNAs across the graft junction may perform some function that affects scion disease severity, but this remains to be seen in the particular case of GLRaV (Chitarra et al., 2017).

Together, these data support several conclusions. (a) The majority of genes differentially expressed as a consequence of infection or between GLRaV (+) plants with different rootstocks were year-specific. A small subset of effects was consistently observed across experimental conditions and in both years. These shared changes in expression involved genes associated with pathogen detection, ABA signalling and transport, ROS-related signalling, cytoskeleton remodelling, vesicle trafficking, phenylpropanoid metabolism, sugar transport and conjugation, and leucine biosynthesis. (b) The impacts of GLRaV-1,2 dual infection on Kober 5BB-grafted vines were the most distinctive and severe. Though there was variation between GLRaV infections observed, only the effects of GLRaV-1,2 were distinguishable overall from those of other infections. (c) The particular effects of GLRaVs in plants grafted to different rootstocks were distinguishable overall at every developmental stage. ABA-related variables were among those that best distinguished the responses to GLRaVs in different rootstock conditions. This included the abundance of ABA, the abundance of ABA-GE, and the expression of genes associated with ABA and other hormone signalling pathways. Finally, this work alone is insufficient to recommend the use of one rootstock or another, but the disparity in sensitivity and symptom severity observed in berries from Cabernet Franc vines grafted to different rootstocks suggests that rootstock selection should be further explored as a strategy to mitigate some of the negative consequences of leafroll virus infections, should vectors of the virus encroach upon a vineyard.

4 | EXPERIMENTAL PROCEDURES

4.1 | Vineyard establishment

The experimental vineyard used in this study was established in 2010 and consists of Cabernet Franc clone 04 (UC Davis, Foundation Plant Services, https://fps.ucdavis.edu/fgrdetails.cfm?varietyid=355; accessed 2 March 2021) grapevines grafted on different rootstocks and infected with zero, individual, or pairs of GLRaVs. The rootstock portion of these plants was inoculated with chip buds carrying each virus in 2009 (Rowhani et al., 2015). All rootstocks and Cabernet Franc scions were tested for grapevine pathogens. Total nucleic acid (TNA) extracts were prepared from all rootstocks and Cabernet Franc scions as described by Al Rwahnih et al. (2017). Extracted TNA samples were analysed by reverse transcription quantitative PCR (RT-qPCR) using TaqMan probes on the QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific) as described previously (Klaassen et al., 2011; Osman et al., 2008; Rwahnih et al., 2017). The samples were screened for the following pathogens: GLRaV-1, GLRaV-3, GLRaV-4 (plus strains 5, 6, 9, Pr, and Car; genus *Ampelovirus*); GLRaV-2 (plus strain 2RG; genus *Closterovirus*); GLRaV-7 (genus *Velarivirus*); grapevine fleck virus (genus *Maculavirus*); grapevine rupestris vein feathering virus (genus *Marafivirus*); grapevine fanleaf virus, tobacco ringspot virus, and tomato ringspot virus (genus *Nepovirus*); grapevine virus A, grapevine virus B, grapevine virus D, grapevine virus E, and grapevine virus F (genus *Vitivirus*); grapevine red blotch virus (genus *Grablovirus*); and grapevine rupestris stem pitting-associated virus (genus *Foveavirus*), phytoplasmas, and *Xylella fastidiosa*.

In autumn 2008, Cabernet Franc grapevines were bench-grafted onto rootstocks, including MGT 101-14 and Kober 5BB. These plants

were subsequently grown in a greenhouse. Between 2009 and 2011, the rootstock portions of these plants were inoculated with two chip buds from single leafroll-infected plants. Infected plants used for chip buds were reconfirmed by RT-qPCR. Plants infected with a single species of GLRaV received two identical chip buds. Plants infected with two species of GLRaVs also received two chip buds, each carrying a single virus. Plants infected with GLRaV-1, GLRaV-2, and/or GLRaV-3 were inoculated with two or more isolates of each species of GLRaV. The inoculated plants were kept in a greenhouse for approximately 1 month, acclimatized, and then planted in the field. Healthy controls included nonchip budded plants and plants chip budded from a healthy source.

The vines were planted in a randomized complete block design, with 7 feet (2.1 m) between vines and 9 feet (2.7 m) between rows. One group of five vines was planted per rootstock × infection condition in each of three blocks. Healthy vines were distributed throughout each block to monitor the spread of viruses, and experimental vines were sampled yearly to test and reaffirm the vines' infection status. A buffer zone of healthy vines was planted as a barrier between the leafroll vineyard and other vineyards in the area. Vines were trained with a bilateral cordon and spur pruned.

4.2 | Cabernet Franc genome sequencing and assembly

High-quality genomic DNA was isolated from grape leaves using the method described in Chin et al. (2016). SMRTbell libraries were prepared for Cabernet Franc clone 04 as described by Massonnet et al. (2020). Final libraries were evaluated for quantity and quality using a Bioanalyzer 2100 (Agilent Technologies) and sequenced on a PacBio RS II (DNA Technology Core Facility, University of California, Davis).

De novo assembly of Cabernet Franc clone 04 (UC Davis, Foundation Plant Services, https://fps.ucdavis.edu/fgrdetails.cfm?varietyid=355; accessed 2 March 2021) was performed using FALCON-Unzip (v. 1.7.7; Chin et al., 2016) as described in Minio et al. (2019a). Repetitive sequences were masked before and after read error correction using the TANmask and REPmask modules in Damasker (Myers, 2014). Contigs were polished with Quiver (Pacific Biosciences, bundled with FALCON-Unzip v. 1.7.7). The primary assembly was scaffolded to reduce sequence fragmentation. First, primary contigs were scaffolded with SSPACE-LongRead v. 1.1; (Boetzer & Pirovano, 2014). Junctions supported by at least 20 reads ("-l 20") were allowed. Hi-C data and the proprietary HiRise software (v. 1.3.0-1233267a1cde) were used for hybrid scaffolding. A Dovetail Hi-C library was prepared by Dovetail Genomics (Scotts Valley, CA, USA) as described in Lieberman-Aiden et al. (2009) and sequenced on an Illumina platform, generating 2 × 150-bp paired-end reads. The repeat and gene annotation were performed as reported in Vondras et al. (2019). Briefly, RepeatMasker (v. open-4.0.6; Smit et al., 2015) and a custom *V. vinifera* repeat library (Minio et al., 2019b) were applied to identify repetitive elements in the genome. Publicly available data sets were used as evidence for gene prediction. Transcriptional evidence included *Vitis* expressed sequence tags, Cabernet Sauvignon corrected Iso-Seq reads (Minio et al., 2019b), Tannat (Da Silva et al., 2013), Corvina (Venturini et al., 2013), and Cabernet Sauvignon transcriptomes (Massonnet et al., 2020), and previously published RNA-Seq data (PRJNA260535). Swissprot Viridiplantae data and *Vitis* data were used as experimental evidence. Each RNA-Seq sample was trimmed with Trimmomatic v. 0.36 (Bolger et al., 2014), assembled with Stringtie v. 1.3.3 (Pertea et al., 2015), and mapped onto the genome using Exonerate v. 2.2.0 (transcripts and proteins; Slater & Birney, 2005) and PASA v. 2.1.0 (transcripts; Haas et al., 2003). Alignments and ab initio predictions generated with SNAP v. 2006-07-28 (Korf, 2004), Augustus v. 3.0.3 (Stanke et al., 2006), and GeneMark-ES v. 4.32 (Lomsadze et al., 2005) were used as input for EVidenceModeler v. 1.1.1 (Haas et al., 2008). EVidenceModeler was used to identify consensus gene structures. A functional annotation was obtained using the RefSeq plant protein database (ftp://ftp.ncbi.nlm.nih.gov/refseq, retrieved 17 January 2017; Jones et al., 2014) as in Minio et al. (2019a).

4.3 | Sampling and sample preparation

Berries from Cabernet Franc grapevines grafted to Kober 5BB or MGT 101-14 and infected with GLRaV-1 (GLRaV-1 [+]), GLRaV-3 (GLRaV-3 [+]), GLRaV-4 (strain 5; GLRaV-4 [+]), GLRaV-1 and GLRaV-2 (GLRaV-1,2 [+]), or GLRaV-1 and GLRaV-3 (GLRaV-1,3 [+]) were sampled during ripening in 2017 and 2018. In both years, fruits were sampled at prévéraison, véraison, mid-ripening, and at commercial harvest. These stages correspond to modified Eichhorn–Lorenz (Coombe, 1995) stages 34 (green berries begin to soften and sugar content [unit, °Brix] starts increasing), 35 (berries begin to change colour and enlarge, c.50% of berries within clusters show colour transition), 36/37 (berries with intermediate sugar content/berries are not quite ripe), and 38 (berries are harvest-ripe). Fruits in 2018 were sampled at developmental stages comparable to 2017 as determined by TSS. In 2017, berries were sampled on 7 July, 31 July, 14 August, and 31 August. In 2018, berries were sampled on 28 June, 30 July, 13 August, and 30 August.

On each sampling date, six biological replicates were taken, with berries from one plant constituting one biological replicate. Two biological replicates per condition were sampled from each of three blocks. There were two exceptions. For Kober 5BB-grafted GLRaV-1,2 (+), three biological replicates were drawn from each of two plants in one block. For Kober 5BB-grafted GLRaV-3 (+), two of the six biological replicates were drawn from one plant. Approximately 20 berries were sampled per plant, with equal numbers of berries sampled from each side of the vine. Samples were then temporarily cooled on ice. Next, berries were rinsed with deionized water, deseeded, and snap-frozen in liquid nitrogen. Berries were stored at −80 °C until being crushed into a fine powder while frozen using a mechanical mill. TSS were measured in technical triplicate using a digital refractometer. Four of six biological replicates, a total of 192 samples per year, were used for RNA-Seq and liquid chromatography coupled with mass spectrometry (LC-MS).

4.4 | RNA extraction and sequencing library preparation

Total RNA was extracted from 2 g of finely ground berry pericarp tissue as previously described (Blanco-Ulate et al., 2013). RNA purity was evaluated with a NanoDrop 2000 spectrophotometer (Thermo Scientific), quantity with a Qubit 2.0 fluorometer (Life Technologies), and integrity by electrophoresis. RNA-Seq libraries were prepared using the Illumina TruSeq RNA sample preparation kit v. 2 (Illumina) and barcoded individually following the manufacturer's protocol. Final libraries were evaluated for quantity and quality with the High Sensitivity chip in a Bioanalyzer 2100 (Agilent Technologies). Libraries were sequenced as 100-bp, single-end reads, using an Illumina HiSeq 4000 sequencer (DNA Technology Core Facility, University of California, Davis), producing an average of 18.07 ± 4.56 million reads per sample in 2017 and 14.69 ± 2.11 million reads per sample in 2018.

4.5 | RNA-Seq data analysis

RNA-Seq reads were trimmed using Trimmomatic v. 0.36 (Bolger et al., 2014) and the following settings: leading: 2, trailing: 2, sliding window: 4:20, min length: 70. Reads were mapped onto the primary assembly of the Cabernet Franc genome using HISAT2 (-k 1; v. 2.0.5; Kim et al., 2015) and counts were generated using htseq-count with default parameters (v. 0.9.0; Anders et al., 2015).

All subsequent analyses were done using R (R Core Team, 2020) in the R Studio environment (R Studio Team, 2020). Data normalization and differential expression analyses were performed using DESeq2 v. 1.24.0 (Love et al., 2014). A variance-stabilizing transformation (VST) was applied to expression data using DESeq2. VST data were centred in each GLRaV (+) condition relative to the mean expression per gene in GLRaV (−) given the same time, rootstock, and year.

Centred data were used for MFA with the FactoMineR R package (Le et al., 2008). Genes were included in the MFA if (a) they were differentially expressed versus GLRaV (−), and/or the effects of an infection differed between rootstocks (b) in at least 1 year ($p < .05$), and (c) the direction of the effect relative to GLRaV (−) was consistent in both years, even if a significant effect was only observed in 1 year. An MFA was repeated for each developmental stage separately. All hormone and metabolite data were included.

Statistical over-representation tests were done using the clusterProfiler R package (Yu et al., 2012) and VitisNet functional categories (Grimplet et al., 2009). To make use of the VitisNet functional annotations, Cabernet Franc genes were used to query Pinot Noir PN40024 sequences with BLASTp (Altschul et al., 1990). The best hits, with no less than 80% reciprocal identity and coverage, were retained.

A curated list of ABA biosynthesis and signalling genes annotated in PN40024 was retrieved from Pilati et al., 2017 (Table S1). As described above, Cabernet Franc genes were used to query Pinot Noir PN40024 sequences. All target–query pairs had no less than reciprocal 97% identity and 86% reciprocal coverage (median coverage, 99%).

4.6 | Hormone extraction and LC-MS/MS

Approximately 50 mg (mean 50.86 ± 3.33 mg) of berry powder was weighed for the extraction and quantitation of ABA, SA, and JA. Exact weights were recorded to later calculate the exact amount of each of these analytes per milligram of fresh tissue. The same samples used for RNA-Seq were used for this analysis. Four biological replicates were used. Extractions and analyses were randomized and performed in technical duplicate.

Hormones were extracted following a method described by Pan et al. (2010) with a few modifications. Samples were subjected to 500 ml 2-propanol:H_2O:HCl (2:1:0.002) and spiked with 50 ng of d_6 ABA, d_5 JA, and d_4 SA (CDN Isotopes). Samples were vortexed, placed in an ultrasonic ice bath for 30 min, washed with dichloromethane, and centrifuged at 16,100 × g for 5 min. Nine-hundred microlitres of the lower phase was taken and dried. Samples were reconstituted in 100 µl 15% methanol and stored at −20 °C until analysis. A detailed description of the chromatographic separation, mass spectrometry data acquisition methodology, and multiple reaction monitoring (MRM) transitions is included in Table S2.

4.7 | Water-soluble metabolite extraction and LC-MS

The same samples used for RNA-Seq and hormone analyses were used to measure water-soluble metabolites by LC-MS. Approximately 200 mg (mean 202.12 ± 4.87 mg) of frozen berry powder was weighed. Extractions and analyses were randomized and performed in technical duplicate. Hormones were extracted in 1 ml 1% HCl in HPLC-grade water (prepared in-house, final resistance 18 MΩ, 0.2 µm filtered) and spiked with salicin (Sigma Aldrich) as an internal standard (50 µg/L final concentration). The samples were vortexed, placed in an ultrasonic ice bath for 30 min, and centrifuged. The supernatants were collected and frozen at −80 °C until analysis. A detailed description of the chromatographic separation, mass spectrometry data acquisition methodology, and MRM transitions is included in Table S2.

4.8 | Data visualization

Figures depicting quantitative data were built using the following R packages: ggplot2 (Wickham, 2016), hrbrthemes (Rudis, 2020), wesanderson (Ram & Wickham, 2018), pheatmap (Kolde, 2019), and UpSetR (Gehlenborg, 2019).

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DATA AVAILABILITY STATEMENT

The DNA (BioProject PRJNA701939) and RNA (BioProject PRJNA701940) sequencing data that support these findings are available at the National Center for Biotechnology Information (NCBI). A Cabernet Franc genome browser and on-line BLAST tool are available at http://www.grapegenomics.com. The assembly, annotations, and raw RNA-Seq counts per library and per gene are available at Zenodo (http://doi.org/10.5281/zenodo.4555977).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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