Grapevine Leafroll associated Virus – 3 (GLRaV-3) Seasonal Titer Changes and Effects on Pinot Noir Fruit in Oregon

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Introduction

The Willamette Valley, west of the Cascade mountain range in the state of Oregon, is home to a growing number of vineyards. This rather recent, booming industry of the region has become an important component of the economy of the state's agriculture since the late 1960's, contributing more than $1.56 billion in 2010 (USDA NASS). Disease control is an important element of maintaining the quality of grapes for use in wine and keeping the industry healthy. This can be costly and frustrating for growers and viticulturists. Of the many viruses that affect grapes, *Grapevine leafroll associated virus-3* (GLRaV-3) is the most common (Tsai *et al*., 2008). It is prevalent in many vineyards in California and eastern Washington, and could become an issue in the Willamette Valley.

GLRaV-3 is a member of the genus *Ampelovirus* within the family *Closteroviridae*. There are nine ampeloviruses that infect grapevines (GLRaV-1, -3, -4, -5, -6, -9, -Car, -Pr and –De) of which GLRaV-3 is the most widespread. Ampeloviruses are transmitted by mealybugs and scale insects. With the recent introduction of the vine mealybug (*Planococcus ficus*) into California (Tsai *et al*., 2008), Grapevine leafroll viruses have become a much greater threat, because this vector is much more prolific than other mealybug vectors that feed on grapevines. GLRaV-3 can also be transferred to healthy plants through grafting if either the scion or rootstock is infected. Infected plants of red fruited cultivars develop reddening of the interveinal lamina of the leaf blades, with the veins remaining green, though a range of symptoms may be observed with GLRaV-3 infection, depending on virus and cultivar. Foliar symptoms begin to develop in late July and early August in the oldest leaves and advance to younger leaves as the season progresses. GLRaV-3 infection results in uneven berry ripening within clusters, as well as reduced sugars and anthocyanins (Lee *et al*., 2009), all leading to reduced quality that may result

in fruit that does not meet the required standards for making quality wines. If a vineyard becomes completely infected with GLRaV-3, the grower can spend up to $40,000 per hectare to remove and replant the infected vines. Preventative measures in the form of vector control (insecticides) can eventually become equally costly; up to $2,400 per hectare can be spent each year to eradicate mealybug vectors and sanitize the vineyard (Atallah *et al*., 2011). Another common grapevine disease, *Grapevine rupestris stem pitting-associated virus* (GRSPaV), is more common in the Willamette Valley, from distribution of GRSPaV infected rootstocks or scions from nurseries or from using infected wood when top-working a vineyard to switch cultivars. Many studies, including one by Gribaudo *et al.* (2006), mention that grafting GLRaV-3 infected vines onto GRSPaV infected rootstocks create even more severe symptoms. Wineries have cut contracts with growers that cannot meet quality standards. Thus a need exists to develop better methods to control GLRaV-3 or minimize the effects it has on fruit quality.

Pinot noir is the most common grape cultivar grown in the Willamette Valley, followed by Pinot gris. The climate and clay soils of the Yamhill County area provide excellent growing potential for grapevines. However, the hot days and cool evenings in the region tend not to favor high populations of mealybugs, or perhaps favor natural predators that keep mealybug populations in check. Based on trapping done by Walton (2010 and 2011, personal comm.) mealybug populations in the Willamette Valley are much lower than in southern Oregon, eastern Washington, the Snake River Appellation (Idaho) and the Columbia Gorge. It is not clear why this is the case, but it results in a reduced risk of leafroll infection in the Willamette Valley. The vector for GLRaV-3 virus, an ampelovirus, is primarily mealybugs (Tsai *et al*., 2008), but it can also be transmitted by soft scale insects and very efficiently through grafting. It has been reported that the titer of GLRaV-3 increases during the growing season and that the most reliable

detection is in the late summer and autumn. This is based on work using serological assays such as ELISA. For this reason, virus detection is usually carried out in the month of September to avoid false negatives as much as possible. Virus detection is also more reliable in older rather than younger leaves. If virus titer is correlated with ease of detection, it would suggest that there are more virus particles in the plant at the end of the growing season than early in the season.

In areas where the virus and its vectors, especially the grape feeding mealybugs, occur, growers control the spread of GLRaV-3 and several other viruses with the use of insecticides during the dormant and growing season. This is a costly, and time-consuming, necessity to maintain the overall health of the vineyard. Additionally, since mealybugs tend to be protected under the bark or in fruit clusters, contact insecticides are not very effective. Insecticides with systemic activity are, therefore, needed to properly control mealybugs in grapevines. Pesticide residues can also build up on fruit, causing downstream wine-making problems (Cesnik *et al*., 2008). Since we expect virus titers to be low in the spring and early summer, it may also be possible that mealybug vectors are less efficient vectors of GLRaV-3 early in the season when virus titers are low. If transmission efficiency by the mealybug requires a specific virus titer, then it may be possible to eliminate pesticide sprays during the early, or during the 'low titer', part of the growing season, saving the growers costs, reducing the pesticide load to the environment, reducing the carbon footprint of grape production, and reducing pesticide residues on the harvested fruit. Previous research dealing with GLRaV-3 transmission through mealybugs lacks much information about the actual mechanism of transmission (Tsai *et al*., 2008). Little research has been done to quantify the virus in grapevines throughout the growing season, or to determine the virus transmission efficacy in relation to virus titer in both vector and plant, and no research on these topics has been done in the Pacific Northwest.

The most serious problems of virus infection are negative effects on glycoside content, flavanols and berry color development in grape clusters (Singh *et al*., 2008). Healthy grape clusters have a synchronized ripening mechanism, in which the green and pink grapes on a red grape cluster all converge to an even red ripeness by the time of harvest. Grape clusters on infected vines do not ripen evenly by the time of harvest, due to a disruption in gene expression throughout various aspects of the plant and fruit (Espinoza *et al*., 2007). Altered grape gene expression has been examined in relation to other grapevine diseases, such as *Xylella fastidiosa* (Doddapaneni *et al*., 2009), and gene expression patterns have been examined in Cabernet Sauvignon infected with GLRaV-3 (Vega *et al.,* 2011), which found a dramatic gene expression change in the mechanism for sugar and anthocyanin metabolism during early fruit development. If the host genes affected by GLRaV-3 were identified, it might be possible to modify the expression of these genes and minimize the effect of GLRaV-3 on fruit quality. The modification of these genes would best be managed through biotechnology approaches, such as is being done with various genes in other crops, to modulate the affects of virus infections.

The purpose of this proposed research project was (1) to quantify the increases of GLRaV-3 RNA weekly during the growing season for two consecutive years and (2) compare differences in sugar level, elasticity, and color index properties between healthy and GLRaV-3 infected fruit during the ripening phase.

Material and Methods

Source of GLRaV-3 Infected Plant Material

A commercial vineyard in Yamhill County, Oregon, where the highest concentration of Pinot noir vineyards is located, was selected for the studies on quantification of GLRaV-3 RNA and fruit quality. A total of 12 plants were non-randomly selected for the experiment; two sets of six plants. All plants were in the same block of the vineyard, receiving the same cultivation practices, on the same southwest facing hill-slope, soil-type, and internal in the production block to avoid edge-effects and other outside differences not pertaining to virus infection. The first set of six plants was self-rooted Pinot noir clone, 'Pommard', and the second set of six plants was Pinot noir clone '114' grafted onto *Vitis riparia,* 'Gloire' rootstock. All twelve plants were tested using RT-PCR and ELISA detection methods to confirm that they were infected with GLRaV-3 before tissue sampling for quantification began. Additionally, six plants of each clone in the same block that tested free of GLRaV-3 in RT-PCR and ELISA tests were also sampled and served as healthy controls.

In the field sampling process, three classes or ages of leaf tissue were collected from each vine: young leaves, middle leaves, and old leaves. Young leaves sampled for this study were soft and fleshy, near the tip of a green vine and only about one-third expanded. Middle aged leaves were selected approximately midway along the cane, and old leaves which had a leathery feel, were collected from the base of the cane. Leaf discs, one cm in diameter, were cut and removed from a young leaf on each set of six plants using a #5 cork borer, and the six leaf discs were combined to form a composite sample. The same protocol was followed for collection of middle and old aged leaves. A total of twelve samples were collected each week; three separate leaf classes from GLRaV-3 infected and healthy plants of each of the two Pinot noir clones in the study.

RNA Extraction

Total RNA from each sample was extracted using a modified Spiegel and Martin method (1993) combined with a glass milk method of Rott and Jelkmann (2001) as described in Tzanetakis *et al*. (2007). Briefly, leaf discs were pulverized using a mortar and pestle in 1.0 mL of extraction buffer consisting of 200 mM Tris base, pH 8.5, 300 mM lithium chloride, 1.5% lithium dodecyl sulfate, 10 mM EDTA, 1% deoxycholic acid, 2% polyvinyl-pyrrolidone molecular weight 30,000 daltons, 1% NP 40 "Tergitol," with an addition of 1% β-mercaptoethanol just before use. After the tissue was thoroughly homogenized, 0.6 mL of the solution was transferred to a microfuge tube and an equal amount of 6 M potassium acetate solution that was 5 M potassium and 6 M acetate, pH 6.5, was added to the tube. The contents of the tube were mixed by inverting the tube 5 or 6 times and then centrifuged at 13,000 rpm for 10 minutes. After centrifugation, 0.75 mL of the supernatant was removed with a 1.0 ml pipette and added to a clean tube containing 0.75 mL of isopropanol. The samples were again mixed by repeated inversion of the tubes and centrifuged for 30 minutes to pellet nucleic acids. The supernatant was drained and pellet reserved. Twenty-five μL of silicon dioxide (glass milk, prepared according to Rott and Jelkmann, 2001) was added to each tube and vortexed to resuspend the nucleic acids. Then, 0.75 mL of binding buffer (10 mM Tris base, pH 7.5, containing 0.5M EDTA, 100 mM NaCl, and an equal amount of 100% ethanol) was added and centrifuged at 10,000 rpm for 10 sec to reform a pellet. The pellet was washed twice with the binding buffer. The final pellet was re-suspended in 100 μL of RNase-free water or an elution buffer (10 mM Tris, pH 7.5 containing 1 mM EDTA). The samples were stored at -80°C until all samples for one complete year had been collected and extracted.

Designing Primers for Quantitative PCR

A reverse transcriptase (RT) reaction with final volume of 50 µL that contained 5 µL of RNA extract as template and random hexamers as primers was carried out following manufacturer's (Fermentas) protocol for RevertAid™ M-MuLV reverse transcriptase with RiboLock™ RNase inhibitor kit at 50°C for 60 min. Five µL of the RT reaction was used as template for a 50 µL PCR reaction that included forward primer, CGC TAG GGC TGT GCA AGT ATT and reverse primer, GTT GTC CCG GGT ACC AGA TAT (Ling *et al*., 1998) to amplify the major coat protein gene of GLRaV-3, following manufacturer's protocol (Invitrogen, Life Technologies™). Amplicons were separated by electrophoresis though a 2% agarose gel stained with ethidium bromide and visualized using UV light. Amplicons from eight samples were sequenced on an ABI 373 OXL DNA analyzer by Macrogen (Seoul, South Korea). To begin primer design for qPCR, the resulting sequences were compared to find highly conserved regions in the coat protein of GLRaV-3 in relation to other GLRaV-3 coat protein sequences from GenBank. The available sequences from GenBank and personal material were copied and pasted into Clustal-W, v.2.1 from EMBL-EBI. Each sequence was compared, and individual nucleotide matches among all sequences were denoted with an asterisk by the program (Fig. 1). Highly conserved nucleotide regions within 0-200 and 800-942 nucleotides were selected for use in coat protein determination. Regions of about 18-30 nucleotides within the coat protein gene, with a high degree of conservation among the analyzed sequences, were then chosen to create forward and reverse primers and probe. Integrated DNA Technologies® (IDT) ensured the sequences had at least 50% GC nucleotide content (to avoid denaturation), among other criteria, and then created the designed primers for the quantitative PCR assays.

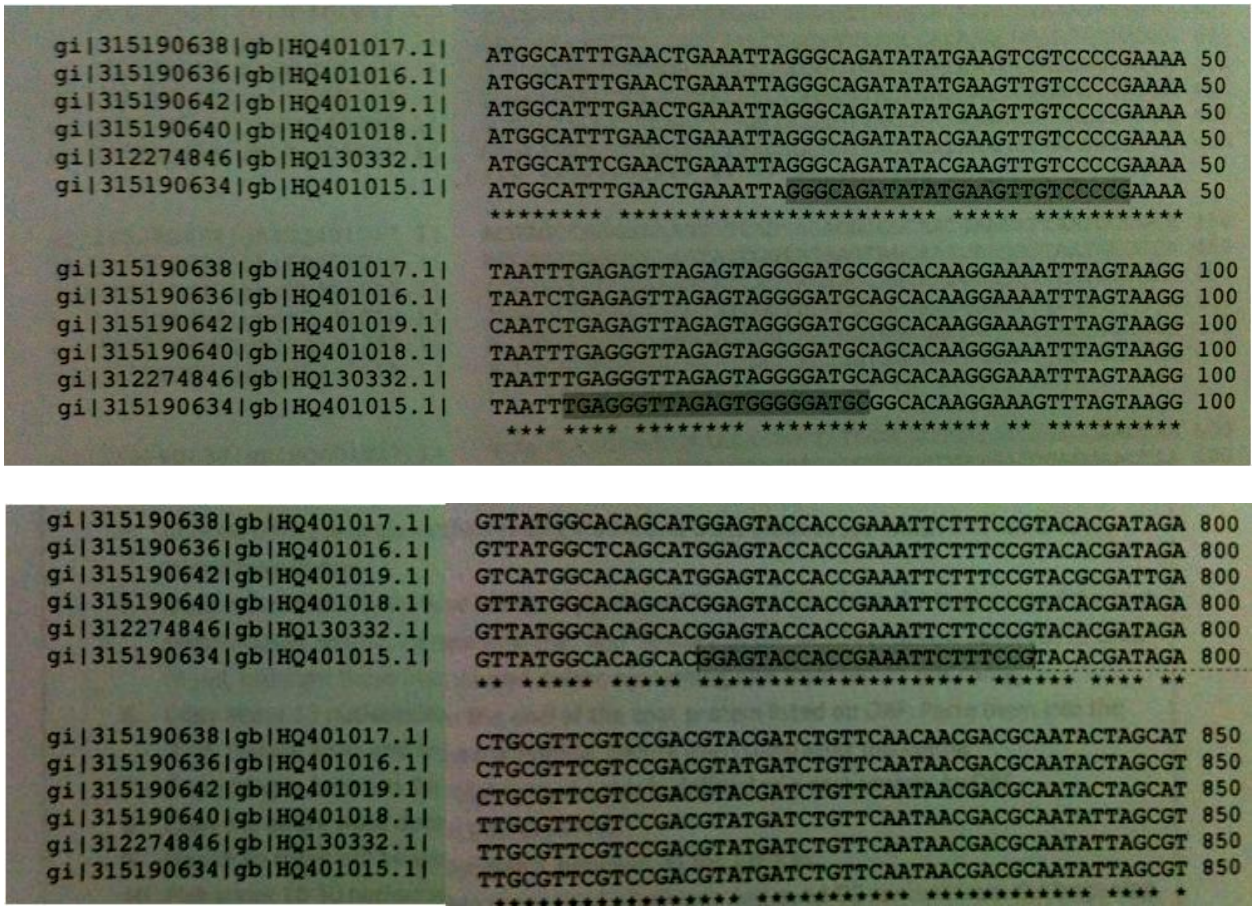

Figure 1 Nucleotide sequence alignment from Clustal W. Positive alignments are regions with * below each nucleotide. Highlighted regions between 0 and 100 bp and regions between 800 and 942 were selected for coat protein gene identification. Forward and reverse primers and probe sequences taken from within coat protein.

Quantitative PCR (qPCR)

Before beginning any processes of qPCR, all RNA samples to be analyzed were standardized to concentrations of 50 ng/µL before the assay. The manufacturer's recommendations for the AgPath-ID™ One-Step RT-PCR Kit (Life Technologies™, USA) was followed to carry out RT and qPCR in one step, using a StepOnePlus™ Real-Time PCR System (Applied Biosystems), and the primers and probe designed for GLRaV-3 qPCR described above, as well as a primer/probe for ndhB (ndhB forward primer: AAG CAA AAG TTC CTA GAT TCA TGG, reverse primer: TTG CGT ATT CGT CCA TAG GTC, probe: TGC TTG CAT ATC CAC CAT TTG AGT CTCC that resulted in a 132 bp amplicon (Quito-Avila *et al*., 2012).

Comparative efficiency (ΔΔCt) analysis of the virus and ndhB amplification was done for each sample to account for variation in initial total RNA concentration (50 ng/µL) and variation in inhibitors in the samples. The reference sample for virus concentration for each of the three tissue types within each clone was the sample from week one. The ΔΔCt calculation assumes 100% PCR efficiency, in that each cycle; there occurs a complete doubling of concentration. See Figure 2 for an example of common initial data output curves from the thermocycler. In order to further ensure that samples are accurately compared, the plates were arranged with each sample in duplicate and a plate contained all samples from one leaf-type, over one growing season. Duplicates for ndhB were done the same way and on the same plate as GLRaV-3. Any errors or losses affecting one sample, should therefore, affect all samples that were compared. The same protocol was followed for each six groups, and for both years of the study.

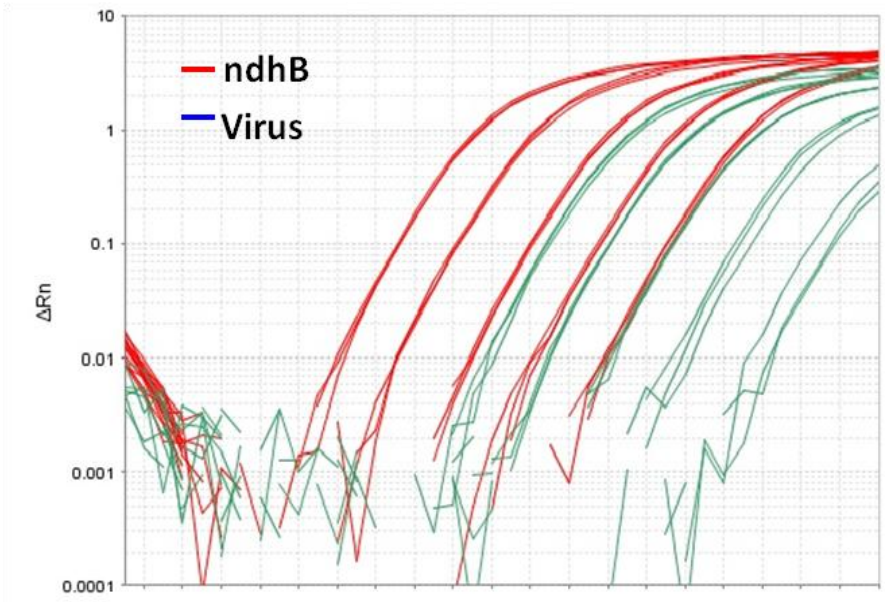

Figure 2 An example of GLRaV-3 qPCR amplification plot with ndhB control gene in 10-fold dilutions.

Berry Composition

To assess the effects of GLRaV-3 on berry composition, analyses of sugar content, elasticity, and color index were monitored for differences during the berry ripening phase for one year. Six separate vines from those used for virus titer measurements were non-randomly selected. Three vines were from the block area containing the GLRaV-3 infected 'Pommard' vines, and the other three were from the part of the block containing healthy 'Pommard' vines. All plants were in the same block, receiving the same cultivation practices, on the same hill-slope, soil-type and internal in the production block to avoid edge-effects. The plants selected had to contain at least six grape clusters. Because grapes are characterized by a significant ripening asynchrony, four berry classes – representative of the ripening transitions (green/hard, green/soft, pink/soft, and red/soft berries) –were monitored from *mid-véraison*, the point of the growing season in which one-half of most grape clusters have become adequately ripe, to harvest. This was to ensure a fine-tuned monitoring of the grapes to determine the effects of GLRaV-3 on the ripening dynamics of grape berries across the ripening phase. Each individual berry belonging to the four classes aforementioned were tagged (colored strings) for identification at the maximum of ripening variability (*mid-véraison)* in healthy and infected plants. Then, samplings were conducted at the same day and time each week until harvest. A total of 864 berries were collected from one week after *mid- véraison* to harvest (5 weeks) during year 2011 for measurements; corresponding to six berries per class (n=4) per cluster (n=6) per plant (n=6). For each cluster, one grape per class, per plant was removed each week, with the pedicel attached to berry.

To measure the berry deformation of each grape, adapted skinfold calipers (Baty International, UK) were used. A formula for elasticity outlined by Thomas *et al*. (2008), was applied to the pressure measurements for each grape. Berry skin color intensity was determined

using a chromameter (CR310) from Minolta Inc. (USA) to measure CIELAB parameters (lightness, hue angle and chroma) of dark red grapes (Carreno *et al*., 1995). Lastly, the total soluble solids (brix degree) of the berries were measured using a digital refractometer (SPER Scientific, USA). These phenological measurements were compiled in average values relative to each berry class. Multivariate analyses (Principal Component and discriminant analyses) will be performed to estimate the effect of GLRaV infection on the berry compositional variability, and to identify best-fit berries per berry class for downstream applications (gene expression experiments).

Results

Primer Design

The GLRaV-3 amplicons sequenced by Macrogen were comparable to sequences of GLRaV-3 available from GenBank, so the available sequences from GenBank were used for the coat protein alignment process, due to more ease in program use. There were six coat protein sequences used from GenBank (Fig. 1). About 18-30 nucleotides were then chosen that displayed good alignment among all coat protein sequences, as well as at least 50% GC nucleotide content to avoid denaturation. Conserved regions of about 20 nucleotides were used to design the forward (TCT AGC ATA CTT CAC GCC AAC) and reverse (ATT GAA CAG ATC GTA CGT CGG) primers for real time PCR, and a conserved internal region of about 20 nucleotides to the amplification primers was selected for the Taqman probe for the real time PCR (/56-FAM/TCA CCA GTT /ZEN/TAC CAT TCA GGG TCG C/3IABkFQ/).

Quantification

In the trial involving young leaf tissue, GLRaV-3 titer quantified for 2010 illustrated a 2^{10}-, or 1,000-fold total increase over the duration of the growing season (Fig. 3a), and 2^{12}-, or 4,000-fold increase for the year 2011 (Fig. 3b). A substantial part of this increase occurred during the early season month of June, accounting for a 2^{4}-fold increase in June for 2010, and over 2^{6}-fold increase in June of 2011. For 2011, this amount accounted for one-half of the total increase established throughout the season. In both years, the Pommard clone displayed a lower general virus titer accumulation than the grafted clones (114/Gloire).

Middle aged leaves had a total GLRaV-3 titer increase of around 2^{7}-fold for both years of the study (Fig. 3c,d), with the month of June contributing, once again, to more than one-half of the total virus titer increase established. In comparison to results from the young leaf tissue trial, middle –aged leaves displayed less of an increase in virus titer over the season, and instead of the exponential curvature displayed in the graph of young tissue, trends in the middle tissue virus titer created a parabolic curve throughout the season.

Mature tissue trials illustrated a virus titer increase comparable to that observed in middle-aged tissue throughout the growing seasons of both years (Fig. 3e,f). In 2010, the Pommard clone GLRaV-3 titer increased more than 2^{8}-fold, while the grafted clones showed a virus titer increase of only 2^{3}-fold (Fig. 3e). The 2011 mature tissue data showed another parabolic curve when graphed, and more similar to middle-aged tissue, with a total GLRaV-3 titer increase of around 2^{7}-fold. The Pommard clone also displayed a lower titer increase than the grafted clones (Fig. 3f).

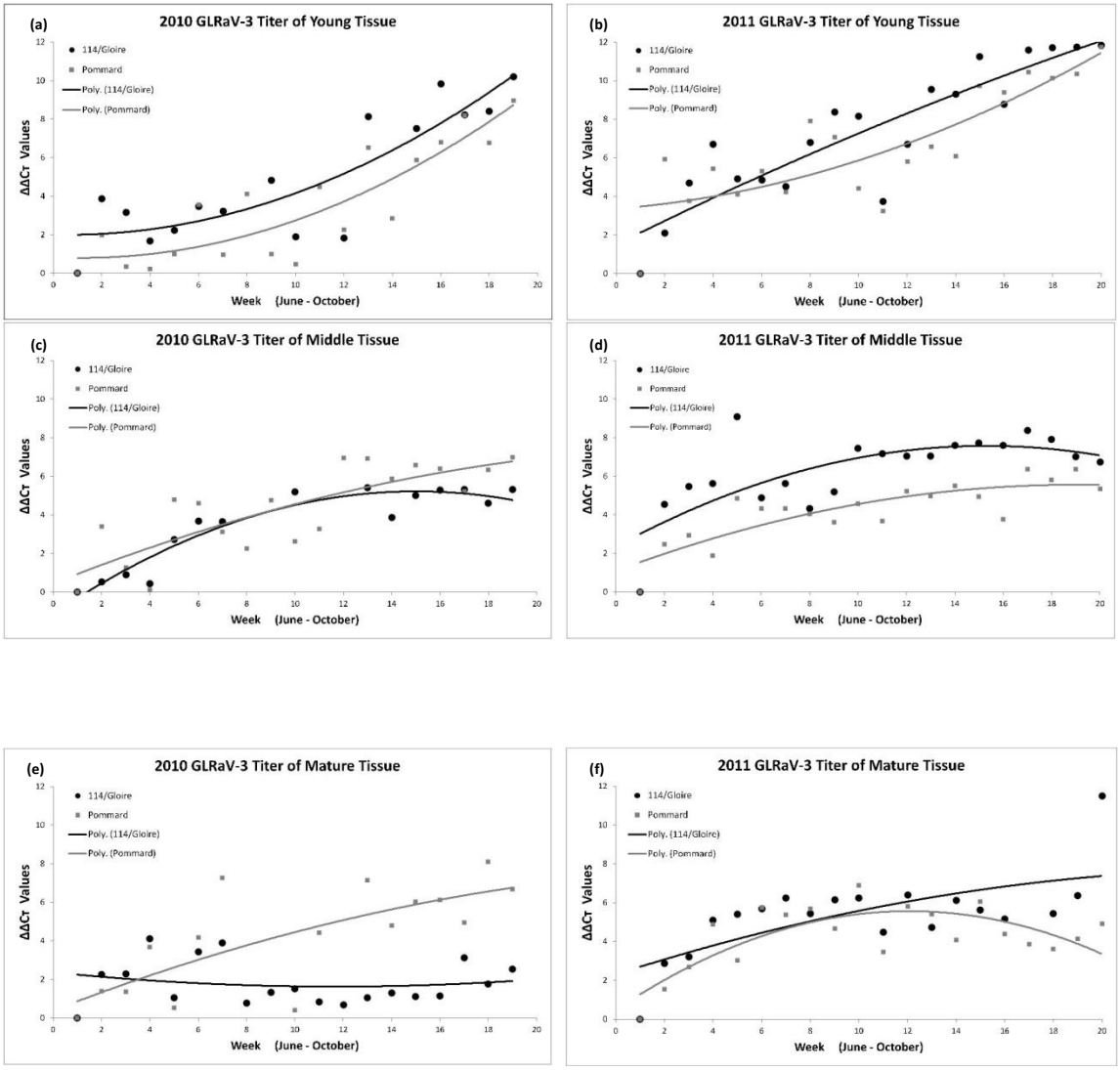

Figure 3 Graphed results of infected Pommard and 114/Gloire grafted clones GLRaV-3 titer comparisons to week one in each season of 2010 and 2011. Graphs are fitted with a polynomial regression trend line. Note titer increases occurring within first four weeks.

Berry Analysis

Mean berry measurements were taken from *healthy* vines, over 5 weeks after*mid-véraison* to harvest. At harvest, Brix° ranged from 26.5% to 27.4% sugar content among all four berry classes (Fig. 4a). The graph for this Brix° analysis includes lines respective to the amount of days required during the season for green grapes to achieve a stage of ripeness equal to that of red grapes for each week. From Figure 4a, about 11 days were required for mean sugar content in healthy green-hard grapes to gather the same mean sugar level of red grapes present in the first

collection at *mid-véraison* +1 week, then a shorter window of only 6 additional days were required to match the next mean sugar content for red grapes during the second collection, two weeks after *mid-véraison*. The increments became shorter, down to 4 additional days to meet the mean sugar content of red grapes each week thereafter. The trend displayed a nearly linear increase of mean sugar content in healthy red grapes, while the green grapes displayed a greater rate of increase and were synchronized by harvest.

As for GLRaV-3 infected vines, mean berry sugar content values ranged from 24.3% to 26.6% sugar content, a 1.4% larger range than healthy grapes (Fig. 4a,b). The ripening trend comparisons between green and red grapes from *mid-véraison* +1 week to harvest were also highly variable; green grapes required only a 6 day window to match the mean sugar content of the red grapes at *mid-véraison* +1 week. The mean sugar content present in red grapes at week two required 11 additional days to develop in green grapes. By harvest, the mean values of sugar content in infected green grapes had not converged to the same values as infected red grapes, nor did the ripening process display the same synchronization trend observed in healthy grapes.

Mean skin elasticity in healthy berries ranged from 1.9 to 2.1 MPa (Fig. 4c). Because of the variation in the graph, lines were not applied to indicate relationships between green and red grapes, such as the ones applied to the Brix° analyses, see discussion.

Skin elasticity ranged from 1.8 to 2.1 MPa; a 0.1 MPa larger range than that of healthy berries (Fig. 4c,d). Again, lines were not used to compare the exact differences between green and red grapes in this graph; see discussion. The elasticity results, however, display less synchronization/convergence of mean MPa at harvest in GLRaV-3 infected grapes than what is observed in healthy grapes.

Mean color index measurements ranged from 22.6 to 30.2 CIELAB units (Fig. 4e). Lines were also integrated into this graph to show the number of days required until mean color units of green grapes synchronized to respective mean amounts of red grapes; a total of 15 days were required after *mid-véraison* +1 week for green grapes to achieve the same CIELAB parameter units of red color as the red grapes had during the first collection. The increments then became much shorter as the color rapidly increased in green grapes to synchronize with the red, requiring only 3 days to match the second week of red-soft color, and then 6 additional days to match the final mean color of the healthy red grapes. The red-soft, mean color, CIELAB units remained at a linear increase from *mid-véraison* to harvest, while the green grapes had a more rapid incline and were synchronized at harvest as expected.

Color indices from berries of infected grapevines ranged from 20.7 to 27.7 CIELAB units indicating strong "red" color common of Pinot noir grapes ready for harvest (Fig. 4f). This is actually a 0.6 CIELAB-unit smaller range than that described in healthy berries (Fig. 4e), indicating lighter color. The days required for green grapes to achieve the ripened color of red grapes at two weeks after *mid-véraison,* the lowest measured mean color value for the red-soft class, were 17; similar with that observed for berries from healthy grapevines. Then, 3 additional days were required for green-soft grapes to achieve the next red color stage from red-soft grapes, which was actually the collection at *mid-véraison* +1 week in this case. The red-soft class continued to increase in mean color units throughout the remaining 8 days of the growing season, and the green-hard class did not synchronize any further, leaving a larger variation in ripening color than that observed in healthy berries.

Color index in infected berries, even with the smaller range in values, were generally lower values for the desired "red" color than healthy grapes do (Fig. 4e,f). Elasticity holds

slightly lower MPa values as well (Fig. 4c,d). Of the healthy berry classes (green-hard, green-soft, pink-soft, and red-soft), the green-hard and green-soft grapes displayed steep increases in the property measurements (aside from elasticity results), to converge at a more even ripeness with the red and pink classes measured.

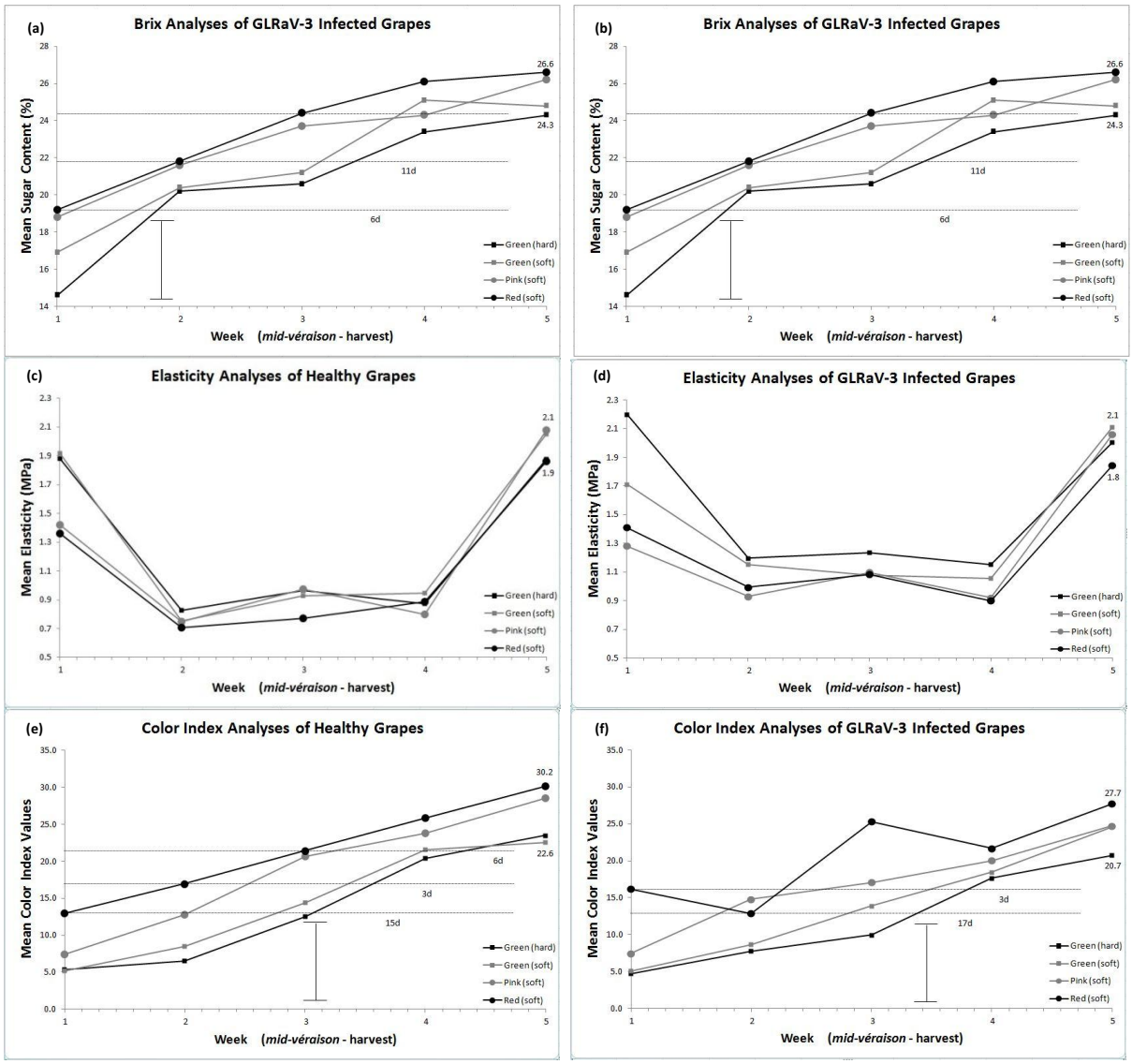

Figure 4 Sugar content of grapes from healthy (a) and GLRaV-3 infected (b) vines. Elasticity measurement from healthy (c) and GLRaV-3 infected (d) vines. Color index measurements from healthy (e) and infected (f) vines. Lines applied (if any) are to indicate window of days required for green-hard (class) grapes to reach a previous ripening stage of red-soft (class) grapes. Week five after *mid-véraison* lowest and highest measurements are displayed next to the point of corresponding berry class.

Discussion

Quantification

GLRaV-3 titer in young leaf tissues displayed the greatest increase throughout the growing season in all plants relative to initial titer at week one (Fig. 3a,b). This is most likely due to a reduced rate of plant growth as the season progressed in the young leaves, allowing for virus titer increase on a tissue weight basis. . Middle-aged and mature leaves develop a leathery feel because of a more developed cuticle, which offers more protection to the plant from biotic and abiotic elements, but is harder to wash and separate from RNA in the extraction process. As seen in Figure 4e, mature leaf tissue from the 114/Gloire grafted clone displayed little amplification. Leaf protecting cuticles could be the cause of this from inhibition of RNA extraction, or inhibition of enzymatic activity during the RT or PCR steps; however, the same trend was not observed in 2011 (Figure 4f). Since Figure 4f does not display this trend, the most likely cause of varying results from mature tissue in 2010 was from amplification problems. All mature tissue samples from the 114/Gloire grafted clone from 2010 were analyzed in one plate with duplicates, suggesting that the problem came from the qPCR assay process.

Overall results from GLRaV-3 titer analyses displayed a strong increase within the month of June (Fig. 3), and in some cases, nearly all of the observed titer increase throughout the season occurred in June. These high titers of GLRaV-3 early in the season suggest mealybugs would be equally efficient GLRaV-3 vectors throughout the season and pest management methods to control mealybugs should be a season long activity once the crawler stages are present in vineyards. This research will be followed with an analysis of GLRaV-3transmission efficacy of individual mealybugs in a colony. . It still remains possible that mealybugs may not be able to transmit GLRaV-3 in the early growing season (June) due to other biological inefficiencies.

Berry Analysis

Mean sugar content analysis of grapes from healthy (control) vines displayed the expected cluster ripening convergence of green to red grapes common in most healthy red-wine varieties. This happens due to a synchronization mechanism, which causes half of the cluster to develop steadily to a red color, while the other half develops the color at a slower pace at first, then rapidly toward harvest. The divisions within graphs in Figure 4 relate the amount of days required for green-hard grapes to reach each level of red-soft ripening (at any property). A polynomial regression cannot be fit to graphs in Figure 4, because each biological function does not behave in the polynomial, nor linear, fashion; therefore, inferences are best made between the individual points of analysis. Note: sugar levels may seem higher than what is usual for other Pinot noir vineyards. This particular vineyard is in a warmer location than others, and commonly experiences higher sugar levels, corresponding to higher alcohol content in its wine products when compared to other Pinot noir vineyard products.

Color index results also displayed the expected convergence at a strong red color acceptable of ripe Pinot noir grapes in healthy control vines (Fig. 4e). Healthy berry color results did not converge as closely together in units as sugar content of healthy grapes, but the convergence was tighter than that observed in GLRaV-3 infected grapes (Fig. 4f).

Sound inferences cannot be drawn from the berry elasticity results (Fig. 4c,d). Week one translates into the highest elasticity value of the season, with large variation before harvest. There could be an unknown biological reason for this occurrence, but the most likely explanation for the varying elasticity results would be that the calipers used to make elasticity measurements are sensitive to whoever operates them. A different operator made elasticity measurements with the calipers for the collection at *mid-véraison* than the operator who made the measurements for

the remaining weeks. If week one (*mid-véraison*) is removed from the analysis, it would be possible to see a trend with the resulting four collections of the study. Another addition to the inconclusiveness of the elasticity results is the upper (2.1 MPa) and lower (1.8 MPa) harvest measurements of infected berry elasticity are very close to the upper (2.1 MPa) and lower (1.9 MPa) harvest measurements of healthy berry elasticity. A larger range in infected berries would be expected. The only indicator of GLRaV-3 infected berries differing from healthy are that the final harvest measurements of infected grapes do not converge together at all (Fig. 4d) , while the healthy (control) grapes do show convergence among the color-classes (Fig. 4c).

Very high elasticity can indicate an un-ripened grape, while low elasticity indicates a raisin-like or tender grape. Grape skin elasticity does not contribute much to quality of wine. Skin color and total berry sugar content compose the quality elements of wine, and are the most important parts of the study. Focusing on the two most important berry properties of sugar content and color index, it can be concluded that green berries at *mid-véraison*, and specifically green-hard berries, have a natural synchronization mechanism that allows them to obtain even ripeness with red-soft berries from *mid-véraison*. It can also be concluded that green-hard berries from infected vines have a disrupted synchronization mechanism that inhibits their even ripening with red-soft grapes at *mid-véraison*.

Future research will examine GLRaV-3 grapes at *mid-véraison* for specific genes inside grapes that are turned off or altered. If these genes can be isolated and restored through the use of biological assays, it could save growers with GLRaV-3 infected vineyards much time and money by eliminating the need to eradicate the virus. Additionally, restoring healthy gene activity in grapes while allowing the virus to infect other areas of the vines may inhibit further rapid

evolution of GLRaV-3 into something new, and possibly, more serious. The data from this study could be used as a reference for typical healthy and infected grape development characteristics.

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