Hop stunt viroid: Effect on Host (*Humulus lupulus*) Transcriptome and Its Interactions With Hop Powdery Mildew (*Podosphaera macularis*)

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Viroids are the smallest known plant pathogens that exploit host systems for their replication and cause diseases in many hosts. In this study, the host response of hop plants to *Hop stunt viroid* (HSVd) infection was studied through transcriptome analysis. RNA sequence analysis of hop leaves infected with HSVd revealed dynamic changes in hop gene expression. Defense-related genes and genes involved in lipid and terpenoid metabolism are the major categories that showed differential expression due to HSVd infection. Additionally, the effect of HSVd on development of hop powdery mildew (*Podosphaera macularis*) (HPM) was studied. Transcriptome analysis followed by quantitative reverse transcription-polymerase chain reaction analysis showed that transcript levels of pathogenesis-related (PR) genes such as PR protein 1, chitinase, and thaumatin-like protein genes are induced in leaves infected with HPM alone. The response in these genes to HPM is significantly down-regulated in leaves with HSVd-HPM mixed infection. These results confirm that HSVd alters host metabolism, physiology, and plant defense responses. Nevertheless, in detached leaf assays, HPM consistently expanded faster on HSVd-negative leaves relative to HSVd-positive leaves. Although HSVd infection suppresses elements associated with the host immunity response, infection by HSVd is antagonistic to HPM infection of hops.

Viroids are single-stranded, circular, nonencapsidated RNA molecules that do not code for any known proteins (Diener 2003). Molecular mechanisms through which viroids induce plant diseases and host responses remain elusive. Two widely accepted modes of pathogenicity through which viroids induce diseases are i) interaction between viroid motifs and host proteins (Owens et al. 2001) that might affect the downstream pathways in which the particular protein is involved, and ii) viroid-induced RNA interference (RNAi) gene silencing (Gómez et al. 2009; Papaefthimiou et al. 2001; Sano et al. 2010; Wang et al. 2004). Whether through RNAi, DNA methylation, or direct interaction with plant proteins, viroids alter host gene expression and physiology, resulting in disease. Itaya et al. (2002) demonstrated that tomato plants inoculated with either mild or severe strains of *Potato spindle tuber viroid* (PSTVd) induced expression of pathogenesis-related (PR) genes *PR1*, *PR2* (β -1,3-glucanase) and *PR3* (chitinase).

Although both strains induced all three PR genes, induction was higher when plants were infected with the severe strain relative to infection with the mild strain. Tessitori et al. (2007) showed that Etrog citron (*Citrus medica* cv. Etrog) infected with *Citrus viroid III* (CVdIIIb) led to downregulation of β -1, 3-galactosidase involved in cold and osmotic stress response, yet ethylene-responsive element-binding protein and peroxidases were up-regulated. These studies illustrate the diverse host pathways that can be altered in response to viroid infection.

Hop stunt viroid (HSVd) is a major threat to hop cultivation globally (Eastwell and Nelson 2007; O’Neal 2015; Parkinson and Reed 2013), yet the impact of HSVd is highly variable among different cultivars (Kappagantu et al. 2017). HSVd infection decreased cone yield and altered alpha/beta acid concentration in some HSVd-sensitive hop cultivars, such as ‘Glacier’ and ‘Willamette’. Vegetative growth and biomass of these cultivars were also severely affected by HSVd infection. None of these effects were observed in the cultivar Nugget. The underlying variation in genotype that leads to sensitivity versus tolerance to HSVd infection is yet to be identified. In addition to effects on growth and yield, HSVd infection has been shown to down-regulate the naringenin-chalcone synthase H1 gene in cultivar Admiral, a key enzyme in the phenylpropanoid pathway (Füssy et al. 2013). However, many aspects of HSVd-induced changes in the host transcriptome were not previously studied. Detailed transcriptome analysis will help illuminate pathways that are altered during HSVd infection and that result in reduced yields of sensitive cultivars.

HSVd frequently occurs in mixed infections with other pathogens in several host species, including peaches (*Prunus persica*) (Tessitori et al. 2002), grapevines, and citrus (Ito et al. 2002). In hops, HSVd occurs in mixed infections with carla-viruses (*Hop mosaic virus*, *Hop latent virus*, and *American hop latent virus*). *Apple mosaic virus* (genus *Ilarvirus*) and *Hop latent viroid* (HLVd) also frequently occur in mixed infections with HSVd (Pethybridge et al. 2008). In addition to virus and virus-like pathogens, HSVd occurs in mixed infection with fungal pathogens such as canker-causing *Fusarium sambucinum* (Cerruti 2010) and hop powdery mildew (HPM) *Podosphaera macularis* (M. Nelson personal communication).

HPM is an economically important disease for hop production worldwide (Gent et al. 2008; Neve 1991). *P. macularis* was first reported in 1997 in the Pacific Northwest, the major hop-growing region of the United States (Ocamb et al. 1999). Annual losses due to yield reduction and investments toward disease control make powdery mildew the most critical disease for economic sustainability of the hop industry (Mahaffee et al. 2003). Changes in the hop transcriptome infected with HPM have not been reported. Identifying the changes in the gene-

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expression pattern of a susceptible cultivar in hops provides valuable information about pathogen biology, the route of pathogenicity, and host pathways that are responsive to HPM infection. Also, knowledge of the potential interaction of HSVd and HPM is important for the development of disease management strategies.

Almost all the hops grown in different regions of the world are ubiquitously infected with HLVd (Barbara and Adams 2003). Although some effects of HLVd on hop resins have been reported (Patzak et al. 2001), HLVd was taken as a common denominator among healthy and HSVd- or HPM-infected plants. That is, all plants used in our current study were uniformly infected with HLVd.

The objective of this study is to identify alterations in the transcriptome of hop leaves resulting from HSVd infection of a HSVd-sensitive cultivar. This study also aims to determine if the prior presence of HSVd in hop leaves would alter susceptibility to HPM infection. The expression of major *PR* genes that were altered during single or mixed infections by one or both HSVd or HPM are quantified.

RESULTS

Transcriptome changes resulting from HSVd infection.

To determine changes in gene expression patterns as a consequence of HSVd infection, high-throughput sequencing of total RNA isolated from HSVd-negative (control) and HSVd-positive ‘Willamette’ leaves was performed. Relative expression levels of each contig based on reads per kilobase per million mapped reads (RPKM) normalized for total counts (Mortazavi et al. 2008) were estimated. Hop contigs that showed fold changes greater than absolute value 2 and corrected *P* value < 0.05 were retrieved. BLAST searches for each of the differentially expressed

contigs were performed using The Arabidopsis Information Resource (TAIR) BLASTX tool with the *Arabidopsis* genome as reference. All contigs that mapped to unknown, hypothetical proteins, uncharacterized proteins, or sequences that poorly mapped to *Arabidopsis thaliana* genes were filtered out. From this selection process, 365 differentially expressed hop contigs were identified, of which 270 contigs showed upregulation and 95 contigs showed downregulation upon HSVd infection (Supplementary Table S1). In a few instances, multiple hop contigs mapped to the same gene of *Arabidopsis*; this could be the result of sequence similarity of multiple contigs from the hop transcriptome with conserved regions of the same *Arabidopsis* gene. With subsequent analysis, 204 unique *Arabidopsis* differentially expressed gene (DEG) sequences were identified. Of these, 140 genes showed upregulation and 65 genes showed downregulation resulting from HSVd infection. One DEG belonged to both sets. The presence of a DEG in both up- and down-regulated datasets could result from the presence of multiple isoforms of the same gene.

Functional classifications of DEGs due to HSVd infection.

Genes were clustered into functional categories as shown in Figure 1. Most of the gene transcript levels that were diminished in HSVd-infected tissue belonged to gene ontology categories of defense response, photosynthesis, lipid biosynthesis, and terpenoid biosynthesis. In contrast, the gene categories that were enhanced as a result of infection included defense response, protein phosphorylation, and response to abiotic stress.

Growth of HPM on healthy versus HSVd-infected leaves.

Detached leaf assays (Fig. 2) of HPM growth and sporulation were conducted with leaves of the ‘Willamette’. The experiment was repeated at seven different timepoints from April to November

Fig. 1. Functional categorization of genes that exhibit altered transcript levels as a result of *Hop stunt viroid* (HSVd) infection. **A**, Transcript levels that increased during infection; **B**, Transcript levels that decreased during infection.

2015 and disease ratings were taken at 18 days postinoculation (dpi). HPM growth was higher on control (HSVd-negative) leaves compared with HSVd-positive leaves at six timepoints (Fig. 3). On average over all seven timepoints, the development of visual symptoms (sporulation) of HPM were 16 times greater

on HSVd-negative leaves relative to HSVd-infected leaves (P value = 2.80×10^{-11}).

RNA isolated from leaves 18 dpi was used as template in quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to determine the concentration of HSVd and HPM

Fig. 2. Measurement of hop powdery mildew growth on 'Willamette' leaves. **A**, Leaves placed on 1% agar gel and inoculated with powdery mildew spores diluted in distilled water with 0.001% Tween-20. **B**, 'Willamette' leaf free of *Hop stunt viroid* with powdery mildew growth. **C**, A *Hop stunt viroid*-infected leaf of 'Willamette'. Picture was taken 18 days postinoculation.

Fig. 3. Disease-ratings of 'Willamette' leaves inoculated with Hop powdery mildew (HPM). Disease rating of a particular leaf is the percentage of leaf covered with HPM with respect to total leaf at 18 days postinoculation. Each bar represents the mean $\pm$ standard error. An asterisk (*) indicates the statistical significant difference (P value < 0.05) of HPM growth on leaves tested negative for *Hop stunt viroid* (HSVd) versus those positive for HSVd. The dark gray bars represent HSVd-free and the light gray bars represent HSVd-positive.

in each sample. To minimize variation during the experiment process, qRT-PCR amplification assays of all the samples were done at a single time, after completing RNA collection at different timepoints. Results revealed a 2,341-fold greater concentration of HPM RNA on HSVd-free leaves relative to HSVd-infected leaves (P value = 0.011).

The relationship between HSVd and HPM based on qRT-PCR data were evaluated by Pearson correlation and linear regression analysis. The P value = 0.0439 ($\alpha < 0.05$) was calculated, using linear model fit (RStudio 0.99.902 software; R Studio Inc., Boston). The concentrations of HSVd and HPM showed a negative correlation (Pearson correlation coefficient r value = -0.238) (Table 1), suggesting that a higher concentration of HSVd in the leaf sample resulted in reduced growth of HPM (Supplementary Table S2).

Changes in gene expression due to the cumulative effect of HSVd and HPM infection compared with single infections of HPM.

Analysis of high-throughput sequencing data of RNA from samples inoculated with HPM (HSVd-free versus HSVd-infected leaves) revealed differential expression of 390 unique genes (DEGs) (Supplementary Table S3). Corrected P value < 0.05 and fold change greater than absolute value 10 was taken as cutoff value to retrieve differentially expressed contigs in this dataset, which was then used in BLASTX search to identify these DEGs. Of 390 DEGs, 61 genes showed upregulation and 329 genes showed downregulation due to HSVd-HPM mixed infection compared with HPM alone. Six DEGs were found in both datasets. Genes were clustered into functional categories; most of the enriched genes based on gene ontology belonged to the categories of defense response, oxidative stress, and protein phosphorylation.

Validation of high-throughput sequencing data using qRT-PCR.

To confirm results suggested by RNA sequence analysis (Table 2), three *PR* genes, namely, those for *PR1* (*PR1* gene family), hop chitinase (*HCH1*) (*PR3* gene family), and thaumatin-

like protein (*TLP*) (*PR5* gene family) were selected for further analysis by qRT-PCR.

In HSVd-sensitive 'Willamette', *PR1* expression levels were significantly higher in hop leaves inoculated with HPM compared with all other treatments (one-way analysis of variance [ANOVA] P value = 0.0024). *PR1* transcript levels in leaves inoculated with HPM were 746% higher compared with those of noninoculated controls (Table 3). No significant difference was observed in *PR1* transcript levels between noninoculated control samples and HSVd singly infected samples. However, HSVd suppressed the accumulation of *PR1* transcript levels by 89% in HSVd-HPM mixed infection samples, thereby inhibiting the defense response to HPM alone.

HCH1 transcript levels paralleled the trends exhibited by *PR1* in all four treatments (one-way ANOVA, P value = 0.001). Relative to noninoculated control plants, no significant difference was detected in the presence of *HCH1* transcripts with plants inoculated with HSVd only, yet *HCH1* transcript levels were 202% higher in samples inoculated with HPM. *HCH1* transcript levels were reduced by 79% in samples with mixed infection compared with noninoculated control samples (Table 3).

TLP transcript levels were not significantly different between noninoculated control samples and those with single inoculations of either HSVd or HPM, but a significant reduction of *TLP* transcripts (67%) was observed in plants with HSVd-HPM mixed inoculations (Table 3).

Overall, in 'Willamette', transcript levels of all three candidate genes showed a similar trend of increased accumulation or remained unchanged during HSVd or HPM single infection and depletion in HSVd-HPM mixed infection samples. Mixed infections resulted in transcript changes of all three genes at greater magnitudes compared with either single pathogen. This suggests greater suppression of plant immunity in mixed infection compared with a single infection of either pathogen alone (Fig. 4). Additionally, transcript accumulation of *PR1* showed similar results in both high-throughput sequence analysis and qRT-PCR (Supplementary Fig. S1). The magnitude of *HCH1* and *TLP* transcript accumulation varied between the two analytical methods used in this study, especially in samples with HSVd-HPM mixed infection. One possible explanation would be the presence of multiple isoforms of these genes in hops that could affect the RPKM values in high-throughput sequence analysis, whereas the primers for qRT-PCR were designed based on the coding sequences of a specific hop gene. Also, qRT-PCR analyses were performed with at least 15 samples from each treatment, thus giving more statistical power over high-throughput sequence analysis.

Among the three candidate genes considered, accumulation of *PR1* transcript levels showed strong correlation with HPM colony size (Table 1). Strength of the linear relationship between two variables (i.e., *PR1* transcript accumulation and HPM colony size) is represented by Pearson correlation coefficient r that is 0.899 at P value = 2.2×10^{-16} . The transcript levels of *HCH1* and

Table 1. Correlation analysis of transcript levels of genes of interest with transcript levels of *Hop stunt viroid* and hop powdery mildew in 'Willamette'^a

Gene	HSVd	HPM	HCH1	PR1	TLP
HSVd	1				
HPM	-0.238	1			
HCH1	-0.210	0.707	1		
PR1	-0.164	0.899	0.723	1	
TLP	-0.050	0.876	0.725	0.832	1

^a Transcript levels were measured using quantitative real-time polymerase chain reaction. Correlation between each pair is expressed through Pearson correlation coefficient (r) value. HSVd = *Hop stunt viroid*, HPM = hop powdery mildew, *PR1* = pathogenesis-related gene 1, *HCH1* = hop chitinase gene, and *TLP* = thaumatin-like protein.

Table 2. List of differentially expressed contigs homologous to pathogenesis-related genes^a

Hop contig	Mean expression values (RPKM)				BLASTX results		
	Control ^b	HSVd ^c	HPM ^d	HSVd-HPM ^e	TAIR ^f accession	Gene name	E value
LA456086	23	7	2100	23	AT2G14580	Basic pathogenesis-related protein 1	2E-58
LA393696	11	33	757	69	AT3G54420	EP3-3 chitinase	3E-82
LA570170	32	2	779	57	AT1G75030	Thaumatococcus like protein	9E-40

^a Expression values are calculated in RPKM (reads per kilobase of transcript per million mapped reads) units.

^b Control samples are not inoculated with either *Hop stunt viroid* (HSVd) or hop powdery mildew (HPM).

^c Samples with single infection of HSVd.

^d Samples with single infection of HPM.

^e Samples with mixed infection of HSVd and HPM.

^f TAIR = The Arabidopsis Information Resource.

TLP followed a slightly weaker correlation, although a correlation of $r > 0.7$ was observed between HPM and transcript levels of *PR1*, *HCH1*, and *TLP* (Table 1). That is, transcript levels of all three PR genes are proportionally higher in HPM-infected samples.

In the HSVd-tolerant ‘Nugget’, no significant difference of *PR1* was observed between HSVd-negative and HSVd-positive samples; yet, in HSVd-positive samples, *HCH1* transcript levels are reduced by 65% (P value = 0.0002) and *TLP* transcript levels were reduced by 81% (P value = 0.0001) compared with HSVd-negative samples (Table 3). Downregulation of *TLP* was not observed in ‘Nugget’ plants that were grown using tissue culture and were inoculated with an HSVd in-vitro transcript (Bullock 2016). In that situation, although the inoculated plants tested positive for HSVd, tissue culture conditions may have suppressed HSVd levels and its impact on gene expression. ‘Nugget’ plants used in the current study were inoculated with HSVd isolate hKFKi in 2007 and were grown under field conditions for 7 years before analysis.

In conclusion, qRT-PCR analysis revealed no significant changes in *PR1* transcript levels between HSVd-positive samples and noninoculated control samples in either sensitive or tolerant cultivars ‘Willamette’ and ‘Nugget’, respectively. Significant reduction of *HCH1* and *TLP* transcript levels was observed only in the HSVd-infected ‘Nugget’ (Table 2); expression of these additional PR genes were not affected by HSVd in ‘Willamette’. However, HSVd infection effectively suppressed the accumulation of transcripts of all three potential defense genes when ‘Willamette’ was challenged by inoculation with HPM.

DISCUSSION

RNA sequence data analysis detected dynamic changes in the hop transcriptome with single-infections of HSVd or HPM and with HSVd-HPM mixed infections. Downregulation of defense response genes associated with biological stress shows that HSVd could suppress some plant defense mechanisms, leading to possible enhanced infection from other pathogens. This relationship was also evident with downregulation of the same defense response genes, during mixed infection by HSVd-HPM, that were up-regulated in HPM infections. This strongly suggests that the presence of HSVd could suppress plant immunity. Potential suppression of plant immunity by HSVd infection was also suggested by the response of several other gene families. *TPS-CIN* (terpene synthase-like sequence-1,8-cineole) involved in defense response and mono terpene biosynthesis was down-regulated in HSVd-positive samples. The suppression of defense response genes was more evident in samples with HSVd-HPM mixed infection. Defense response genes such as *OSM34* (osmotin-like protein thaumatin related) gene, PR protein-coding

genes *PR1* and *PR4*, and several peroxidases were down-regulated during HSVd-HPM mixed infection. In contrast, other pathways that may provide protection under some circumstances were up-regulated. For example, lipoxygenase (*LOX1*, *LOX2*, *LOX3*, and *LOX4*) genes are suggestive of induction of the jasmonate pathway and changes in lipid metabolism during HSVd infection. RNA-seq analysis also showed HSVd infection resulted in greater accumulation of ethylene responsive genes (*ERF9*, *ERF4*, *ERF104*, and *RRTF1*), *AOS* (allene oxide synthase) and *HCH1B* (basic endochitinases B); all of which are involved in defense response. *HCH1B* is a chitin-binding enzyme that provides defense against fungal pathogens and was up-regulated in HSVd-infected tissue.

Basic metabolic pathways were also altered by infection. Photosynthetic genes such as *FD1* (ferredoxin 1), F-type H+-transporting *ATPD* (ATPase subunit delta), *PSAD-2* (photosystem I subunit D-2), *PSAL* (photosystem I subunit L), and *PSB27* (photosystem II family) protein were down-regulated in HSVd-positive samples. Genes with oxidoreductase activity, such as *FNR2* (ferredoxin-NADP[+]-oxidoreductase 2), *FAD2* (fatty acid desaturase 2), *ALDH2B7* (aldehyde dehydrogenase 2B7) and *2OG* (2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein) that are involved in maintaining the redox status of the cells were also down-regulated during HSVd infection. Further investigation is required to determine the relative role that each of these genes play in response to HSVd and the susceptibility of the HSVd-infected plant to other pathogens.

RNA sequencing was conducted with limited sample numbers; thus, results are suggestive only. Detailed analyses of three PR candidate genes (*PR1*, *HCH1*, and *TLP*) that exhibited, by high-throughput sequencing, the greatest differential expression in response to HSVd infection were pursued. qRT-PCR analysis showed no significant alteration in *PR1* levels between noninoculated controls (HSVd-free and HPM-free) and HSVd-infected samples in either ‘Willamette’ or ‘Nugget’. Since *PR1* gene expression is salicylic acid (SA) dependent and commonly used as a marker for systemic acquired resistance in plants, the absence of a *PR1* transcript level response suggested that the SA-dependent pathway was not triggered in hop by HSVd infection. A similar result was observed in tobacco plants infected with PSTVd (Hammond and Zhao 2009). In contrast to the above results, induction of defense response genes like *PR1b* was observed in dwarfed tomato plants infected by mild and severe strains of PSTVd (Itaya et al. 2002). Thus, induction of a *PR1* gene during viroid infection could be associated with specific host-pathogen interaction rather than a general phenomenon. Another possible explanation for the lack of *PR1* gene transcript accumulation during HSVd infection could be possible degradation of *PR1* transcripts through RNA silencing

Table 3. Summary of pathogenesis-related gene expression analysis determined using quantitative real time polymerase chain reaction analysis^a

Gene	‘Willamette’						‘Nugget’	
	HSVd ^b		HPM ^c		HSVd-HPM ^d		HSVd ^b	
	% Relative to control	<i>P</i> value	% Relative to control	<i>P</i> value	% Relative to control	<i>P</i> value	% Relative to control	<i>P</i> value
<i>PR1</i> ^e	136	0.2200	746	0.0171	11	0.0005	56	0.1955
<i>HCH1</i> ^f	92	0.7790	202	0.0927	21	0.0042	35	0.0002
<i>TLP</i> ^g	67	0.3535	125	0.6930	33	0.0320	19	0.0001

^a Student’s *t* test was used to compute significant differences between noninoculated control and infected samples. Control samples are not inoculated with either *Hop stunt viroid* (HSVd) or hop powdery mildew (HPM).

^b Samples with single infection of HSVd.

^c Samples with single infection of HPM.

^d Samples with mixed infection of HSVd and HPM.

^e Pathogenesis related gene 1.

^f Hop chitinase gene.

^g Thaumatin-like protein.

mechanisms. In this present study, the response of the *PR1* gene is superimposed over any effect that the uniform infection by HLVd may have contributed to all sample categories.

Prior infection by HSVd reduced the incidence and intensity of HPM sporulation on hop leaves; this trend was substantiated by disease ratings and by qRT-PCR results. However, the magnitude of HPM inhibition associated with HSVd infection was higher, as measured by qRT-PCR analysis (2,341-fold) relative to changes measured as visual disease ratings (16-fold). Since qRT-PCR analysis considers all RNA transcribed from the internal transcribed spacer 1 (*ITS1*) region of *P. macularis*, it provides a snapshot of all active cells of *P. macularis* on the leaf at the time

of RNA isolation, whereas visual disease ratings estimate production of spores and colony size on the leaf surface only.

Powdery mildew is a biotrophic pathogen that requires the living host tissue to complete its life cycle. During compatible interactions, powdery mildews acquire plant metabolites from the living tissues through haustoria. Several genes that are involved in race-specific and broad-spectrum resistance that confer resistance to powdery mildew have been identified in *Arabidopsis* and barley (Schulze-Lefert and Vogel 2000). Resistance to powdery mildew is usually associated with activation of a host response that constricts the spread of biotrophic pathogens. High-throughput sequence analysis suggested upregulation of

Fig. 4. Expression levels of three pathogenesis-related genes among treatments, calculated using quantitative real-time polymerase chain reaction analysis in ‘Willamette’. Each bar represents the mean $\pm$ standard error. Statistical significant differences between noninoculated control and infected samples were computed using Student’s *t* test (one asterisk [*], *P* value < 0.05; two asterisks [**], *P* value < 0.01). HSVd = *Hop stunt viroid*, HPM= hop powdery mildew; HSVd-HPM= *Hop stunt viroid*-hop powdery mildew mixed infection. **A**, Pathogenesis-related gene 1 (*PR1*); **B**, Hop chitinase gene (*HCH1*); and **C**, thaumatin-like protein (*TLP*). Note that different scales are used in the graphs based on expression levels.

genes involved in response to osmotic stress and ethylene-responsive protein factors in addition to downregulation of oxidoreductases resulting from HSVd infection; this may have led to alterations in the osmotic balance and redox status of the cells, making them unsuitable for the growth of HPM.

Transcripts of several defense-response genes, including three specific genes (*PR1*, *HCH1*, and *TLP*) considered in this study, accumulated to higher levels during HPM infection. The SA-dependent defense system pathway usually associated with expression of *PR1* is highly induced during powdery mildew infection of other hosts (Fung et al. 2008; Xin et al. 2012). Furthermore, the accumulation of *PR1* and *PR5* gene transcripts during compatible interactions of *Arabidopsis* with powdery mildew suggest the induction of the SA pathway (Reuber et al. 1998). In contrast, the powdery mildew-resistant *Arabidopsis* mutants (*pmr-1* to *pmr-4*) did not show induction of the *PR1* gene (Vogel and Somerville 2000), suggesting that the SA-induced defense response pathway may not be associated with resistance to powdery mildew. Although the *PR1* gene and, presumably, the SA pathway are highly induced in hop upon powdery mildew infection alone, this may not be sufficient to induce resistance toward hop powdery mildew.

Miehle and Seigner (2007) introduced the *HCH1* gene from powdery mildew resistance hop cultivar Zenith to the susceptible cultivar Saazer by agroinfiltration. Some races of HPM showed reduced growth on 'Saazer' plants expressing the *HCH1* gene, suggesting the possible role of *HCH1* in powdery mildew resistance. The current study shows that, in the presence of HSVd, the response of the *HCH1* gene to HPM is dramatically down-regulated and, yet, these leaves exhibited greater resistance to HPM growth.

This study demonstrates that HSVd infection acts as a negative regulator of several genes that are typically elicited by other pathogens as part of the defense response. The presence of HLVd may modulate the effects of HSVd on the expression of defense-related genes; further study of this relationship is necessary to resolve this question. However, the presence of HLVd represents the status of most commercial hop plants currently grown in North America (Eastwell and Nelson 2007) and provides a valid and practical background in which to measure the effects of HSVd. Unexpectedly, the prior presence of HSVd reduces the ability of HPM to replicate and sporulate on hop leaves. It is not known at this time if the suppression of PR proteins by HSVd affects the development of disease caused by other pathogens. However, the data presented herein are critical findings in gaining further insights into HSVd pathogenesis and the host responses that it solicits.

MATERIALS AND METHODS

Cultivar selection.

'Willamette' is moderately sensitive to both HSVd infection (Kappagantu et al. 2017) and HPM infection (Mahaffee et al. 2003). This cultivar provided an ideal platform to study the transcriptome changes in hosts during single and mixed infections of these two pathogens. 'Nugget' is tolerant to HSVd infection (Kappagantu et al. 2017) and resistant to the HPM isolate 166 infection. All plants used in this study tested positive for HLVd. 'Nugget' and 'Willamette' plants were propagated through tissue culture and were inoculated in 2007 with an extract from a single hop plant infected with HSVd, these plants and their corresponding noninoculated control plants were grown under field conditions until 2014, when they were propagated through green cuttings and were subsequently maintained under greenhouse conditions. 'Zeus' plants were propagated through green cuttings (Bullock 2016) from virus-tested stock plants. All plants were grown at 25 to 28°C with a 14-h-light and 10-h-dark cycle.

HPM inoculations.

HPM was maintained by pressing leaves of 'Zeus' infected with HPM isolate 166 onto young leaves of three 'Zeus' plants. The powdery mildew-inoculated plants were maintained in isolation at 28°C with 14-h-light and a 10-h-dark period.

Ten fully expanded leaves of uniform size from noninoculated HSVd-negative control and HSVd-positive 'Willamette' plants were collected. Leaves were sterilized with calcium hypochlorite solution (0.86 gm/liter) for 5 min and were washed thrice with sterile distilled water to remove traces of the bleaching agent. Leaves were dried for 5 min by gently dabbing with sterile tissue paper. Leaves were then placed on petri dishes with 1% plant cell culture-tested agar (Sigma-Aldrich, St.Louis) with the adaxial surface touching the agar to keep leaves hydrated (Fig. 2A).

'Zeus' leaves heavily infected with powdery mildew were chopped into small pieces and were transferred into 50-ml centrifuge tubes containing 5 ml of distilled water with 0.001% Tween-20 (Sigma-Aldrich); the tube was vortexed for 1 min. Spore concentration was measured using a hemocytometer and was adjusted to approximately 20,000 spores/ml with sterile water. This inoculum (35 µl) was placed on each of three spots on each leaf, i.e., 105 µl of total inoculum per leaf (Fig. 2A). After 5 min, excess liquid was carefully removed with a sterile cotton swab. Inoculated leaves were incubated at 22°C for 18 days, with 14 h of light daily. Disease ratings were determined 18 dpi as follows (Fig. 2B). Pictures of each leaf inoculated with HPM were captured, using Nikon D5200 24.1 MP CMOS digital SLR with a 18-135 mm NIKKOR Zoom Lens (Nikon Inc., Melville, NY, U.S.A.) and were imported into GeneTools software (Syngene, London). The total area of the leaf was assigned the calibrator value of 100 and manual band quantification was used to calculate the area of each spot on the leaf infected with HPM. Thus, the disease rating of a particular leaf is the percentage of leaf covered with HPM spores.

The experiment was repeated at monthly intervals with 'Willamette' leaves (from May 2015 through Nov 2015); at least ten noninoculated control and ten HSVd-inoculated leaves were collected at each timepoint. In total, data were collected at seven different timepoints. As, 'Zeus' is highly susceptible to HPM and 'Nugget' is resistant to HPM, one HSVd-negative leaf of 'Zeus' and one HSVd-negative leaf of 'Nugget' were also inoculated with HPM at each point, as positive and negative controls, respectively. Data analyzed, using Microsoft Excel (Microsoft Inc., Redmond, WA, U.S.A.), is presented as a mean with standard error; a *P* value < 0.05 was considered as statistically significant.

RNA isolations and quality testing.

Total RNA was isolated from leaf tissues with the RNeasy plant mini kit (Qiagen, Valencia, CA, U.S.A.). Each leaf was treated as a separate sample. Each leaf was detached from the petiole and was homogenized in a mesh-lined grinding bag (Agdia Inc., Elkhart, IN, U.S.A.) containing 3 ml of RLT buffer (Qiagen) amended with 3% 2-mercaptoethanol, 2.5% polyvinylpyrrolidone (PVP-40), and 0.1% sodium metabisulfite. The lysate was incubated at 65°C for 10 min and was centrifuged at 18,400 × *g* for 2 min. The lysate was transferred to a QIAshredder spin column (Qiagen) and was centrifuged at 21,130 × *g* for 2 min. One-half volume of 100% ethanol was added to the supernate, was mixed immediately, was transferred to a RNeasy Plant mini spin column (Qiagen), and was centrifuged at 8,000 × *g* for 30 s. RW1 buffer (700 µl; Qiagen) was added to the column and it was centrifuged at 8,000 × *g* for 30 s. The column was treated with 80 µl of DNase (10 µl of RNase-Free DNase with 70 µl of RDD buffer [Qiagen]) and was incubated for 15 min at room temperature. Each column was washed twice with 500 µl of RPE buffer (Qiagen). RNase-free

water (60 µl) was added to each column and was centrifuged at $8,000 \times g$ for 1 min to elute total RNA.

RNA concentration and quality was determined from fixed wavelength readings at 230, 260, and 280 nm (Genesys 10 Bio UV-Visible Spectrophotometer with a nanocell accessory [Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.]). Ratios 260/280 and 260/230 were calculated to measure contamination due to protein (absorbance at 280) and carbohydrates/phenols (absorbance at 230 nm), respectively.

Total RNA (1 µl) was analyzed by agarose gel electrophoresis to check the integrity of RNA. RNA samples with a 260/280 and 260/230 ratio of 1.8 or greater and with clear 28S and 18S rRNA bands in agarose gels were stored at -80°C until further analysis.

Sample 11796 $\times$ 3 was diluted from 640 ng to 447 ng per microliter, using distilled water, before high-throughput sequencing to obtain an RNA concentration comparable to other samples used in this study.

High-throughput sequencing.

Samples of noninoculated leaves, leaves infected with HSVd only, leaves inoculated with HPM only, and HSVd-infected leaves inoculated with HPM (HSVd-HPM mixed infection) were used for sequencing. Thus, a total of 12 samples (three samples of each treatment) were used for high-throughput sequencing (Supplementary Table S4). Each sample used for sequencing was RNA-pooled from two individual leaves. Library construction and sequencing was performed by Huntsman Cancer Institute, Core Centre for High Throughput Genomics, Salt Lake City, UT, U.S.A. Libraries were constructed using Illumina TruSeq Stranded Total RNA sample prep with RiboZero (plant) kit (Illumina, San Diego, CA, U.S.A.). Library quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, U.S.A.). Sequencing was performed with a HiSeq 50 Cycle Single-Read sequencing cluster kit v4 and Illumina HiSeq 2000 sequencer, and raw sequencing data were retrieved in Illumina FASTQ format. Raw sequences were deposited and are available in Gene Expression Omnibus database under accession numbers GSM2705476 to GSM2705487.

Data preprocessing, read mapping, and differential expression analysis.

Sequencing data analysis was performed using CLC Genomics Workbench 9.0.1 (CLC-Bio-Qiagen, Aarhus, Denmark). The following quality control for raw reads was conducted: i) adapter sequences were trimmed; ii) low-quality reads and reads with ambiguous nucleotides were trimmed based on PHRED quality scores (Q-score), using default parameters, and iii) reads smaller than 15 nt and greater than 1,000 nt were removed. The average number of reads per sample after trimming was 19,279,022; the average length of each read was 50 bases.

Trimmed raw reads were used in RNA sequence expression analysis. Expression analysis was performed using the hop transcriptome, available in the National Center for Biotechnology Information (NCBI) database (GenBank Transcript BioProject 270039) (387,929 transcript contigs), as the reference genome. RNA sequence analysis was performed with default settings in CLC Genomics Workbench and expression values for each contig were calculated in total counts and RPKM (normalized for total counts) (Mortazavi et al. 2008). Distribution of expression values among different samples was represented by box plot, variability among different samples was represented by principal component analysis (PCA) (Supplementary Fig. S2). $\log_{10}$ transformed expression values were used for both box plot and PCA plot. Since none of the samples showed atypical distribution and samples exhibited less variability within the group compared with between groups, RPKM

values between all the samples were directly used to calculate fold change of contigs. Two contigs that were not represented in all samples were removed from further analysis. Statistical analysis of RNA-seq data were performed using the empirical analysis of DGE (edge) tool in CLC Genomics Workbench, v. 9.0.1., with total count filter cutoff set at 5. Contigs that showed false discovery rate (FDR) corrected P value < 0.05 were selected for further analysis.

RNA from the samples inoculated with HPM (HSVd-free and HSVd-infected leaves inoculated with HPM) was collected from agar plates 18 dpi after inoculation with HPM, whereas RNA for noninoculated control and HSVd-positive leaves were collected directly from plants. Therefore, the changes in RNA sequence levels were calculated separately for the samples with and without HPM inoculations. To further filter the differentially expressed contigs to a manageable number, fold change greater than absolute value 2 was used for the noninoculated control and HSVd-positive leaves dataset (samples without HPM inoculations). Fold change greater than absolute value of 10 was used to filter contigs in samples inoculated with HPM.

Gene ontology and functional clustering.

BLAST searches for each of the differentially expressed contigs were performed using the TAIR BLASTX tool with the *Arabidopsis* genome as reference. Only contigs with an E value ≤ 0.05 in the BLASTX search were selected for further analysis. Contigs with high sequence similarity to annotated *Arabidopsis* gene sequences were retrieved, and the corresponding TAIR accession was used to run functional clustering and pathway analysis using Database for annotation, visualization and integrated discovery (DAVID; Bioinformatics Resources 6.7 tool, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, U.S.A.) with the *Arabidopsis* genome as background. Fisher's exact test was used to measure the gene enrichment scores and annotation terms to functionally categorize candidate genes with the DAVID functional categorization tool with default settings.

Sequence analysis and primer design for quantification of candidate genes.

High-throughput sequencing analysis showed an approximately 300-fold change for hop contig LA456086 when all four treatments were compared (Table 1). Mapping the contig LA456086 sequence using BLASTX tool in the TAIR database indicated a sequence match with basic PR1 (TAIR accession: AT2G12480) with an E value of 2×10^{-58} . To further confirm the contig LA456086 sequence specificity to PR1, contig LA456086 was used for a BLASTX search in the NCBI database. NCBI BLASTX results suggested a match between contig LA456086 and *Citrus sinensis* PR1 protein (E value = 7×10^{-82}). In both cases, the BLASTX tool was used to search protein databases using translated nucleotide query (i.e., contig LA456086 nucleotide sequence), and the results confirmed sequence similarity between the protein sequence of translated contig LA456086 and PR1 proteins from other plant species.

The ORFfinder tool was used to identify the open reading frame (ORF) of the PR1 coding sequence on contig LA456086. An ORF was identified between positions 249 and 700 (492 nt coding for 163 amino acids) on the contig LA456086 nucleotide sequence that showed sequence similarity with the *PR1* gene of *Gossypium raimondii* (E value = 2×10^{-60}). The ORF (Supplementary Fig. S3, highlighted) coding the *PR1* gene was used to design primers for qRT-PCR analysis.

Humulus lupulus endochitinase precursor gene (*HCH1*) sequence from the NCBI database (AF147497.1, GenBank) was retrieved. Primers were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA, U.S.A.) to amplify a 220-bp

product of *HCHI* gene exon 3. Coding sequences for the *TLP* gene were identified on hop scaffold LD139544 and were used to design primers for qRT-PCR analysis, using the NCBI primer BLAST tool (Bullock 2016).

qRT-PCR.

All qRT-PCR reactions were performed with a thermocycler (Rotor-Gene 6000 instrument [Corbett Research, Mortlake, Australia] equipped with 6000 series software, version 1.7), set to capture fluorescent readings at 520 nm with a gain setting of 7. Wavelength of excitation and emission spectrum of EvaGreen (Biotium, Inc., Fremont, CA, U.S.A.) dye-bound to PCR amplicon (i.e., double-stranded DNA) is 500 and 530 nm, respectively. qRT-PCR reactions were performed with the primers listed in Supplementary Table S5. A minimum of 15 samples of 'Willamette' and 'Nugget' from each treatment were used in qRT-PCR assays to conduct statistical analysis. Sample collection was limited to control and HSVd-positive treatments for the 'Nugget'. RNA concentrations of samples were adjusted to 75 ng/μl to maintain uniformity among the samples.

HSVd qRT-PCR.

Each reaction consisted of 3 μl of sample RNA (adjusted to a concentration of 75 ng/μl), 22 μl of master mix buffer (12.5 μl 2× buffer [Ambion, Thermo Fisher Scientific Inc.], 4.75 μl of RNase-free H₂O, 0.25 μl of forward primer [HSVd-F, 20 μM], and 0.25 μl of reverse primer [HSVd-R, 20 μM]), 2 μl of 7.5 μg nonacetylated bovine serum albumin (BSA) (New England Biolabs Inc., Ipswich, MA, U.S.A.) per microliter, 1 μl of Super Script III reverse transcription and Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.), and 1.25 μl of 20× EvaGreen (Biotium). First-strand synthesis was performed at 60°C for 30 min, followed by 94°C for 2 min and 40 cycles of 94°C for 15 s, 53°C for 60 s, and 68°C for 30 min, with a final extension of 68°C for 5 min.

HPM qRT-PCR.

An assay was previously described using primer set HPMF2 and HPMR4 to successfully amplify 123 bp of the ITS1 region of *Podospaera macularis* DNA, based on a SYBR green qPCR detection assay (Peetz 2007). To adjust the assay to amplify polycistronic precursor RNA of the ITS1 region, conditions were modified to contain 3 μl of sample RNA (adjusted to a concentration of 75 ng/μl), 22 μl of master mix buffer (12.5 μl 2× buffer [Ambion], 4.75 μl of RNase-free H₂O, 0.25 μl of forward primer [HPMF2] and 0.25 μl of reverse primer [HPMR4], 20 μM each), 2 μl of 7.5 μg/μl nonacetylated BSA, 1 μl Super Script III reverse transcription and Platinum *Taq* DNA polymerase (Invitrogen), and 1.25 μl of 20× EvaGreen (Biotium). First-strand synthesis was performed at 55°C for 30 min, followed by 94°C for 2 min; 40 cycles of 94°C for 20 s, 65°C for 44 s, 72°C for 30 s, and a final extension of 72°C for 10 min.

qRT-PCR amplification of candidate hop genes.

For the HSVd-sensitive 'Willamette', RNA samples collected from control, HSVd-infected, HPM-infected (control leaves inoculated with HPM), and HSVd-HPM (HSVd positive leaves inoculated with HPM) leaves were analyzed by qRT-PCR reactions to measure changes in transcript levels of the candidate *PR* genes. These data were compared with those obtained from qRT-PCR analysis of the HSVd-tolerant 'Nugget'. Since the HPM strain 166 used in this study does not infect 'Nugget' (Gent et al. 2009), only RNA collected from control and HSVd-positive leaves were used for qRT-PCR analysis. The relative expression changes of candidate genes measured by qRT-PCR assays were represented as percent relative to the noninoculated control group.

To calculate the relative concentration of *PR1* and *HCHI* genes in each treatment, the following protocol was used to amplify the specific gene of interest. The reaction mixture contained 3 μl of sample RNA, 22 μl of master mix (12.5 μl 2× buffer [Ambion], 4.75 μl of RNase-free H₂O, 0.25 μl of forward primer and 0.25 μl of reverse primer, 20 μM each), 2 μl of 7.5 μg/μl nonacetylated BSA, 1 μl of Super Script III reverse transcription with Platinum *Taq* DNA Polymerase [Invitrogen], and 1.25 μl of 20× EvaGreen (Biotium). First-strand synthesis was performed at 55°C for 30 min, followed by 94°C for 2 min and 40 cycles of 94°C for 15 s, 60°C for 1 min, 68°C for 1 min and a final extension of 68°C for 5 min. qRT-PCR analysis for the *TLP* gene was performed using the following conditions. First-strand synthesis was performed at 58°C for 30 min, followed by 94°C for 2 min and 40 cycles of melting 94°C for 15 s, annealing at 58°C for 30 s, extension at 68°C for 1 min, and a final extension of 68°C for 5 min. All qRT-PCR reactions were performed with a thermocycler (Rotor-Gene 6000 instrument equipped with 6000 series software, version 1.7), and amplified products were sequenced to confirm identity of amplified product (ELIM Biopharmaceuticals, Hayward, CA, U.S.A.).

qRT-PCR amplification of reference genes and normalization.

Reference genes used for normalization of expression analysis were the *7SL RNA* component of the signal recognition particle (*7SL RNA*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Maloukh et al. 2009; Natsume et al. 2015; Štajner et al. 2013). For reference gene amplification, qRT-PCR conditions were similar to those used to amplify the *PR1* gene, with their respective primers, except specific annealing temperature of 55°C for *GAPDH* and 57°C for *7SL RNA* were used.

Confirmation of specificity of qRT-PCR.

Each qRT-PCR amplification was followed by a high-resolution melt curve analysis ranging between 50 to 99°C, raising 1°C at each step. All qRT-PCR products were resolved through high voltage capillary gel electrophoresis (QIAxcel, Qiagen) with a 50- to 800-bp DNA ladder (Qiagen) as a reference.

qRT-PCR statistical analysis.

Relative gene expression analysis was performed using the two standard curve method. The cycle threshold (Ct) values of standard curves of genes of interest (*HSVd*, *HPM*, *PR1*, *HCHI*, *TLP*) were normalized to Ct values of standard curves of reference genes. Results were analyzed in Microsoft Excel. The *F* test was used to calculate equality among variances of samples belonging to different groups, followed by the Student's *t* test to compute significant differences between noninoculated control and HSVd samples.

Differences between gene of interest levels among all four groups (noninoculated control, HSVd, HPM, and HSVd-HPM) were calculated by single-factor ANOVA at significance *P* value <0.05. Correlation and linear regression analysis was performed using RStudio 0.99.902 software (R Studio Inc.).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Arabidopsis Information Resource (TAIR) BLASTX tool:

<https://www.arabidopsis.org/Blast/index.jsp>

The ORFfinder tool: www.ncbi.nlm.nih.gov/orffinder