Identification and Characterization of Verticillium Nonalfalfae-responsive Micrornas in the Roots of Resistant and Susceptible Hop Cultivars

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Abstract

Background

Micro RNAs are 21- to 24-nucleotide-long, non-coding RNA molecules that regulate gene expression at the post-transcriptional level. They can modulate various biochemical and physiological processes, including plant response and resistance to fungal pathogens, by regulating the expression of numerous genes. Hops are grown for use in the brewing industry and have recently attracted the attention of the pharmaceutical industry. Severe Verticillium wilt disease caused by the phytopathogenic fungus *Verticillium nonalfalfae* is the main factor in yield loss in many crops, including hops (*Humulus lupulus* L.).

Results

In our study, we identified miRNAs in hops and their expression patterns in the roots of susceptible and resistant hop cultivars in the early stages of infection with the fungus *V. nonalfalfae*. In total 56 known and 43 novel miRNAs were predicted. In response to infection with *V. nonalfalfae*, we found five known and two novel miRNAs that are differentially expressed in the susceptible cultivar and six known miRNAs in the resistant cultivar. Differentially expressed miRNAs target 49 transcripts and their gene ontology enrichment analysis showed that the susceptible cultivar responds by modulating the processes of protein localization and pigment synthesis, whereas the resistant cultivar responds by modulating transcription factors and hormone signalling.

Conclusion

The results of our study suggest that the susceptible and the resistant cultivar respond differently at the miRNA level to infection with *V. nonalfalfae* and that miRNAs may contribute to the successful defence of the resistant cultivar.

Background

Hops (*Humulus lupulus* L.) are traditionally cultivated for use in the brewing industry as an essential ingredient that provides flavour and acts as a stabilizer or preserver of the beer [1]. In recent years, the bioactive compounds of hops have also become increasingly attractive for use in the pharmaceutical industry [2, 3]. One of the main limiting factors in hop production are fungal diseases, especially those caused by the soil-borne plant pathogenic fungus *Verticillium nonalfalfae* (formerly known as *Verticillium albo-atrum*) [4]. The symptoms of Verticillium wilt in hops caused by *V. nonalfalfae* vary depending on the pathogenicity of the fungal strain and the sensitivity of the cultivar. Susceptible hop cultivars can suffer from severe symptoms (e.g. leaf chlorosis and necrosis) and also complete dieback of rootstock caused by a highly virulent strain of *V. nonalfalfae* [5].

To prevent infections with various phytopathogens, plants have evolved multi-tiered defence mechanisms. The first layer of defence is represented by pattern recognition receptors (PRRs) present at the cell membrane surface that recognize conserved pathogen-associated molecular patterns (PAMPs). This phenomenon is called PAMP-triggered immunity (PTI) or basal defence. PTI comprises both physical and chemical defence responses, e.g. the deposition of lignin-like compounds in the cell wall, the production of reactive oxygen species (ROS) and the activation of signalling cascades that modulate gene expression. Successful pathogens can counteract the plant basal immune response by deploying the effectors into the cytoplasm of plant cells to attenuate defence. Their presence in the cytoplasm is directly or indirectly detected by receptors, so-called resistance (R) proteins, or nucleotide-binding leucine-rich repeat (NB-LRR) proteins encoded by *R*-genes. Resistance mediated by *R*-genes is considered to be the second layer of defence and confers an enhanced type of defence, known as effector-triggered immunity (ETI) [6, 7]. To achieve the best effectiveness of the defence response, all defence mechanisms must be well regulated. In this dynamic and complex network of gene regulatory pathways during the immune response, short non-coding RNAs, so-called micro RNAs (miRNAs), play a pivotal role [8, 9]. miRNAs are a class of small endogenous non-coding RNA molecules with a length of 21 to 24 nucleotides, which act as post-transcriptional regulators of gene expression and are thus involved in various biological processes [10]. Recently, Soto-Suarez, Baldrich, Weigel, Rubio-Somoza and San Segundo [11] demonstrated that miR396 mediates PAMP-triggered immune response against necrotrophic and hemibiotrophic fungal pathogens in *Arabidopsis*. Navarro, Dunoyer, Jay, Arnold, Dharmasiri, Estelle, Voinnet and Jones [12] discovered that in *Arabidopsis* plants treated with PAMP (Flagellin fragment 22), miR393 was upregulated and as a result the expression of *F-box auxin receptors* was silenced, leading to suppression of the auxin signalling pathway and enhanced PTI. The upregulation of miR393 was also detected in soybean in response to infection with the pathogenic fungus *Phytophthora sojae*. Moreover, miR393-knockdown soybean plants show increased susceptibility to infection [13]. However, miRNAs other than miR393 can modulate the auxin signalling pathway in various pathogen infections and are involved in plant immune response. For example, miR160 regulates auxin response factors (ARFs) in potato and thereby indirectly affects the expression of genes that modulate salicylic acid–auxin cross-talk, which is associated with local defence and systemic acquired resistance to *Phytophthora infestans* [14]. NB-LRR proteins, products of *R*-genes that mediate ETI in plants, are targeted by several miRNAs, such as miR2118 in *Medicago truncatula* [15] and in cotton infected with *Verticillium dahliae* [16], by gma-miR1510a/b, which contributes to resistance to *Phytophthora sojae* [17] and by ptc-MIR482, ptc-MIR1447 and ptc-MIR1448 in *Populus trichocarpa* [18, 19].

Although there is increasing evidence that miRNAs play an important role in the regulation of gene expression during the immune response in plants, there is a scarcity of information on miRNA-mediated gene silencing during the pathogenesis of fungal diseases, such as Verticillium wilt, in various crops. In hops, a single quantitative trait loci (QTL) has been identified, which explains 26% of the phenotypic variance for Verticillium wilt resistance [20], and potential EST-RGA markers have been developed [21]. A well-studied example of Verticillium wilt resistance to date relates to the tomato's *Ve1* gene, which codes

for a leucine-rich repeat (LRR) receptor-like protein that confers the resistance to a strain of *V. dahlia* race 1 or *V. nonalfalfae* [22–25]. *Ve1* orthologue was also characterized in hops and it is suggested that it provides the resistance to *V. dahliae* strain 1 by detecting an effector protein Ave1 [26]. In the proteomic study, Mandelc, Timperman, Radisek, Devreese, Samyn and Javornik [27] observed an accumulation of defence-related proteins, such as chitinase, β-glucanase, thaumatin-like protein, peroxidase and germin-like protein in the compatible interactions between *V. nonalfalfae* and hops, while such response was not detected in the incompatible interaction. Similarly, increased expression of genes involved in innate immunity, the jasmonic acid pathway and wounding was observed in the roots and shoots of the susceptible hop cultivar [28] while Cregeen, Radisek, Mandelc, Turk, Stajner, Jakse and Javornik [29] observed increased expression of genes involved in ubiquitination (SKP1), vesicle trafficking (cdc48), protein degradation (puromycine-sensitive aminopeptidase), protein-protein interactions (syntaxin and Fk506), transport (acyl-CoA-binding protein) and morphogenesis (furry protein) in the resistant cultivar, and decreased expression in the susceptible cultivar.

In the present work, we characterized miRNAs in hops and identified *V. nonalfalfae*-responsive miRNAs in the roots of the susceptible and resistant hop cultivars. Furthermore, we used an *in silico* approach to predict transcripts targeted by *V. nonalfalfae*-responsive miRNAs and discussed their potential role in the defence response to *V. nonalfalfae* in susceptible or resistant hop cultivar.

Results

High-throughput sequencing of H. lupulus miRNAs

To investigate a miRNA response in roots of the susceptible and resistant hop cultivar after inoculation with *V. nonalfalfae*, small RNA libraries were constructed from 3 control and 3 treated root samples of both hop cultivars. A total of 90,355,033 reads were obtained, ranging from 5,222,013 to 10,059,037 reads per library with a mean read length of 14 bp to 20 bp. After processing the raw sequencing data, we obtained from 1,771,295 to 4,636,681 sequencing reads with a mean read length of 18 bp to 22 bp per library. Processed reads from each library were aligned against the hop draft genome [30, 31] disallowing mismatches (Table 1).

Table 1
Statistics of small RNA sequencing reads from 12 sRNA libraries. First two letters of sample names denote cultivar; CE = susceptible cultivar Celeia; WT = resistant cultivar Wye Target; Ctrl = control; Trt = treated, and numbers denote biological replicates (1–3).

Sample	Num. of raw reads	Mean length of raw reads (bp)	Num. of reads after processing	Mean length of processed reads (bp)	Mapped to hop genome
CE-Ctrl1	10041349	18	4636681	20	2634528 (56.82%)
CE-Ctrl2	6109522	16	2232764	20	1341656 (60.09%)
CE-Ctrl3	8146318	17	3232808	20	2044935 (63.26%)
CE-Trt1	8631607	16	2672954	19	1575284 (58.93%)
CE-Trt2	6991916	18	3466372	20	2000154 (57.70%)
CE-Trt3	6723592	14	1771295	18	1230008 (69.44%)
WT-Ctrl1	6223982	20	3745411	22	2094410 (55.92%)
WT-Ctrl2	5222013	19	2933213	21	1729720 (58.97%)
WT-Ctrl3	7737001	20	4083377	22	2393417 (58.61%)
WT-Trt1	10059037	18	4435862	21	2559244 (57.69%)
WT-Trt2	7537943	18	3698547	20	2125103 (57.46%)
WT-Trt3	6930753	20	3202208	22	1811863 (56.58%)

Table 2. Differentially expressed miRNAs in response to infection with *V. nonalfalfae* and their target transcripts in hops.

miRNA	Log_2FC; adj. $p \leq 0.1$		Sig. interaction; $p < 0.05$	Target transcript (orthologue ID *)
	DE in CE	DE in WT		
hlu-miR156e–f	NS	-0.65	NS	Squamosa promoter-binding-like protein 15 (W9RJ15; hops transcript: GAAW01048142.1) [4], Squamosa promoter-binding-like protein 6 (W9QNN5) [4], Squamosa promoter-binding-like protein 12 (W9RS10) [4], Squamosa promoter-binding-like protein 7 (W9R3S1) [4], Squamosa promoter-binding-like protein 13 (W9QLM6) [4], Squamosa promoter-binding protein 1 (W9SN75) [4], gag-polypeptide of LTR copia-type (3694.POPTR_0008s08210.1), Protein SCO1 homolog 2 (225117.XP_009378785.1), LOB domain-containing protein (W9SE87)
hlu-miR159c–d	1.28	0.95	NS	Putative anion transporter 3 (W9RGS2) [1, 3], Transcription factor GAMYB (W9QVM8) [2, 4], Integrase catalytic domain-containing protein (A0A087HSL5) [1, 2, 3, 4], Acetyltransferase At3g50280-like (M5VT21) [2, 4], RNA polymerase II transcription regulator recruiting activity (102107.XP_008226955.1), Cytochrome p450 (57918.XP_004291627.1)
hlu-miR160b	NS	1.21	NS	Auxin response factor (A0A061FPV2) [3,4], Auxin response factor (W9QUH2) [3,4], Auxin response factor (W9S7Q7) [3,4]
hlu-miR164b	NS	NS	-1.4	NAC domain-containing protein 100 (W9QTW9), NAC domain-containing protein (W9QCM5; hops transcript: GAAW01060518.1)
hlu-	-1.5	NS	1.35	Mediator of RNA polymerase II transcription subunit

miR167a–d				(102107.XP_008227322.1)
hlu-miR167f	-2.1	NS	1.34	Mediator of RNA polymerase II transcription subunit (102107.XP_008227322.1)
hlu-miR169a–d	NS	NS	-2.2	Nuclear transcription factor Y subunit A-8 (W9QJW4), ATPase (W9WDM0), Nuclear transcription factor Y subunit A-1 (W9RR19), Nuclear transcription factor Y subunit A-10 (W9SK30)
hlu-miR169m–n	NS	NS	-1.95	Nuclear transcription factor Y subunit A-10 (W9SK30), Nuclear transcription factor Y subunit A-1 (W9RR19), TATA-binding protein-associated factor (W9R0N8), Exo84_C domain-containing protein (W9SE15)
hlu-miR171g–h	NS	NS	-1.3	Scarecrow-like protein 22(102107.XP_008238556.1), Scarecrow-like protein 6 (57918.XP_004306953.1), GRAS domain-containing protein (A0A251QFM0; hop transcript: GAAW01082848.1), NAD(P)-bd_dom domain-containing protein (M5VYK9)
hlu-miR319c–f	NS	0.85	NS	Transcription factor GAMYB (W9QVM8) [2, 4], Teosinte branched 1, putative isoform 1 (A0A061GDP3) [4]
hlu-miR390a	NS	NS	-2.2	Regulation of response to stimulus (218851.Aquca_1504_00001.1) ARM repeat superfamily protein isoform 1 (A0A061G6D5) Mitochondrial protein (3827.XP_004514187.1) Cation-transporting ATPase (W9RQ63)
hlu-miR408a–b	NS	-1.41	-3.9	Long chain acyl-CoA synthetase 8 (W9QFE0) [4]
hlu-miR828a–b	-3.50	NS	2.5	Serine/threonine-protein phosphatase (W9QNS5) [2], Nuclear pore membrane glycoprotein (W9S8T7), RNA pol II transcription regulator recruiting activity (3649.evm.model.supercontig_96.57), 3-hydroxyisobutyryl-CoA hydrolase (A0A087GEK8) [1]

miRNA-363 miRNA-1427	-2.61	NS	2.4	Polyphenol oxidase (W9S222) [1, 2], ER lumen retaining receptor family (F6HCQ4) [1, 2], Dynamin-related protein 4C (W9QQY9) [2]
miRNA-898 miRNA-2452	-2.49	NS	NS	L-threonine ammonia-lyase activity (161934.XP_010694863.1), gag-polypeptide of LTR copia-type (3750.XP_008361163.1), Vacuolar protein sorting-associated protein 54 (W9SA63) [1]
miRNA-617	NS	NS	-6.35	Wall-associated receptor kinase (F6GSN9), DIS3-like exonuclease 2 (W9QVR2)

Sig. interaction is the difference between the condition effect for the resistant cultivar compared to the condition effect for the susceptible cultivar.

* Orthologue ID is either UniProt (Entry) or STRING identifier.

NS = not significant.

[1] Significant genes identified in topGO analysis of a biological process ontology performed with targets of differentially expressed miRNAs in the susceptible cultivar.

[2] Significant genes identified in topGO analysis of a molecular function ontology performed with targets of differentially expressed miRNAs in the susceptible cultivar.

[3] Significant genes identified in topGO analysis of a biological process ontology performed with targets of differentially expressed miRNAs in the resistant cultivar.

[4] Significant genes identified in topGO analysis of a molecular function ontology performed with targets of differentially expressed miRNAs in the resistant cultivar.

Known and novel miRNAs identified in hop root tissue

Reads perfectly aligned against the hop genome were subjected to miRNA prediction analysis using the miR-PREFeR pipeline [32], which predicted 2621 *MIR* loci and their mature, precursor, and star miRNA sequences. Of the predicted *MIR* loci (miRNA candidates), 30 miRNA candidates were removed as they occurred in duplicates in mature and precursor sequences. Of the remaining 2591 miRNA candidates, 100 miRNA candidates (44 unique mature miRNAs) perfectly aligned against known miRNAs that belong to

27 different miRNA families from miRBase [33], and 20 miRNA candidates (12 unique mature miRNAs) aligned with up to two mismatches against known miRNAs that belong to 10 different miRNA families (Fig. 1, Table S1). Aligning predicted miRNA precursor sequences (pre-miRNAs) against RNA sequences deposited in the RNAcentral database [34] did not result in additional annotations. The names of known miRNAs identified in *H. lupulus* were assigned based on the criteria and conventions for miRNA naming (Supplementary Table S1) [33, 35]. Identified known miRNA families were not evenly represented in the number of members. The most represented families were MIR169 with five members; MIR156 and MIR477 with four members; MIR160, MIR167, MIR171 and MIR319 with three members; MIR159, MIR172, MIR390, MIR393, MIR394, MIR395, MIR399 and MIR482 with two members and the remaining 15 families were represented by a single member (Table S1). Additionally, the same mature miRNAs of the same family derive from a different number of precursor miRNAs or *MIR* loci. For example, MIR169 members derive from two (hlu-miR169m, hlu-miR169n) to six (hlu-miR169g, hlu-miR169h, hlu-miR169i, hlu-miR169j, hlu-miR169k, hlu-miR169l) different miRNA precursors and are altogether coded by 16 *MIR* loci.

Seven miRNA families comprise miRNAs that align perfectly or with up to two mismatches with known miRNAs of the corresponding miRNA family deposited in miRBase. Hlu-miR169o–p, hlu-miR319g–i, hlu-miR390b–c, hlu-miR394c–d, hlu-miR395c, hlu-miR477e, hlu-miR482a–b and hlu-miR482c aligned with one mismatch against known miRNAs from miRBase and are also very similar to other members of their family. With two mismatches, the following miRNAs were aligned against known miRNAs from miRBase; hlu-miR156g, hlu-miR477c–d, hlu-miR5225 and hlu-miR408a–b (Fig. 1).

The most abundant miRNAs in the susceptible cultivar were hlu-miR482a–b and hlu-miR482c with on average 26,633 and 33,356 normalized read counts, respectively, followed by hlu-miR159a–b with on average 23,689 normalized read counts. Aforementioned miRNA families were also the most abundant in the resistant cultivar; hlu-miR482a–b had on average 30,089, hlu-miR482c 29,664 and hlu-miR159a–b 17,212 normalized read counts.

Of the 2471 predicted novel miRNA candidates, those with at least 100 reads mapped to the predicted mature miRNA and at least one read to the corresponding star miRNA were considered as highly reliable predictions. Thus, we obtained 43 reliable predictions of novel mature miRNAs deriving from 152 miRNA precursors. The names of novel miRNAs identified in *H. lupulus* were given as “miRNA-” followed by a consecutive number of the prediction but are not in order because unreliable predictions have been removed (Supplementary Table S2). When novel predicted mature miRNAs are derived from different precursor sequences, these miRNAs are assigned two or more slash-separated precursor names, e.g. miRNA-363/miRNA-1427.

Using CD-HIT-EST [36] to cluster precursor sequences of novel miRNAs and pre-miRNAs from miRBase, we identified 89.01% similarity between the predicted precursor of miRNA-405 and miR156v (MI0022992) from *Malus domestica*. Furthermore, the mature sequence of miRNA-405 aligned with three mismatches (e-value: 0.36) against csi-miR156g-3p (MIMAT0048860) from miRBase. Based on this evidence, we assigned novel miRNA-405 to the MIR156 family and named it as hlu-miR156h.

Additionally, clustering precursor sequences showed 81.37% similarity between one novel miRNA deriving from four different precursors (miRNA-665, miRNA-2226, miRNA-2474, miRNA-2537) and miR395j from *P. trichocarpa* (MI0002324). These miRNA precursors were assigned to MIR395 family, whereas mature miRNA was treated as a novel. The remaining novel miRNA precursors were clustered into thirty-three clusters, which were treated as novel miRNA families (Table S2).

Differentially expressed miRNAs between V. nonalfalfae**-treated and control samples**

The differential expression analysis of known and novel predicted miRNAs between treated and control samples was performed separately for each cultivar. miRNAs with at least 100 read counts in the susceptible or resistant cultivar were included in the differential expression analysis. Mature miRNAs with the FDR corrected *p*-value ≤ 0.1 were considered as significantly differentially expressed. The results of differential expression analysis indicate that different members of the same miRNA family differ significantly in expression levels (Supplementary TableS1). Furthermore, except for hlu-miR477f and hlu-miR159c–d, which show similar expression patterns in the susceptible and resistant cultivar treated with *V. nonalfalfae*, other DE miRNAs differ between the two cultivars.

In *V. nonalfalfae*-treated root samples of the susceptible cultivar Celeia, we identified 7 differentially expressed miRNAs, five of which belong to four different miRNA families (MIR159, MIR828, MIR477 and MIR167) and two novel miRNAs (miRNA-363/miRNA1427 and miRNA-898/miRNA-2452) belong to two different novel miRNA families. A significant upregulation was detected for hlu-miR159c–d ($log_2FC = 1.2$) and a significant downregulation for hlu-miR828a–b ($log_2FC = -3.5$), hlu-miR477 ($log_2FC = -2.1$) and two members of MIR167, i.e., hlu-miR167f ($log_2FC = -2.1$) and hlu-miR167a–d ($log_2FC = -1.5$). Additionally, a significant downregulation was detected for two novel miRNAs, namely miRNA-363/miRNA-1427 ($log_2FC = -2.6$) and miRNA-898/miRNA-2452 ($log_2FC = -2.5$) (Fig. 2A and Table S4).

In *V. nonalfalfae*-treated root samples of the resistant cultivar Wye Target, we identified 6 known differentially expressed miRNAs that belong to 6 different miRNA families (MIR408, MIR477, MIR156, MIR160, MIR319 and MIR159). Three miRNAs were upregulated, i.e., hlu-miR160b ($log_2FC = 1.2$), hlu-miR319c–f ($log_2FC = 0.9$) and hlu-miR159c–d ($log_2FC = 1$), while hlu-miR408a–b ($log_2FC = -1.4$), hlu-miR477f ($log_2FC = -1.6$) and hlu-miRR156e–f ($log_2FC = -0.65$) were downregulated (Fig. 2B and Table S5).

Differentially expressed miRNAs between susceptible and resistant hop cultivars

Compared to the susceptible cultivar CE, the resistant cultivar WT showed higher expression of hlu-miR167a–d and hlu-miR167f ($log_2FC = 1.3$, respectively), hlu-miR828a–b ($log_2FC = 2.5$) and a novel miRNA family with miRNA-363 and miRNA-1427 ($log_2FC = 2.4$). Whereas hlu-miR390a ($log_2FC = -2.2$), hlu-miR169a–d ($log_2FC = -2.2$), hlu-miR169m–n ($log_2FC = -1.9$), hlu-miR164a–c ($log_2FC = -1.4$), hlu-

miR408a–b (log_2FC = -3.9), hlu-miR171g–h (log_2FC = -1.3) and a novel miRNA-617 (log_2FC = -6.3) showed lower expression in response to infection with *V. nonalfalfae* (Fig. 3).

Clustering of samples based on the expression values of DE miRNAs between cultivars in general revealed four clusters. According to expression values of DE miRNAs, the samples of the two cultivars are distinctly separated with the exception of one outlier (CER_Trt2) (Fig. 3). Since the latter sample (CER_Trt2) has similar miRNA expression of MIR828, MIR167, MIR169, MIR171, MIR390, MIR408 and two novel miRNAs (miRNA-617 and miRNA-1427) as WT-control samples, it is nested within the cluster of control and treated samples of WT cultivar. On the other hand, the WT-control sample (WTR_Ctrl3) is clustered with WT-treated samples, since they have a similar expression in MIR167, MIR169 and MIR171, MIR390 and MIR408 (Fig. 3).

qPCR amplification of differentially expressed miRNAs

Differentially expressed miRNAs of the susceptible and resistant cultivars were further validated by stem-loop RT-qPCR (Fig. 4). We have successfully confirmed the amplification of all selected miRNAs and as we expected due to the approach used, the expression of some miRNAs differ from DESeq2 analysis. Contrary to DESeq2 results, the RT-qPCR showed no significant differences between treated and control samples of the susceptible cultivar (Fig. 4A) due to the high biological variability.

In the resistant cultivar, we confirmed the suppression of miR408a–b after infection with *V. nonalfalfae* with both approaches. Stem-loop RT-qPCR (log_2FC = -1.6) and DESeq2 (log_2FC = -1.4) analyses revealed a significant decrease of miR408a–b in the treated samples compared to the control samples. Although miR156e–f, miR319c–f, miR159c–d and miR477f were not significantly differentially expressed between the treated and control samples using stem-loop RT-qPCR, the expression trends were the same as in DESeq2 analysis (Fig. 4B).

miRNA target prediction and GO analysis of miRNA targets

In silico psRNATarget analysis [37] of miRNA target transcripts (mRNA molecules) was performed for differentially expressed miRNAs of susceptible and resistant hop cultivars and revealed 49 transcripts that are potential targets (Table 2 and Table S9). For a single miRNA, psRNATarget has identified from one to up to nine different target transcripts and one transcript can be targeted by more than one DE miRNA (Table 2). Transcription factor GAMYB (W9QVM8) is targeted by two different miRNAs; hlu-miR159c–d, which is 1.28- and 0.95-fold (log_2) upregulated in the susceptible and resistant cultivar, respectively, and by hlu-miR319c–f, which is 0.85-fold (log_2) upregulated in the resistant hop cultivar. Most of the identified targets of DE miRNAs were found to encode transcription factors or transcriptional regulators. For example, auxin response factor (ARF) is targeted by hlu-miR160b, which is upregulated in the resistant cultivar. Moreover, some targets encode proteins involved in effector-triggered immunity, e.g. wall-associated receptor kinase (a target of novel miRNA-617) [38], or are involved in metabolic pathways (polyphenol oxidase; a target of novel miRNA-363/miRNA-1427) [39].

Gene ontology analysis (GO) showed that the targeted transcripts of the susceptible cultivar are enriched for twenty-one GO terms of biological processes with six significant targets, while seven enriched GO terms with eight significant targets were identified for molecular functions (Table 2 and Table S7). In the resistant cultivar, GO analysis revealed fifty-one enriched GO terms with five significant targets in the biological process ontology and six enriched GO terms with sixteen significant targets in the molecular function ontology (Table 2 and Table S8).

It is noteworthy that susceptible and resistant cultivars alter the expression of different miRNAs in response to infection with *V. nonalfalfae* and therefore the biological processes and molecular functions of their targets are expected to differ. In the susceptible cultivar, enriched biological processes of the DE miRNA targets include transportation and localization, i.e. protein retention in ER lumen, maintenance of protein localization, vesicle-mediated transport and cytosolic transport, and pigment biosynthetic process. Enriched molecular functions of the DE miRNA targets are ER retention sequence binding, signal sequence binding and catechol oxidase activity (Table S7). Targets of DE miRNAs in the resistant cultivar are involved in the auxin-activated signalling pathway, various regulatory processes, e.g., regulation of DNA-templated transcription, macromolecule biosynthetic process, nitrogen compound metabolic process, aromatic compound biosynthetic process, etc., and have various binding functions (Table S8).

Discussion

We have sequenced 12 sRNA libraries from three control and three *V. nonalfalfae*-treated roots of the susceptible cultivar-Celeia and the resistant cultivar-Wye Target. Using the miR-PReFER miRNA prediction pipeline, we identified 56 mature miRNAs belonging to 30 known plant miRNA families and 35 novel miRNA families represented by a different number of members. A genome-wide profiling study of miRNAs in *Verticillium*-treated roots of *Gossypium barbadense* (*Verticullium*-tolerant cultivar) and *Gossypium hirsutum* (*Verticillium*-susceptible cultivar) reported the identification of 215 known miRNA families and 14 novel miRNAs and more than 65 miRNA families showing the differences in expression between control and *V. dahliae*-treated samples in both cotton cultivars [16]. In *Populus beijingensis*, Chen, Ren, Zhang, Xu, Zhang and Wang [19] identified 74 conserved miRNAs belonging to 37 miRNA families and 27 novel miRNAs from 35 *MIR* loci. *P. beijingensis* plants treated with a canker disease pathogen (*Dothiorella gregaria*) exhibited differential expression of 33 miRNAs. With the *in silico* analysis, they predicted nine target genes for three conserved miRNAs (pbe-miR477c, pbe-miR482b, and pbe-miR2111) and 63 target genes for 35 novel miRNAs. Targeted genes are involved in plant development, abiotic and biotic stress responses or encode leucine-rich repeats, which are targeted by different miRNAs and are involved in disease resistance in other plants [17, 19, 40, 41].

In our study, a significant upregulation in response to infection with *V. nonalfalfae* was observed for hlu-miR159c–d in both hop cultivars. Similarly, as reported, miR159 was upregulated in *P. beijingensis* treated with *D. gregaria* [19], *P. trichocarpa* treated with *Botryosphaeria dothidea* [42] and in *Triticum aestivum* during *Puccinia graminis* f.sp. *tritici* infection [43]. Recently, Zhang, Zhao, Zhao, Wang, Jin, Chen, Fang, Hua, Ding and Guo [44] discovered that cotton plants treated with *V. dahlia* export miR159 to the fungal hyphae where it targets an isotrichodermin C-15 hydroxylase (HiC-15) which is responsible for fungal

virulence. MIR159 family is conserved in many terrestrial plant species [45, 46] and its main role is a regulation of *GAMYB* or *GAMYB-like* transcription factors that possess highly conserved binding sites for miR159 [47]. *In silico* miRNA target prediction revealed that hlu-miR159c–d (upregulated in both cultivars) and hlu-miR319c–f (upregulated in the resistant cultivar) bind to transcription factor GAMYB but have also other distinct targets. Besides, hlu-miR319c–f targets transcripts of hops *teosinte branched 1* (A0A061GDP3) which belongs to the TCP transcription factor family. While both miRNAs regulate MYB TFs in *Arabidopsis*, miR319 acts predominantly on transcription factors of the TCP family and to a lesser extent on the expression of *MYB*, since the expression levels and domain of miR319 limit its regulation of *MYB*, while the sequence of miR159 prevents binding to *TCP* transcripts [48, 49], which is also observed in our study. Recently, Wu, Qi, Meng and Jin [50] showed that sly-miR319 acts as a positive regulator of tomato resistance to *Botrytis cinerea* infection by repressing the expression *SlTCP29*. Moreover, previous studies show that abscisic acid (ABA) induces the accumulation of miR159 in germinating *Arabidopsis thaliana* seeds, which in turn mediates the cleavage of MYB101 and MYB33 transcripts [51], and that miR159 is also involved in the signalling of the phytohormone gibberellin (GA) during floral development by targeting GAMYB-like transcripts [52]. Hormone signalling is one of the key pathways that modulate plant responses to biotic stress [53].

A significant downregulation was observed in our study for hlu-miR156e–f in the resistant hop cultivar, and *in silico* target prediction showed that it targets transcripts of 6 different SQUAMOSA PROMOTER-BINDING-LIKE proteins (SPB), protein SCO1 homolog 2 and LOB DOMAIN-CONTAINING protein. The interaction between hops' miR156 and SPB15 was previously confirmed by 5' RLM-RACE analysis [54]. Eleven SPB genes have been predicted to be targets of miR156 in *Arabidopsis* [55] and *Oryza sativa* [56], respectively. Furthermore, Bhogale, Mahajan, Natarajan, Rajabhoj, Thulasiram and Banerjee [57] validated the interaction between miR156 and StSPL3, StSPL6, StSPL9, StSPL13 in *Solanum tuberosum* ssp. *andigena* and observed that miR156 can be transported through the plants via the phloem. Upregulation of miR156 and SPB targeting was observed in the compatible interactions of *P. beijingensis* and *P. trichocarpa* treated with *D. gregaria* and *B. dothidea*, respectively [19, 42]. Proteins from SPB family are thought to be transcriptional activators and have roles in leaf development, vegetative phase change, flower and fruit development, plant architecture, shoot maturation, gibberellin signalling and response to fungal toxin [58, 59].

Another interesting target of hlu-miR156e-f that we identified in the miRNA target analysis is the transcript of LATERAL ORGAN BOUNDARIES (LOB) DOMAIN-CONTAINING protein (W9SE87). The latter protein family is involved in a positive feedback loop that promotes the expression of the NAC domain-containing protein 30 (*NAC030*)/VASCULAR-RELATED NAC-DOMAIN PROTEIN7 (*VND7*), which regulates genes associated with the differentiation of tracheary elements in *Arabidopsis*, e.g. genes involved in secondary wall biosynthesis, cell wall modifications such as xylan accumulation and programmed cell death [60]. VASCULAR-RELATED NAC DOMAIN7 TF plays an important role in the response to infection with *V. longisporum* in Arabidopsis, as it induces *de novo* formation of functional xylem elements from bundle sheath cells, which subsequently leads to vein clearing and xylem hyperplasia within the vasculature of the roots as well as to enhanced drought tolerance [61]. Additionally, LOB domain-

containing proteins act as transcriptional activators that directly regulate *EXPANSINA17*, a gene encoding a cell wall-loosening factor that promotes lateral root emergence [62]. A downregulation of miR156 and a significantly lower expression of miR164 (discussed below) in the resistant compared to the susceptible hop cultivar could indicate the role of these two miRNAs in the modulation of GA signalling and processes of root growth and xylem development in the resistant hop cultivar during the infection with *V. nonalfalfae*.

Hlu-miR160b, which is significantly upregulated in infected compared to control root samples of the resistant hop cultivar, is predicted to target transcripts of auxin response factors (ARFs), DNA binding proteins that bind to a specific sequence in promoters of auxin-responsive genes [63]. Upregulation of miR160 and its regulation of ARFs has also been demonstrated during the pathogenesis of stem canker disease in *P. trichocarpa* [42] and in potato, where it targets *St*ARF10, which binds to the promoter in the *StGH3.6* gene, a mediator of salicylic acid–auxin cross-talk, and is thus associated with local defence and systemic acquired resistance to *Phytophthora infestans* [14]. In *A. thaliana*, miR160 controls root cap formation by regulating the expression of *ARF10* and *ARF16*. Disturbed miR160-directed regulation of ARF16 resulted in reduced fertility and fewer lateral roots [64]. In addition, researchers observed defects in root growth in *Arabidopsis* plants expressing a miR160-resistant version of *ARF17* [65].

In response to infection with *V. nonalfalfae*, the resistant cultivar showed significantly lower expression of hlu-miR164b, which targets transcripts of NAC domain-containing proteins. The hlu-miR164b cleavage site within the transcript of hops NAC domain-containing protein was confirmed by 5' RLM-RACE analysis done by Mishra, Duraisamy, Matousek, Radisek, Javornik and Jakse [54]. Hu, Lei, Wang, Liu, Tang, Zhang, Chen, Peng, Yang and Wu [66] observed a significant decrease of ghr-miR164 in the response of cotton plants to infection with *V. dahliae*. Additionally, the researchers showed that ghr-miR164 directly cleaves the mRNA of *GhNAC100*, which in turn increased the resistance of plants to the fungus. The decrease of miR164 was also observed in *Oryza sativa* upon infection with *Magnaporthe oryzae* strain Guy11, and rice plants with the dysfunctional miR164a/OsNAC60 regulatory module developed a significant susceptibility to infection with Guy11 [67]. Auxin-induced expression of miR164 in wild-type *Arabidopsis* plants resulted in decreased levels of the *NAC1* transcripts and reduced lateral root emergence. Additionally, *Arabidopsis mir164a* and *mir164b* mutants that express less miR164 show higher expression of NAC1 and have abundant lateral roots. This evidence may indicate that the auxin-miR164-*NAC1* regulation provides a homeostatic mechanism that modulates auxin signalling during lateral root development [68].

In the susceptible hop cultivar treated with *V. nonalfalfae*, we observed a significant downregulation of hlu-miR167a–d, hlu-miR167f, hlu-miR828a–b and two novel miRNAs, i.e. miRNA-363/miRNA-1427 and miRNA-898/miRNA-2452. All aforementioned miRNAs, except miRNA-898/miRNA-2452, had significantly higher expression in the resistant cultivar in response to infection with *V. nonalfalfae*. Contrary to our results MIR167 showed higher expression in interactions *T. aestivum-P. graminis* f. sp. *tritici* [43], *P. beijingensis-D. gregaria* [19] and *Persicaria minor-Fusarium oxysporum* [69]. The observed differences in the expression pattern could be due to different sampling time, tissue or different functions of the same

miRNA family in different species during infection with different pathogens. Similarly as in our study, a mediator of RNA polymerase II was predicted as a target of MIR167 in *Persicaria minor* [69]. Compared to the susceptible hop cultivar, the resistant cultivar showed a significantly lower expression of MIR169. Similarly, Li, Zhao, Li, Hu, Wang, Cao, Xu, Zhao, Xiao, Yang, et al. [70] observed a higher expression of miR169 in the susceptible *O. sativa* cultivar, but not in the resistant cultivar when infected with *M. oryzae*. By *in silico* analysis of miRNA targets, we identified the nuclear transcription factor Y (NF-YA) as a target of miR169. In rice, miR169 suppresses the expression of NF-YA genes and thus acts as a negative regulator in rice immunity against blast fungus *M. oryzae*, since the transgenic lines that overexpress miR169 became hyper-susceptible to *M. oryzae* due to the reduced expression of defence-related genes [70]. A significantly lower expression of miR169 in the resistant hop cultivar might thus contribute to hop resistance. Additionally, miR169 regulates *NF-YA2* and *NF-YA10* genes, which are involved in the control of primary root growth [71] and regulate leaf growth via auxin signalling in *Arabidopsis* [72].

The resistant hop cultivar shows a significantly lower expression of miR171 after infection with *V. nonalfalfae* compared to the susceptible hop cultivar. In our study we identified three miR171 targets that encode the proteins of the GRAS-domain family; Scarecrow-like protein 22 (SCR22), scarecrow-like protein 6 (SCR6) and GRAS domain-containing protein. The cleavage site of miR171 within the hop transcripts of the GRAS domain-containing protein was confirmed by Mishra, Duraisamy, Matousek, Radisek, Javornik and Jakse [54]. Proteins of the GRAS family are involved in gibberellin (GA) signalling, which regulates various aspects of plant growth and development [73]. In A*rabidopsis*, SCR protein regulates asymmetric cell division of the cortex/endodermal initial cells during root development [74]. In addition, *Arabidopsis* miR171c negatively regulates shoot branching by targeting three different members of the GRAS family [75]. We may assume that the resistant hop cultivar modulates GA hormone signalling via the miR171-GRAS regulatory pathway, which may contribute to the observed resistance to *V. nonalfalfae*.

The resistant hop cultivar shows also a significantly lower expression of miR390 after infection with *V. nonalfalfae* compared to the susceptible hop cultivar. In *Arabidopsis*, miR390 targets precursor RNAs of *TAS3* and thus triggers the biogenesis of trans-acting small interfering RNA or tasiARFs that cleave the transcripts of ARF genes, thereby regulating plant growth and lateral root development [76]. Although miRNA target analysis didn't predict binding sites of hlu-miR390a in the transcripts of hop *TAS3*, we identified its four potential targets. One of the targets is involved in the regulation of the response to a stimulus and encodes proteins with successive leucine-rich repeat motifs. This hop protein might belong to the class of toll-like receptors which bind pathogen- and danger-associated molecular patterns [77].

In the resistant cultivar, we observed a downregulation of hlu-miR408a–b and a significantly lower expression of the latter in response to infection with *V. nonalfalfae* compared to the susceptible hop cultivar. It is reported that miR408 targets laccases and cupredoxins and is involved in various abiotic stress responses in *Arabidopsis* [78], while its role in biotic stress responses has not yet been fully described. Yin, Ramachandran, Zhai, Bu, Pappu and Hulbert [79] observed an increased expression of miR408 in *Arabidopsis* plants that overexpressed the effector protein SR1-a of *Puccinia graminis* f. sp. *tritici* (Pgt), but the increase was not significantly higher in wheat leaves. In our study, the binding site of

miR408 was found only in transcripts of the long-chain acyl-coenzyme synthetase. In *A. thaliana*, long-chain acyl-coenzyme synthetase activates C16 or C18 fatty acids, which represent a substrate for cutin and wax [80]. The downregulation of miR408 may modulate the biosynthetic pathways of cutin and wax, which could lead to the accumulation of these compounds in the roots of resistant hop cultivar when treated with *V. nonalfalfae*.

In the susceptible hop cultivar, we observed a downregulation of miR828 and two novel miRNAs, namely miRNA-363/miRNA-1427 and miRNA-898/miRNA-2452. Recent studies showed that miR828 regulates phenylpropanoid biosynthesis either by direct cleavage of MYB TFs or by cleaving t*rans-acting siRNA gene 4* (*TAS4*) which result in the production of ta-siRNAs that silence the expression of *MYB* gene [81, 82]. In our study, a hlu-miR828a–b cleavage site was not predicted within the transcripts of *MYB* or *TAS4* transcripts, however, it targets transcripts of proteins with RNA pol II transcription regulator recruiting activity. The latter protein contains DNA-binding domains of MYB-related proteins, as well as the SANT domain family which are found in nuclear receptor co-repressors and subunits of chromatin-remodelling complexes [83, 84]. Serine/threonine-protein phosphatase and 3-hydroxyisobutyryl-CoA hydrolase are potential targets of hlu-miR828a–b that were also significant in GO enrichment analysis of molecular functions and biological processes in the susceptible hop cultivar.

Cleavage sites of novel miRNA-363/miRNA-1427 were identified in transcripts of polyphenol oxidase, a protein from the family of the ER lumen retaining receptors and in dynamin-related protein 4C. In previous studies, novel miRNAs targeting polyphenol oxidase were identified in *P. trichocarpa* [18], *Salvia miltiorrhiza* [39], *Solanum tuberosum* [85] and *Vitis vinifera* [86] however, they differ in sequence from miRNA-363/miRNA-1427 identified in our study. The other two potential targets, the protein of the ER lumen retaining receptor family and dynamin-related protein 4C, are both involved in cellular localization or transport.

Additionally, novel miRNA miRNA-898/miRNA-2452 potentially targets transcripts of vacuolar protein sorting-associated protein, which is involved in protein transport between endosomes and the trans-Golgi network [87].

The resistant cultivar showed a significantly lower expression of novel miRNA-617 than the susceptible one after infection. The cleavage site of miRNA-617 was predicted in transcripts of wall-associated receptor kinase from receptor-like kinases (RLK) protein family, which are involved in the detection and signal transduction during pathogen attacks [38].

With RT-qPCR based on the SYBR green approach, we have shown that all targeted miRNAs are amplified *in vitro*, but with this approach, we cannot directly compare expression patterns. There may be many reasons for the differences in the results obtained with different approaches, as has been shown previously [88]. In our case, the templates for miRNA sequencing were enriched samples for small RNAs, whereas we used total RNA for RT-qPCR analysis. Studies have already shown that the library preparation steps create bias in sequencing results [88, 89]. Thus, the differences in the results of DE analysis may be due to different approaches of sample processing and reverse transcription. One of the important

impacts could also be differential expression analysis. While relative quantification by the ΔΔCt method is used for qPCR analysis [90], normalization of read counts by the DESeq2's median of ratios is used in the analysis of RNA-Seq data [91]. However, it is beyond the scope of this study to comment on a possible bias arising from either method and the appropriateness of comparing the results of the two methods.

Conclusions

Hops have become an increasingly agronomically important crop, mainly due to their use in the brewing industry and more recently in the pharmaceutical industry. In our study, we characterized miRNAs in hops and identified differentially expressed miRNAs in the roots of susceptible and resistant hop cultivars 24 hours after infection with the phytopathogenic fungus *V. nonalfalfae*. We identified 56 known miRNAs belonging to 30 different miRNA families and 43 novel miRNAs. In response to Verticillium infection, we identified seven and six differentially expressed miRNAs in the susceptible and resistant hop cultivar, respectively. We observed that hop cultivars respond to infection by altering the expression of different miRNAs. A total of 49 target transcripts were identified for differentially expressed miRNAs by *in silico* target analysis. Gene ontology enrichment analysis showed that the targets of differentially expressed miRNAs in the susceptible cultivar are involved in protein retention in ER lumen, vesicle-mediated transport, pigment biosynthetic process, etc. In the resistant cultivar, however, the targets are participating in the auxin-activated signalling pathway and regulation of DNA-templated transcription. Based on the results obtained, we can assume that the resistant cultivar responded to infection with miRNAs that regulate transcripts that modulate the processes of gene expression and hormonal signalling, which was not observed in the susceptible cultivar. The obtained results suggest that miRNAs might play an important role in hop response and resistance to *Verticillium* in the resistant cultivar.

Methods

Inoculation of hop plants

Hop plants of the susceptible variety Celeia and resistant Wye Target were provided by Slovenian Institute for Hop Research and Brewing. Hop plants were vegetatively propagated as softwood cuttings in a greenhouse or as dormant cuttings from the rootstock. One-year old rooted cutting were used in the experiment. The plants were inoculated by root dipping method using the well-established protocol proposed by Flajsman, Radisek and Javornik [92]. Briefly, the roots of 3 biological replicates of one-year-old rooted cuttings of each cultivar were immersed for 10 minutes in a suspension containing conidia of the highly virulent strain of *V. nonalfalfae* (PV1, isolate T2) (5×10^6 conidia/mL), and the roots of 3 control plants of each cultivar were mock-inoculated using sterile water. Artificially inoculated (treated) and mock-inoculated (control) root tissue was sampled after 24 h post-inoculation. The roots were cut off the stems at the first node, washed, freeze-dried with liquid nitrogen and ground to a fine powder in

mortars and pestles. Following grinding, the samples were stored at -80 °C until total RNA and small RNAs were isolated.

Small RNA isolation, library construction and sequencing

Small RNAs were isolated from 100 mg root tissue of both cultivars in three *V. nonalfalfae*-treated and three control replicates, using mirVana™ miRNA Isolation Kit (Waltham, Massachusetts, USA) according to manufacturer's instructions for the enrichment of small RNAs. The quantity and quality of the small RNA-enriched sample and miRNA fraction were assessed with Agilent® 2100 Bioanalyzer® instrument (Agilent Technologies, Inc., Santa Clara, California, USA) using Bioanalyzer Agilent® Small RNA Kit, following the manufacturer's instruction. Thus, we determined the input amount of small RNAs, to construct three control and three treated small RNA libraries for each cultivar. Small RNA libraries were constructed using the Ion Total RNA-Seq Kit v2 and Ion Xpress™ RNA-Seq Barcode 1–16 Kit following the manufacturer's instructions. Briefly, adaptors were hybridized and ligated to small RNAs, and the reverse transcription was performed. Afterwards, purification and size-selection were performed using magnetic beads to obtain only miRNAs and other small RNAs to which barcodes were added through PCR amplification. The yield and size distribution of amplified cDNA libraries were assessed with Agilent® 2100 Bioanalyzer® instrument (Agilent Technologies, Inc., Santa Clara, California, USA) and Agilent® High Sensitivity DNA Kit to pool equimolar barcoded libraries of each cultivar separately. Three inoculated and three mock-inoculated barcoded libraries of susceptible or resistant cultivars were pooled in equimolar concentration and prepared for sequencing according to the manufacturer's instructions, accompanying Ion PI™ Hi-Q™ OT2 200 Kit and Ion PI™ Hi-Q™ Sequencing 200 Kit. Both prepared samples were sequenced on the Ion Proton™ System (Waltham, Massachusetts, USA). The raw sequencing data were deposited to the SRA archive (BioProject ID PRJNA665133).

Prediction, identification and differential expression analysis of miRNAs in hops

Prior to bioinformatics analysis, barcodes, adapters and low-quality raw sequence reads were removed using FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and high-quality sequencing reads were used for further analysis. Briefly, FASTA files containing sRNA-seq reads were pre-processed using scripts provided by miR-PREFeR pipeline [32] and the reads were aligned with Bowtie [93], disallowing mismatches, against hop draft genome sequences obtained from HopBase [30, 31]. Afterwards, alignment files of processed RNA-Seq reads were used to predict hop miRNAs using a miR-PREFeR pipeline with parameters set according to criteria for plant miRNA annotation [94].

To identify known miRNA families in hops, predicted mature or precursor miRNA sequences were aligned with Bowtie2 [95] against mature or precursor sequences in the microRNA database (miRBase Release 22.1) [33]. Additionally, predicted precursor miRNA sequences were aligned against RNACentral, a non-coding RNA sequence database [34].

Minimum folding energy (MFE) of secondary structures of predicted precursor miRNAs was calculated using RNAfold tool [96] and used to calculate adjusted minimal folding free energy (AMFE), which enables indirect comparison of MFEs among predicted known and novel pre-miRNAs [97].

sRNA counts of predicted mature miRNAs provided by miR-PREFeR output were subjected for differential expression analysis in R version 3.5.1 [98]. Count matrices containing read counts of control and treated samples were constructed for susceptible and resistant cultivar, respectively. Prior to differential expression analysis, predicted mature miRNAs with less than 100 read counts in control and treated samples were discarded. The differential expression analysis of predicted mature miRNAs was performed with DESeq2 [99]. Predicted miRNAs with FDR corrected *p*-value < 0.1 were treated as significantly differentially expressed between treated and control samples. Filtering according to the $\log_2$ fold change parameter was not applied because we wanted to detect low but significant changes in the expression of miRNAs.

To test whether the treatment effect differs across cultivars, the interaction term was added to the model in DESeq2 and the entire read-count matrix containing mature miRNAs with more than 200 read counts in all samples was used in the differential expression analysis. Predicted mature miRNAs with *p*-value ≤ 0.05 were considered as differentially expressed.

Furthermore, predicted precursors of novel miRNAs (novel pre-miRNAs) and known pre-miRNAs from miRBase were clustered using CD-HIT-EST [36] with a global sequence identity threshold 0.8. Predicted novel pre-miRNAs clustered with annotated pre-miRNAs were grouped into corresponding known miRNA families and predicted novel pre-miRNAs that did not show similarity were categorized as novel miRNA families.

Validation of the presence of miRNAs in hops by two-step stem-loop reverse transcription quantitative PCR

To confirm the expression of miRNAs, two-step stem-loop reverse transcription and real-time quantitative PCR (Two-step stem-loop RT-qPCR) of differentially expressed miRNAs was performed according to the protocol proposed by Kramer [100]. Total RNA was extracted from *V. nonalfalfae*-treated and control root samples of both hop cultivars using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA), following manufacturer's instructions. The concentration of total RNA was determined with Agilent® 2100 Bioanalyzer® instrument (Agilent Technologies, Inc., Santa Clara, California, USA) using Agilent® RNA 6000 Nano Kit. Afterwards, selected miRNAs were reverse transcribed with a stem-loop primer specific for each miRNA (Table S3) using TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) according to manufacturer's instructions. Two-step stem-loop RT-qPCR analysis was used to validate the differential expression of miR159c–d, miR167f, miR477f and miRNA-363 in the susceptible cultivar and miR156e–f, miR159c–d, miR319c–f and miR477f in the resistant cultivar. Cycle threshold (Ct) values of miRNAs in the samples were normalized using hlu-miR162 as an internal reference, as it showed the most stable expression between treated and control samples and between both cultivars based on miRNA-Seq and qPCR data. Three independent biological replicates of *V. nonalfalfae*-treated or

control samples of both cultivars were analyzed. An ANOVA test was performed on ΔCt values to evaluate differences in miRNA expression between control and treated samples in susceptible or resistant cultivar, and relative changes (ΔΔCt) were calculated using the method by Livak and Schmittgen [90] to compare the results of miRNA-Seq and RT-qPCR differential expression analyses.

In silico **prediction of miRNA targets of differentially expressed miRNAs**

Micro RNA target analysis was performed on-line using psRNATarget Analysis Server (2017 Update) [37]. Mature miRNA sequences of differentially expressed miRNAs were used in *in silico* miRNA target prediction analysis. The targets of differentially expressed miRNA of susceptible and resistant hop cultivars were predicted in annotated hop transcriptome [101] with the following parameters; max expectation cutoff: 2.5, seed region: 2–13, number of mismatches allowed in seed region: 2, range of mismatch disable slicing: 9–11, HSP length for scoring: 19, penalty for GU pair: 0.5, penalty for other mismatches: 1.0, allowing bulge on target, penalty for opening gap: 2.0, penalty for extending gap: 0.5, weight for seed region: 1.5, calculating UPE around the target site (target accessibility analysis): 17 nt upstream and 13 nt downstream. Afterwards, gene ontology (GO) analysis was performed using R package topGO (version 2.40.0) [102] on targets of differentially expressed miRNAs in order to identify over-represented/enriched GO terms and significant miRNA targets belonging to enriched GO terms. Classical enrichment analysis was performed with Fisher's statistical test (p-value ≤ 0.05).

Declarations

Ethics approval and consent to participate

The study comply with institutional, national, or international guidelines, IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analysed during the current study are available in the NCBI Sequence Read Archive (SRA) repository (https://www.ncbi.nlm.nih.gov/sra/) under the BioProject accession PRJNA665133 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA665133) and SRA accession numbers; SRR12696058, SRR12696057, SRR12696054, SRR12696053, SRR12696052, SRR12696051, SRR12696050, SRR12696049, SRR12696048, SRR12696047, SRR12696056, SRR12696055.

The hop draft genome was obtained from the HopBase genomic resource which is availabe at http://hopbase.org and http://hopbase.cgrb.oregonstate.edu [30, 31].

The computationally annotated hop transcriptome is available from our laboratory and the raw NGS sequences are publicly available at NCBI's SRA archive under BioProject number PRJNA342762, BioSample SAMN05767836, SRA run SRR4242068: https://www.ncbi.nlm.nih.gov/sra/?term=SRR4242068 [101].

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization: NŠ and JJ; design of the work: UK, JJ, SR, and NŠ; acquisition, formal analysis and interpretation of data: UK; NS, JJ; funding acquisition: UK and JJ; investigation: UK; project administration: NŠ; resources: JJ, SR, and NŠ; supervision: NŠ; validation: UK, JJ, SR, and NŠ; visualization: UK; writing—original draft: UK; writing—review and editing: UK, JJ, SR, and NŠ. All authors read and approved the final manuscript.

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References

1. Steenackers B, De Cooman L, De Vos D. Chemical transformations of characteristic hop secondary metabolites in relation to beer properties and the brewing process: A review. Food Chem. 2015;172:742-756.
2. Hrncic MK, Spaninger E, Kosir IJ, Knez Z, Bren U. Hop Compounds: Extraction Techniques, Chemical Analyses, Antioxidative, Antimicrobial, and Anticarcinogenic Effects. Nutrients. 2019;11(2).
3. Liu Y, Gao X, Deeb D, Arbab AS, Dulchavsky SA, Gautam SC. Anticancer agent xanthohumol inhibits IL-2 induced signaling pathways involved in T cell proliferation. J Exp Ther Oncol. 2012;10(1):1-8.
4. Radišek S. Verticillium Wilt. In: Compendium of Hop Diseases and Pests. Mahaffee W, Pethybridge S, Gent DH (editors). St. Paul (Minnesota): The American Phytopathological Society; 2009: 33-36.

5. Radisek S, Jakse J, Javornik B. Genetic variability and virulence among *Verticillium albo-atrum* isolates from hop. Eur J Plant Pathol. 2006;116(4):301-314.
6. Chisholm ST, Coaker G, Day B, Staskawicz BJ. Host-microbe interactions: Shaping the evolution of the plant immune response. Cell. 2006;124(4):803-814.
7. Jones JDG, Dangl JL. The plant immune system. Nature. 2006;444(7117):323-329.
8. Fei QL, Zhang Y, Xia R, Meyers BC. Small RNAs Add Zing to the Zig-Zag-Zig Model of Plant Defenses. Mol Plant Microbe Interact. 2016;29(3):165-169.
9. Li F, Pignatta D, Bendix C, Brunkard JO, Cohn MM, Tung J, Sun HY, Kumar P, Baker B. MicroRNA regulation of plant innate immune receptors. P Natl Acad Sci USA. 2012;109(5):1790-1795.
10. D'Ario M, Griffiths-Jones S, Kim M. Small RNAs: Big Impact on Plant Development. Trends Plant Sci. 2017;22(12):1056-1068.
11. Soto-Suarez M, Baldrich P, Weigel D, Rubio-Somoza I, San Segundo B. The *Arabidopsis* miR396 mediates pathogen-associated molecular pattern-triggered immune responses against fungal pathogens. Sci Rep. 2017;7(44898).
12. Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JDG. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science. 2006;312(5772):436-439.
13. Wong J, Gao L, Yang Y, Zhai JX, Arikit S, Yu Y, Duan SY, Chan V, Xiong Q, Yan J *et al*. Roles of small RNAs in soybean defense against *Phytophthora sojae* infection. Plant J. 2014;79(6):928-940.
14. Natarajan B, Kalsi HS, Godbole P, Malankar N, Thiagarayaselvam A, Siddappa S, Thulasiram HV, Chakrabarti SK, Banerjee AK. MiRNA160 is associated with local defense and systemic acquired resistance against *Phytophthora infestans* infection in potato. J Exp Bot. 2018;69(8):2023-2036.
15. Jagadeeswaran G, Zheng Y, Li YF, Shukla LI, Matts J, Hoyt P, Macmil SL, Wiley GB, Roe BA, Zhang WX *et al*. Cloning and characterization of small RNAs from *Medicago truncatula* reveals four novel legume-specific microRNA families. New Phytol. 2009;184(1):85-98.
16. Yin ZJ, Li Y, Han XL, Shen FF. Genome-Wide Profiling of miRNAs and Other Small Non-Coding RNAs in the *Verticillium dahliae*-Inoculated Cotton Roots. Plos One. 2012;7(4).
17. Cui XX, Yan Q, Gan SP, Xue D, Dou DL, Guo N, Xing H. Overexpression of gma-miR1510a/b suppresses the expression of a NB-LRR domain gene and reduces resistance to *Phytophthora sojae*. Gene. 2017;621:32-39.
18. Lu SF, Sun YH, Chiang VL. Stress-responsive microRNAs in *Populus*. Plant J. 2008;55(1):131-151.
19. Chen L, Ren YY, Zhang YY, Xu JC, Zhang ZY, Wang YW. Genome-wide profiling of novel and conserved Populus microRNAs involved in pathogen stress response by deep sequencing. Planta. 2012;235(5):873-883.
20. Jakse J, Cerenak A, Radisek S, Satovic Z, Luthar Z, Javornik B. Identification of quantitative trait loci for resistance to Verticillium wilt and yield parameters in hop (*Humulus lupulus* L.). Theor Appl Genet. 2013;126(6):1431-1443.

21. Majer A, Javornik B, Cerenak A, Jakse J. Development of novel EST-derived resistance gene markers in hop (*Humulus lupulus* L.). Mol Breed. 2014;33(1):61-74.
22. Diwan N, Fluhr R, Eshed Y, Zamir D, Tanksley SD. Mapping of Ve in tomato: a gene conferring resistance to the broad-spectrum pathogen, *Verticillium dahliae* race 1. Theor Appl Genet. 1999;98(2):315-319.
23. Kawchuk LM, Hachey J, Lynch DR, Kulcsar F, van Rooijen G, Waterer DR, Robertson A, Kokko E, Byers R, Howard RJ *et al*. Tomato Ve disease resistance genes encode cell surface-like receptors. P Natl Acad Sci USA. 2001;98(11):6511-6515.
24. Fradin EF, Abd-El-Haliem A, Masini L, van den Berg GCM, Joosten MHAJ, Thomma BPHJ. Interfamily Transfer of Tomato Ve1 Mediates Verticillium Resistance in Arabidopsis. Plant Physiol. 2011;156(4):2255-2265.
25. Zhang Z, Fradin E, de Jonge R, van Esse HP, Smit P, Liu CM, Thomma BPHJ. Optimized Agroinfiltration and Virus-Induced Gene Silencing to Study Ve1-Mediated *Verticillium* Resistance in Tobacco. Mol Plant Microbe Interact. 2013;26(2):182-190.
26. Song Y, Zhang Z, Seidl MF, Majer A, Jakse J, Javornik B, Thomma BPHJ. Broad taxonomic characterization of Verticillium wilt resistance genes reveals an ancient origin of the tomato Ve1 immune receptor. Mol Plant Pathol. 2017;18(2):195-209.
27. Mandelc S, Timperman I, Radisek S, Devreese B, Samyn B, Javornik B. Comparative proteomic profiling in compatible and incompatible interactions between hop roots and Verticillium albo-atrum. Plant Physiol Biochem. 2013;68:23-31.
28. Progar V, Jakse J, Stajner N, Radisek S, Javornik B, Berne S. Comparative transcriptional analysis of hop responses to infection with *Verticillium nonalfalfae*. Plant Cell Rep. 2017;36(10):1599-1613.
29. Cregeen S, Radisek S, Mandelc S, Turk B, Stajner N, Jakse J, Javornik B. Different Gene Expressions of Resistant and Susceptible Hop Cultivars in Response to Infection with a Highly Aggressive Strain of Verticillium albo-atrum. Plant Mol Biol Report. 2015;33(3):689-704.
30. Hill ST, Sudarsanam R, Henning J, Hendrix D. HopBase: a unified resource for *Humulus* genomics. Database-Oxford. 2017.
31. Padgitt-Cobb LK, Kingan SB, Wells J, Elser J, Kronmiller B, Moore D, Concepcion G, Peluso P, Rank D, Jaiswal P *et al*. A phased, diploid assembly of the Cascade hop (*Humulus lupulus*) genome reveals patterns of selection and haplotype variation. bioRxiv. 2019;786145.
32. Lei JK, Sun Y. miR-PREFeR: an accurate, fast and easy-to-use plant miRNA prediction tool using small RNA-Seq data. Bioinformatics. 2014;30(19):2837-2839.
33. Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res. 2011;39:D152-D157.
34. Sweeney BA, Petrov AI, Burkov B, Finn RD, Bateman A, Szymanski M, Karlowski WM, Gorodkin J, Seemann SE, Cannone JJ *et al*. RNAcentral: a hub of information for non-coding RNA sequences. Nucleic Acids Res. 2019;47(D1):D221–D229.

35. Ambros V, Bartel B, Bartel DP, Burge CB, Carrington JC, Chen XM, Dreyfuss G, Eddy SR, Griffiths-Jones S, Marshall M *et al*. A uniform system for microRNA annotation. RNA. 2003;9(3):277-279.
36. Li W, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics. 2006;22(13):1658-1659.
37. Dai XB, Zhuang ZH, Zhao PXC. psRNATarget: a plant small RNA target analysis server (2017 release). Nucleic Acids Res. 2018;46(W1):W49-W54.
38. Yang K, Qi L, Zhang Z. Isolation and characterization of a novel wall-associated kinase gene TaWAK5 in wheat (*Triticum aestivum*). Crop J. 2014;2(5):255-266.
39. Li CL, Li DQ, Li J, Shao FJ, Lu SF. Characterization of the polyphenol oxidase gene family reveals a novel microRNA involved in posttranscriptional regulation of PPOs in *Salvia miltiorrhiza*. Sci Rep. 2017;7.
40. de Vries S, Kukuk A, von Dahlen JK, Schnake A, Kloesges T, Rose LE. Expression profiling across wild and cultivated tomatoes supports the relevance of early miR482/2118 suppression for *Phytophthora* resistance. Proc Royal Soc B. 2018;285(1873).
41. Ji HM, Zhao M, Gao Y, Cao XX, Mao HY, Zhou Y, Fan WY, Borkovich KA, Ouyang SQ, Liu P. FRG3, a Target of slmiR482e-3p, Provides Resistance against the Fungal Pathogen *Fusarium oxysporum* in Tomato. Front Plant Sci. 2018;9.
42. Zhao JP, Jiang XL, Zhang BY, Su XH. Involvement of microRNA-Mediated Gene Expression Regulation in the Pathological Development of Stem Canker Disease in *Populus trichocarpa*. Plos One. 2012;7(9).
43. Gupta OP, Permar V, Koundal V, Singh UD, Praveen S. MicroRNA regulated defense responses in *Triticum aestivum* L. during *Puccinia graminis* f.sp. *tritici* infection. Mol Biol Rep. 2012;39(2):817-824.
44. Zhang T, Zhao YL, Zhao JH, Wang S, Jin Y, Chen ZQ, Fang YY, Hua CL, Ding SW, Guo HS. Cotton plants export microRNAs to inhibit virulence gene expression in a fungal pathogen. Nat Plants. 2016;2(10).
45. Montes RAC, Rosas-Cardenas FD, De Paoli E, Accerbi M, Rymarquis LA, Mahalingam G, Marsch-Martinez N, Meyers BC, Green PJ, de Folter S. Sample sequencing of vascular plants demonstrates widespread conservation and divergence of microRNAs. Nat Commun. 2014;5.
46. You CJ, Cui J, Wang H, Qi XP, Kuo LY, Ma H, Gao L, Mo BX, Chen XM. Conservation and divergence of small RNA pathways and microRNAs in land plants. Genome Biol. 2017;18.
47. Millar AA, Lohe A, Wong G. Biology and Function of miR159 in Plants. Plants-Basel. 2019;8(8).
48. Palatnik JF, Wollmann H, Schommer C, Schwab R, Boisbouvier J, Rodriguez R, Warthmann N, Allen E, Dezulian T, Huson D *et al*. Sequence and expression differences underlie functional specialization of *Arabidopsis* MicroRNAs miR159 and miR319. Dev Cell. 2007;13(1):115-125.
49. Allen RS, Li JY, Stahle MI, Dubroue A, Gubler F, Millar AA. Genetic analysis reveals functional redundancy and the major target genes of the *Arabidopsis* miR159 family. P Natl Acad Sci USA. 2007;104(41):16371-16376.

50. Wu F, Qi J, Meng X, Jin W. miR319c acts as a positive regulator of tomato against Botrytis cinerea infection by targeting TCP29. Plant Sci. 2020;300:110610.
51. Reyes JL, Chua NH. ABA induction of miR159 controls transcript levels of two MYB factors during *Arabidopsis* seed germination. Plant J. 2007;49(4):592-606.
52. Achard P, Herr A, Baulcombe DC, Harberd NP. Modulation of floral development by a gibberellin-regulated microRNA. Development. 2004;131(14):3357-3365.
53. Bari R, Jones J. Role of plant hormones in plant defence responses. Plant Mol Biol. 2009;69(4):473-488.
54. Mishra AK, Duraisamy GS, Matousek J, Radisek S, Javornik B, Jakse J. Identification and characterization of microRNAs in *Humulus lupulus* using high-throughput sequencing and their response to Citrus bark cracking viroid (CBCVd) infection. BMC Genomics. 2016;17.
55. Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP. Prediction of plant microRNA targets. Cell. 2002;110(4):513-520.
56. Xie K, Wu C, Xiong L. Genomic organization, differential expression, and interaction of SQUAMOSA promoter-binding-like transcription factors and microRNA156 in rice. Plant Physiol. 2006;142(1):280-293.
57. Bhogale S, Mahajan AS, Natarajan B, Rajabhoj M, Thulasiram HV, Banerjee AK. MicroRNA156: A Potential Graft-Transmissible MicroRNA That Modulates Plant Architecture and Tuberization in *Solanum tuberosum* ssp. *andigena*. Plant Physiol. 2014;164(2):1011-1027.
58. Chen X, Zhang Z, Liu D, Zhang K, Li A, Mao L. SQUAMOSA promoter-binding protein-like transcription factors: star players for plant growth and development. J Integr Plant Biol. 2010;52(11):946-951.
59. Stone JM, Liang X, Nekl ER, Stiers JJ. Arabidopsis AtSPL14, a plant-specific SBP-domain transcription factor, participates in plant development and sensitivity to fumonisin B1. Plant J. 2005;41(5):744-754.
60. Soyano T, Thitamadee S, Machida Y, Chua NH. ASYMMETRIC LEAVES2-LIKE19/LATERAL ORGAN BOUNDARIES DOMAIN30 and ASL20/LBD18 Regulate Tracheary Element Differentiation in Arabidopsis. Plant Cell. 2008;20(12):3359-3373.
61. Reusche M, Thole K, Janz D, Truskina J, Rindfleisch S, Drubert C, Polle A, Lipka V, Teichmann T. *Verticillium* Infection Triggers VASCULAR-RELATED NAC DOMAIN7-Dependent de Novo Xylem Formation and Enhances Drought Tolerance in *Arabidopsis*. Plant Cell. 2012;24(9):3823-3837.
62. Lee HW, Kim J. EXPANSINA17 Up-Regulated by LBD18/ASL20 Promotes Lateral Root Formation During the Auxin Response. Plant Cell Physiol. 2013;54(10):1600-1611.
63. Tiwari SB, Hagen G, Guilfoyle T. The roles of auxin response factor domains in auxin-responsive transcription. Plant Cell. 2003;15(2):533-543.
64. Wang JW, Wang LJ, Mao YB, Cai WJ, Xue HW, Chen XY. Control of root cap formation by microRNA-targeted auxin response factors in Arabidopsis. Plant Cell. 2005;17(8):2204-2216.

65. Mallory AC, Bartel DP, Bartel B. MicroRNA-directed regulation of Arabidopsis AUXIN RESPONSE FACTOR17 is essential for proper development and modulates expression of early auxin response genes. Plant Cell. 2005;17(5):1360-1375.
66. Hu G, Lei Y, Wang L, Liu J, Tang Y, Zhang Z, Chen A, Peng Q, Yang Z, Wu J. The ghr-miR164 and GhNAC100 module participates in cotton plant defence against *Verticillium dahliae*. bioRxiv. 2018:440826.
67. Wang Z, Xia Y, Lin S, Wang Y, Guo B, Song X, Ding S, Zheng L, Feng R, Chen S *et al*. Osa-miR164a targets OsNAC60 and negatively regulates rice immunity against the blast fungus *Magnaporthe oryzae*. Plant J. 2018.
68. Guo HS, Xie Q, Fei JF, Chua NH. MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for *Arabidopsis* lateral root development. Plant Cell. 2005;17(5):1376-1386.
69. Samad AFA, Rahnamaie-Tajadod R, Sajad M, Jani J, Murad AMA, Noor NM, Ismail I. Regulation of terpenoid biosynthesis by miRNA in Persicaria minor induced by *Fusarium oxysporum*. BMC Genomics. 2019;20.
70. Li Y, Zhao SL, Li JL, Hu XH, Wang H, Cao XL, Xu YJ, Zhao ZX, Xiao ZY, Yang N *et al*. Osa-miR169 Negatively Regulates Rice Immunity against the Blast Fungus *Magnaporthe oryzae*. Front Plant Sci. 2017;8.
71. Sorin C, Declerck M, Christ A, Blein T, Ma L, Lelandais-Briere C, Njo MF, Beeckman T, Crespi M, Hartmann C. A miR169 isoform regulates specific NF-YA targets and root architecture in *Arabidopsis*. New Phytol. 2014;202(4):1197-1211.
72. Zhang M, Hu X, Zhu M, Xu M, Wang L. Transcription factors NF-YA2 and NF-YA10 regulate leaf growth via auxin signaling in *Arabidopsis*. Sci Rep. 2017;7(1):1395.
73. Schwechheimer C. Gibberellin signaling in plants - the extended version. Front Plant Sci. 2012;2.
74. Pysh LD, Wysocka-Diller JW, Camilleri C, Bouchez D, Benfey PN. The GRAS gene family in Arabidopsis: sequence characterization and basic expression analysis of the *SCARECROW-LIKE* genes. Plant J. 1999;18(1):111-119.
75. Wang L, Mai YX, Zhang YC, Luo QA, Yang HQ. MicroRNA171c-Targeted SCL6-II, SCL6-III, and SCL6-IV Genes Regulate Shoot Branching in *Arabidopsis*. Mol Plant. 2010;3(5):794-806.
76. Marin E, Jouannet V, Herz A, Lokerse AS, Weijers D, Vaucheret H, Nussaume L, Crespi MD, Maizel A. miR390, Arabidopsis TAS3 tasiRNAs, and Their AUXIN RESPONSE FACTOR Targets Define an Autoregulatory Network Quantitatively Regulating Lateral Root Growth. Plant Cell. 2010;22(4):1104-1117.
77. Dodds PN, Rathjen JP. Plant immunity: towards an integrated view of plant-pathogen interactions. Nat Rev Genet. 2010;11(8):539-548.
78. Ma C, Burd S, Lers A. miR408 is involved in abiotic stress responses in Arabidopsis. Plant J. 2015;84(1):169-187.

79. Yin CT, Ramachandran SR, Zhai Y, Bu CY, Pappu HR, Hulbert SH. A novel fungal effector from *Puccinia graminis* suppressing RNA silencing and plant defense responses. New Phytol. 2019;222(3):1561-1572.
80. Pulsifer IP, Kluge S, Rowland O. *Arabidopsis* LONG-CHAIN ACYL-COA SYNTHETASE 1 (LACS1), LACS2, and LACS3 facilitate fatty acid uptake in yeast. Plant Physiol Biochem. 2012;51:31-39.
81. Deng YX, Lu SF. Biosynthesis and Regulation of Phenylpropanoids in Plants. CRC Crit Rev Plant Sci. 2017;36(4):257-290.
82. Bonar N, Liney M, Zhang RX, Austin C, Dessoly J, Davidson D, Stephens J, McDougall G, Taylor M, Bryan GJ *et al*. Potato miR828 Is Associated With Purple Tuber Skin and Flesh Color. Front Plant Sci. 2018;9.
83. Aasland R, Stewart AF, Gibson T. The SANT domain: a putative DNA-binding domain in the SWI-SNF and ADA complexes, the transcriptional co-repressor N-CoR and TFIIIB. Trends Biochem Sci. 1996;21(3):87-88.
84. Boyer LA, Latek RR, Peterson CL. The SANT domain: a unique histone-tail-binding module? Nat Rev Mol Cell Biol. 2004;5(2):158-163.
85. Chi M, Liu CM, Su YQ, Tong YW, Liu HY. Bioinformatic prediction of upstream microRNAs of PPO and novel microRNAs in potato. Can J Plant Sci. 2015;95(5):871-877.
86. Ren GH, Wang BJ, Zhu XD, Mu Q, Wang C, Tao R, Fang JG. Cloning, expression, and characterization of miR058 and its target PPO during the development of grapevine berry stone. Gene. 2014;548(2):166-173.
87. Schindler C, Chen Y, Pu J, Guo XL, Bonifacino JS. EARP is a multisubunit tethering complex involved in endocytic recycling. Nat Cell Biol. 2015;17(5):639-U229.
88. Git A, Dvinge H, Salmon-Divon M, Osborne M, Kutter C, Hadfield J, Bertone P, Caldas C. Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. RNA. 2010;16(5):991-1006.
89. Baran-Gale J, Kurtz CL, Erdos MR, Sison C, Young A, Fannin EE, Chines PS, Sethupathy P. Addressing Bias in Small RNA Library Preparation for Sequencing: A New Protocol Recovers MicroRNAs that Evade Capture by Current Methods. Front Genet. 2015;6.
90. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods. 2001;25(4):402-408.
91. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010;11(10).
92. Flajsman M, Radisek S, Javornik B. Pathogenicity Assay of *Verticillium nonalfalfae* on Hop Plants. Bio-Protoc. 2017;7(6).
93. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009;10(3).

94. Axtell MJ, Meyers BC. Revisiting Criteria for Plant MicroRNA Annotation in the Era of Big Data. Plant Cell. 2018;30(2):272-284.
95. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9(4):357-359.
96. Lorenz R, Bernhart SH, Siederdissen CHZ, Tafer H, Flamm C, Stadler PF, Hofacker IL. ViennaRNA Package 2.0. Algorithms Mol Biol. 2011;6.
97. Zhang BH, Pan XP, Cox SB, Cobb GP, Anderson TA. Evidence that miRNAs are different from other RNAs. Cell Mol Life Sci. 2006;63(2):246-254.
98. R Core Team: R: A Language and Environment for Statistical Computing. In., 3.5.1 edn. Vienna, Austria: R Foundation for Statistical Computing; 2017.
99. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12).
100. Kramer MF. Stem-loop RT-qPCR for miRNAs. Curr Protoc Mol Biol. 2011;95(1):15.10.11-15.10.15.
101. Pokorn T, Radisek S, Javornik B, Stajner N, Jakse J. Development of hop transcriptome to support research into host-viroid interactions. Plos One. 2017;12(9).
102. Alexa A, Rahnenfuhrer J: topGO: Enrichment Analysis for Gene Ontology. In., 2.40.0 edn; 2020.

Figures

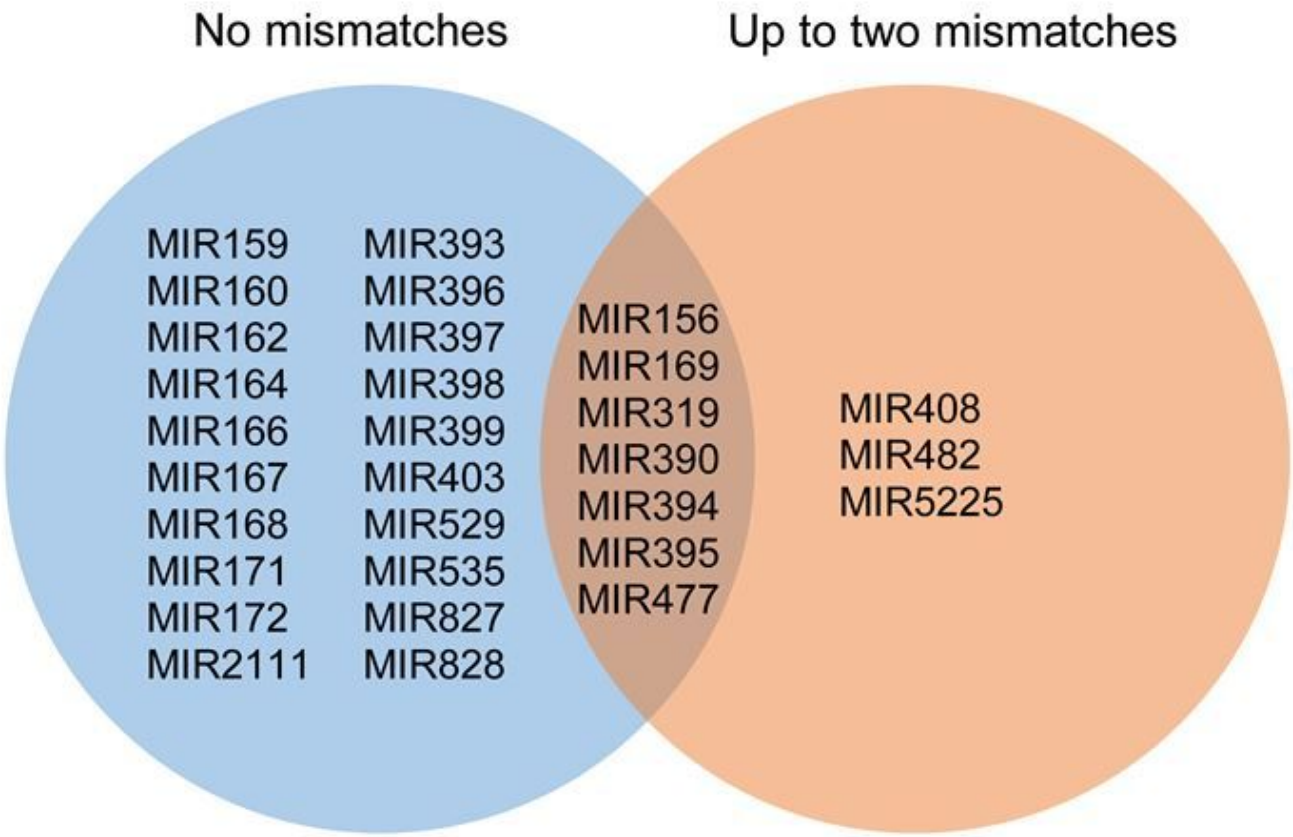

Figure 1

Predicted hop miRNA families that align with known miRNAs deposited in miRBase. Hop miRNA families with members that align without mismatches are in the blue circle (20 miRNA families) and those that align with up to two mismatches are in the orange circle (3 miRNA families). Seven families comprise members that align perfectly or with up to two mismatches.

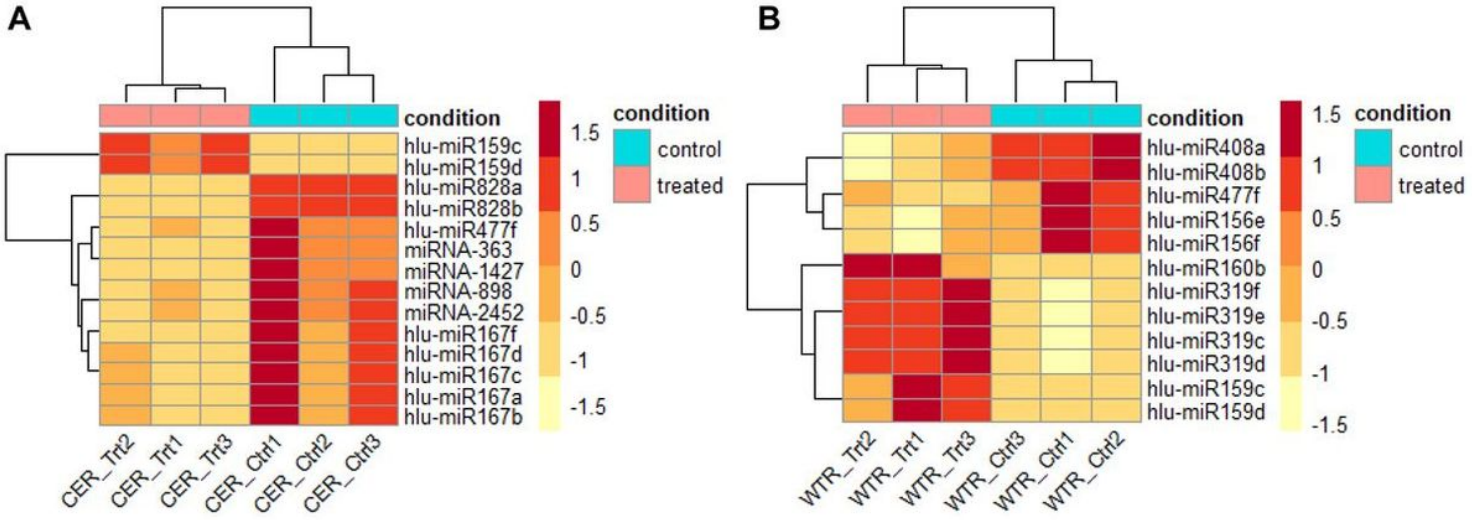

Figure 2

Heat maps of differentially expressed miRNAs between V. nonalfalfae-treated (Trt) and control (Ctrl) root samples. (A) DE miRNAs in roots of the susceptible cultivar Celeia (CER) and (B) DE miRNAs in roots of the resistant cultivar Wye Target (WTR). The colour scale is in log2.

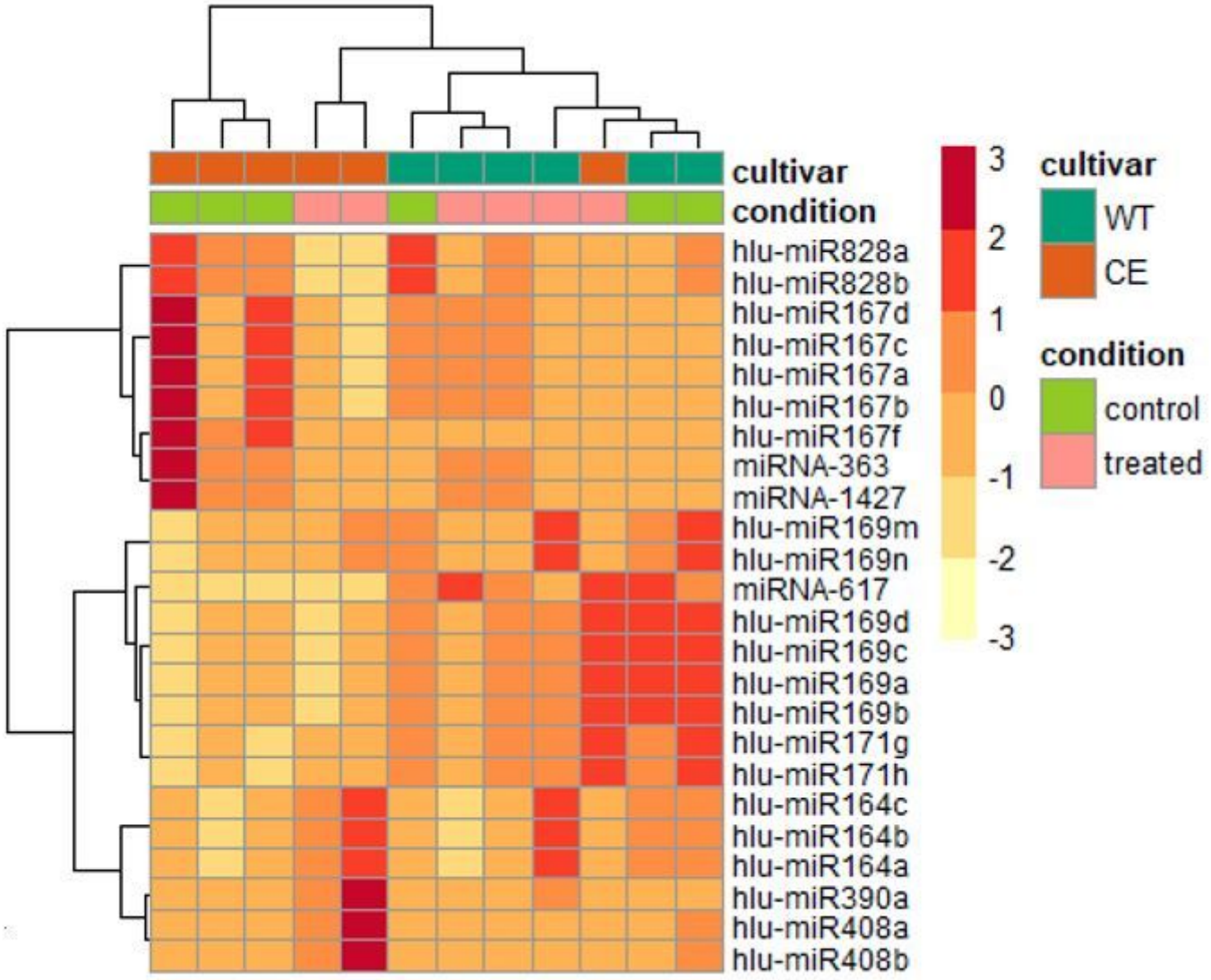

Figure 3

Heat map of differentially expressed miRNAs between the susceptible cultivar Celeia (CE) and the resistant cultivar Wye Target (WT) in response to infection with V. nonalfalfae. The samples were clustered based on miRNA expression values using the Euclidean distance measure and Ward clustering method.

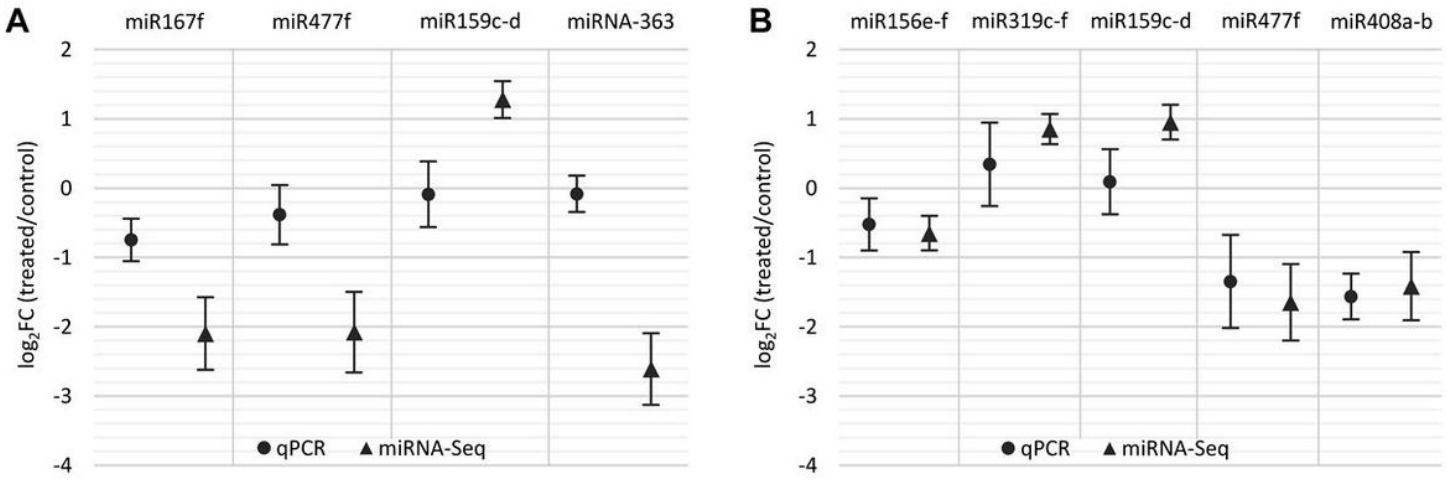

Figure 4

Expression of miRNAs after infection with V. nonalfalfae (log2FC (treated/control)) obtained with stem-loop RT-qPCR (●) and miRNA-Seq (DESeq2) (▲) methods. (A) Expression of selected DE miRNAs in the susceptible cultivar Celeia and (B) expression of selected DE miRNAs in the resistant cultivar Wye Target.

Supplementary Files

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- SupplementaryMaterial.xlsx