Identification and functional prediction of long non-coding RNAs (lncRNAs) involved in immune response in *Lupinus luteus* under *Colletotrichum lupini* infection

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Abstract

Background:

Long non-coding RNAs (lncRNAs) are increasingly recognized as important regulators in plant defense mechanisms and potential targets for crop improvement. In this study, we present the first comprehensive identification and characterization of lncRNAs associated with anthracnose resistance in *Lupinus luteus*, a crop of ecological and economic significance. We performed RNA-seq analysis on 32 libraries from resistant and susceptible genotypes post-inoculated with the fungal pathogen *Colletotrichum lupini*.

Results:

Our analysis identified 6,737 lncRNA transcripts originating from 2,785 loci, predominantly classified as long intergenic non-coding RNAs (~ 90%). Differential expression analysis revealed that the resistant genotype showed a higher number of differentially expressed genes following infection, including 20 lncRNAs consistently upregulated across all time points. Co-expression network analysis identified two lncRNAs, *Llu_lncRNA_25730* and *Llu_lncRNA_6595*, that showed promising associations with immune-related genes. Functional prediction linked these lncRNAs to diverse plant defense mechanisms, including phenylpropanoid biosynthesis, pathogen recognition, transcriptional regulation, transport, cell wall modification, and hydrolase activity related to GDSL-type esterase/lipase proteins (GELPs).

Conclusion:

These findings support the potential involvement of lncRNAs in the molecular basis of *L. luteus* resistance to *C. lupini*, providing novel insights into fungal pathogen resistance mechanisms through an lncRNA-centric perspective.

1. Background

Non-coding RNAs (ncRNAs) constitute up to 90% of the eukaryotic transcriptome, whereas only 1–2% corresponds to protein-coding genes [1]. Most ncRNAs are constitutive and ubiquitous, such as transfer RNA (tRNA) and ribosomal RNA (rRNA) [1–3]. A smaller fraction of ncRNAs regulates the genome; among these, long non-coding RNAs (lncRNAs, > 200 nt) have recently been recognized as key regulators of gene expression across a wide range of biological processes and organisms [4, 5]. In plants, lncRNAs participate in growth, flowering, reproduction, and even in regulating plant defense response against both biotic and abiotic stresses [1, 3, 6, 7]. Several studies on plant-pathogen interactions have revealed a predominant regulatory role of lncRNAs in response to pathogen and fungal infections, often showing strong co-expression with protein-coding genes involved in plant immune

resistance [8–12]. Consequently, lncRNAs have emerged as key regulatory molecules with potential applications in agriculture [6].

Lupin crops have been recognized as promising resources for enhancing food security due to their high seed protein content and their potential to promote sustainable agriculture [13]. *L. luteus* offers a notable advantage related to good adaptability to different climate conditions and protein content, reaching 75–95 g of protein in 100 g of seeds [14]. Unfortunately, the expansion of its crops has been limited globally by the anthracnose caused by *Colletotrichum lupini* [15–17]. This genus is part of the top 10 endophytic fungi infecting ~3,000 plant species [15–17]. Most *Colletotrichum* species exhibit a hemibiotrophic lifestyle, characterized by an initial biotrophic phase in which the pathogen colonizes living host cells without causing immediate tissue destruction, followed by a necrotrophic phase associated with host cell death [18]. The specific trigger that induces the switch from biotrophic to the necrotrophic phase remains unclear [15]. However, in experimental conditions of *L. luteus* radicles infected by *C. lupini*, the necrotrophic phase may occur between 60 and 84 hpi post-infection [19]. In this stage, the pathogen induces host cell death, resulting in necrotic lesions and visible symptoms [18]. Plants have evolved sophisticated defense mechanisms that enable pathogen recognition, activation of immune signaling, and in some cases, the establishment of genetic resistance [20]. Curiously, numerous studies have demonstrated that lncRNAs are closely associated with plant resistance to anthracnose [10, 21–23].

Regarding lupins and lncRNAs research, published studies have primarily linked lncRNAs to abiotic stress response [24, 25], whereas the role of lncRNAs in biotic stress and anthracnose resistance remains largely unexplored. Recently, a *L. luteus* genotype resistant to anthracnose was identified [26]. Nonetheless, the molecular mechanisms underlying its resistance remain poorly understood [27]. Previous research has focused primarily on the regulation of resistance through protein-coding genes [19], with little attention paid to non-coding genes, despite their growing recognition in plant resistance regulation [8, 10–12].

In this study, we present the first comprehensive identification and characterization of lncRNAs associated with anthracnose resistance in *L. luteus*, using RNA-seq analysis of 32 libraries from resistant and susceptible genotypes previously infected by *C. lupini*. We also explored regulatory networks between lncRNAs and protein-coding genes involved in the immune response. To the best of our knowledge, this study is the first to report *L. luteus* lncRNAs involved in anthracnose resistance, providing novel insights into fungal pathogen defense mechanisms through an lncRNA-centric perspective.

2. Methods

2.1 *L. luteus* RNA-seq data source

This study was based on a publicly available dataset of *L. luteus* RNA-seq data deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1169352 [19]. In the original

study, resistant and susceptible genotypes of *L. luteus*, along with *C. lupini* spores, were obtained from the germplasm bank of the Agriaquaculture Nutritional Genomics Center (CGNA), La Araucanía, Chile. Briefly, hypocotyl inoculations were performed by applying a conidial suspension dropped at the base of the radicle emerging from the seed [19]. Samples for RNA extraction were collected at key stages of infection: early infection stage (24 hours post-infection, hpi), the putative switch between the biotrophic and necrotrophic phases (60 hpi), and the necrotrophic phase (84 hpi). Uninoculated radicles emerging from the seed (0 hpi) served as controls [19]. Libraries were paired-end sequenced (2 x 150 bp) on a NovaSeq 6000 (Illumina®) platform at CD Genomics Inc. (NY, USA) [19].

A total of 32 RNA-seq libraries were analyzed, corresponding to the following sampling times: 0, 24, 60, and 84 hpi for the resistant and susceptible *L. luteus* genotypes, with four biological replicates per treatment (Table S1).

2.2 Mapping and transcriptome assembly

Quality control was performed with FastQC v0.11.9 [28], and summary reports were aggregated using MultiQC v.1.23 [29]. Adapters, low-quality reads (Phred quality cutoff Q = 30), and sequences shorter than 50 nt were removed using Fastp v0.23.2 [30] as described in [31]. The cleaned reads were mapped to the *L. luteus* reference genome (Number access: GCA_964019355.1) [32], using Hisat2 v2.2.165 [33]. Subsequently, transcriptome assembly was performed using Stringtie v2.2.2 [34]. To compare datasets, assembled transcripts were contrasted with the annotated genes in the *L. luteus* reference genome [32] using Gffcompare v0.11.2 [35].

2.3 lncRNAs identification

The coding potential of the assembled transcripts was assessed using two computational tools: CPC2 v1.0.1 [36] and FEELnc v3 [37]. Only transcripts consistently classified as lncRNAs by both tools were considered lncRNAs according to default criteria: coding potential score < 0.5, ≥ 200 nt, ORF coverage percentage < 50%, and present two or more exons, which is similar to previous studies focused on plant lncRNAs identification [10, 12, 38]. Remaining lncRNAs were classified according to their genome localization in relation to neighbor genes by the classifier module of FEELnc using the gene location information from the *L. luteus* reference genome [32].

2.4 Differential expression analysis

To prepare the data, counts for all genes (protein-coding and lncRNAs) were obtained using FeatureCounts v1.22.2 [39]. Then, to minimize biases related to library sizes and compositional RNA-seq samples, a Trimmed Mean of M-values (TMM) normalization [40] was applied using the edgeR v4.6.2 package [41] in R v4.5.1, as described in [31]. The filterByExpr function from the edgeR package was applied to remove low-expressed genes, and the remaining count data were used to perform the differential expression analysis.

Differentially expressed genes (DEGs) were calculated using two computational tools: edgeR v4.6.3 [41] and DESeq2 v1.48.1 [42] in R v4.5.1. To evaluate transcriptomic differences between resistant and

susceptible genotypes under *C. lupini* infection, a contrast design comparing non-inoculated samples versus samples collected at each hour post-infection, for each genotype was applied. The following thresholds were established for DEGs: a p-value < 0.001, false discovery rate (FDR) < 0.05, and a |log2FC| >= 2 for upregulated or |log2FC| >= -2 for downregulated genes, both classifying as differentially expressed genes (DEGs). Consensus results between edgeR and DESeq2 were reported as DEGs in this study. In this manner, we identified DEGs associated with the infection response for each genotype and hour post-infection.

To predict the principal biological functions of DEGs, a Gene Ontology (GO) enrichment analysis was performed using the BiNGO tool [43] integrated in Cytoscape v3.10.2 [44], with the GO annotation information from the *L. luteus* reference genome. Finally, the GO terms redundancy was minimized using GOATOOLS [45] in Python v3.10.

2.5 Co-expression network analysis

To identify gene modules associated with the different infection stages of *C. lupini* in the resistant and susceptible genotypes, a co-expression network analysis was performed using the WGCNA package v.1.73 [46] in R v4.5.1, following [31] with some modifications. Counts for all genes (protein-coding and lncRNAs) normalized in TMM were used as input for WGCNA. The blockwiseModules function of the WGCNA library was used to automate the process. A soft thresholding power (β) was chosen to achieve a scale-free topology. In this study, β was set to 12, yielding an R^2 of 0.9. A weighted adjacency matrix was created by raising the absolute value of the Pearson correlation coefficient between gene pairs to the selected soft-threshold power. From this matrix, a topological overlap matrix (TOM) was constructed to calculate network connectivity. Genes were hierarchically clustered based on TOM dissimilarity, and modules were determined using the dynamic tree-cut algorithm with a minimum module size of 30 genes[47]. The relationships between modules and the different infection conditions were assessed using Pearson correlation coefficients, comparing modules with both genotype conditions and post-infection time points. Modules with significant correlations (R2 > |0.7|, p < 0.05) were considered associated with their respective condition. To continue, module membership (MM) and gene significance (GS) metrics were used to identify genes strongly associated with a module and with an infected condition, according to the following statistical criteria: MM ≥ 0.8 and p-value ≤ 1e-5, and GS ≥ 0.5 and *p-value* ≤ 1e-2.

Finally, the construction and visualization of the co-expression network were carried out as follows: first, an extensive network was constructed, including all co-expressed gene connections with values > 0.02. Second, the network weights were filtered using a threshold at the 0.95 quantile, retaining the 5% strongest connections. Third, genes previously identified as differentially expressed in the resistant genotype were identified in the network. The visualization was performed using Cytoscape v3.10.2 [44].

2.6 Functional prediction of lncRNAs through co-expressed protein-coding genes

The lncRNAs previously identified as DEGs in the resistant genotype under *C. lupini* infection were mapped to the final co-expression network. Strongly co-expressed protein-coding genes with lncRNAs were recognized as putative lncRNA targets. These protein-coding genes were annotated, providing a basis for inferring lncRNA gene function based on the principle that highly correlated expression patterns are associated with similar functions and regulatory relationships [48, 49]. Thus, target genes were subjected to Gene Ontology enrichment analysis using the BiNGO tool [43] integrated in Cytoscape v3.10.2 [44]. Along with the anterior, these targets were also annotated with the *L. luteus* reference genome [32], whose annotations were obtained from many databases such as InterProscan v5.62-94.0 [50], EggNOG v5.0.2 [51], BLAST v2.14.1 [52], and UniProt (SwissProt + Trembl)[53], considering an e-value <1e-10.

Finally, a rigorous selection of lncRNA-targets was carried out based on two main criteria: (i) a functional association with immune responses supported by annotation and published literature, and (ii) exclusive upregulation in the resistant genotype across all post-infection time points. Furthermore, each target sequence was subjected to BLASTx analysis [54, 55] to identify sequence similarities between our target genes and annotated genes from other plant species in the NCBI database. Only hits with plant sequences, a query cover > 50%, sequence identity > 70%, and conserved domains were retained for further *in silico* quantification analysis.

2.7 *In silico* quantification of lncRNA-targets expression levels across infection

To quantify gene expression levels of selected lncRNA targets, the count data were normalized by applying the natural logarithm with a pseudocount, computed as log(TMM + 1). Subsequently, a student's t-test was performed to identify statistically significant differences in gene expression of a select gene between the resistant and susceptible genotype at each time point. Genes with p-values < 0.05 were considered significantly differentially expressed between the two genotypes at this time point.

2.8 Prediction of the regulation mechanism between lncRNA-targets

The lncRNA regulatory mechanism can be categorized as *cis* or *trans*, depending on whether the target is located nearby on the same chromosome or at a distant location, respectively [56]. To investigate the genomic distances between our selected lncRNAs and their protein-coding targets, we retrieved their chromosomal coordinates from the GTF annotation file generated during transcriptome assembly on the *L. luteus* reference genome [32]. Some studies have reported that a *cis-regulatory* mechanism was considered when the distance between an lncRNA and its target does not exceed 100 kb upstream or downstream; by contrast, the regulation mechanism was classified as *trans* [38, 57]. In our study, a 100 kb distance threshold was used to classify putative regulatory mechanisms between specific lncRNAs and their targets. Additionally, to calculate the probability of physical interactions between selected lncRNAs and their potential targets, an interaction predictor, LncTar v1.0 [58], was used. An interaction

with normalized energy levels (ndG) > -0.1 was classified as a probable direct interaction between the lncRNA and its target.

All codes employed in this work are available at https://github.com/dtomicslab/lncRNAs_study_lupinus.

3. Results

3. 1 LncRNAs identification from L. luteus RNA-seq libraries

To identify lncRNAs in the *Lupinus luteus* RNA-seq data set published in [19] and to explore their association with anthracnose resistance, we applied the workflow outlined in Fig. 1A. First, raw reads from the 32 libraries were cleaned according to Section 2.2. Approximately half of the raw read data remained after the cleaning step (Table S1). Consequently, a total of 109,443 transcripts derived from 41,646 genes were obtained. In contrast to the 36,895 protein-coding genes annotated in the reference genome of *L. luteus* [32], our results identified 5,198 unannotated genes from the hypocotyl libraries used.

Our lncRNA prediction identified 20,598 putative transcripts from CPC2 and 6,932 from FEELnc. Further analysis included only lncRNAs consistently predicted by both tools, yielding 6,737 transcripts from 2,785 lncRNA genes (Fig. 1B). The lncRNAs were subsequently classified by their genomic locations relative to gene partners. A remarkable difference between the number of lncRNAs classified as intergenic (1,947) and genic (838) was observed (Fig. 1C).

3.2 Differentially expressed *L. luteus* lncRNAs and protein-coding genes under *C. lupini* infection

To investigate transcriptional responses to *C. lupini* infection, a contrast design comparing the non-infected with 24, 60, and 84 hpi for both genotypes was used. Transcriptomic analysis revealed 388 upregulated and 154 downregulated protein-coding genes across all times post-infection in the resistant genotype (Fig. S1A, B). A remarkable difference was observed in the susceptible genotype, with only 21 protein-coding genes upregulated and 8 downregulated across all time points post-infection (Fig. S1C, D).

Notably, only the resistant genotype exhibited a gradual increase in DEGs throughout disease progression (Fig. 2A, B). In contrast, the susceptible genotype displayed a transient peak in DEGs at 24 hpi, followed by a sharp decline at 60 hpi and a subsequent increase at 84 hpi, including upregulated protein-coding genes (Fig. 2A) and lncRNAs (Fig. 2B). Furthermore, these expression patterns were also observed for downregulated protein-coding genes and lncRNAs (Fig. S2A, B). It is also worth noting that protein-coding and lncRNA DEGs exhibited similar temporal patterns across all experiments.

Analysis focused exclusively on lncRNAs differentially expressed in the resistant genotype identified 20 upregulated (Fig. 2C, Table S2) and 17 downregulated lncRNAs that were consistently induced across all

infection stages (Fig. S3A, B). This observation was absent in the susceptible genotype, in which no shared lncRNAs across all infection times were identified as upregulated, and only one was annotated as downregulated at all times post-infection (Fig. S3C, D).

Additionally, to identify the principal putative functions of upregulated protein-coding genes in response to *C. lupini* infection in both genotypes, a GO enrichment analysis was performed. Based on our top 10 GO term analysis for the resistant (Fig. 2D) and susceptible genotype (Fig. S4), we found common enriched categories related to plant defense responses at 24 hpi, such as response to chemical stimulus (GO:0004221), response to endogenous stimulus (GO:0009719), and response to stress (GO:0006950). However, in susceptible plants, these categories were no longer top enriched by 60 hpi (Fig. S4). Some of the top 10 GO terms observed only in resistant plants and related to immune response, included the flavonoid biosynthetic process (GO:0009813), enriched at 24 hpi and re-activated at 84 hpi; suberin biosynthetic process (GO:0010345) and plant-type cell wall organization (GO:0009664) at 60 hpi; and response to hydrogen peroxide (GO:0042542) and ethylene-mediated signaling pathway (GO:0009873) at 84 hpi (Fig. 2D). Interestingly, the phenylpropanoid metabolic process (GO:0009698) was the only GO term enriched consistently across all infection stages, suggesting a sustained involvement in plant defense throughout the course of infection **(**Fig. 2D**)**.

3.3 Co-expression analysis between lncRNAs and protein-coding genes associated with *C. lupini* resistance response

The weighted gene co-expression network was constructed from 2,357 lncRNAs and 30,898 protein-coding genes, yielding 21 distinct modules. These modules exhibited variability in size, ranging from 76 genes (Modules 20 and 21) to 1,889 genes (Module 1) (Fig. 3A). A comprehensive breakdown of the gene composition per module, including the relative proportions of protein-coding and lncRNA genes, is presented in Fig. 3A.

Subsequently, the relationships between the 21 modules with the traits of resistance and susceptibility, and the temporal response, were calculated with a module-trait correlation analysis. Thus, a key module was identified as highly and exclusively associated with resistance conditions: Module 5 (Fig. 3B). Module 5 (constituted by 1,009 upregulated genes in resistance, including 432 lncRNAs and 577 protein-coding genes), exhibited the strongest and consistent associations with the resistance treatments (R^2 = 0.99, p = 8e − 31) in contrast with the rest of the modules (Fig. 3B). Furthermore, their association with temporal responses in resistant samples was maintained across all post-infection stages, including non-infected ($R^2 > 0.34$, $p \leq 0.05$; Fig. 3B). Interestingly, this module presented the highest proportion of lncRNAs in contrast with the rest of the modules (Fig. 3A). GO enrichment analysis revealed a predominant association between Module 5 and immune-related processes. Specifically, enriched GO categories in this module included cellular response to hormone stimulus (GO:0032870), response to endogenous stimulus (GO:0009719), response to other organism (GO:0051707), and cellular response to stress (GO:0033554), among other stimulus-related terms (Fig. 3C).

On the other hand, the 20 lncRNAs identified as exclusively upregulated in the resistant genotype across all post-infection time points (Fig. 2C, Table S2) were mapped onto our co-expression results to elucidate their relationships with other genes. Among them, two lncRNA genes, *Llu_lncRNA_6595* and *Llu_lncRNA_25730*, stood out for their strong co-expression with other target genes and with each other. Both lncRNAs and the majority of their target genes were notably part of Module 5, which had been previously identified as the module most strongly correlated with the resistant response (Fig. 3B). In particular, *Llu_lncRNA_6595* showed significant co-expression with 221 protein-coding genes (220 within Module 5, and one within Module 11). In comparison, *Llu_lncRNA_25730* co-expressed with 329 protein-coding genes (315 within Module 5, 13 within Module 11, and one within Module 14). Regarding protein-coding targets, all *Llu_lncRNA_6595* targets were also co-expressed with *Llu_lncRNA_25730*.

To explore the functional associations among lncRNA-target genes and immune responses, we performed GO enrichment analysis. The results revealed a clear link between the targets and plant defense mechanisms, as indicated by enriched categories such as jasmonic acid (JA) and ethylene (ET)-dependent systemic resistance (GO:0090861), flavonol metabolic process (GO:0051554), cellular response to biotic stimulus (GO:0070497), and phenylpropanoid metabolic process (GO:0009698) (Fig. 4A). This result suggests that both lncRNAs are associated with immune-related processes. To validate and refine these functional predictions, each target gene was subsequently annotated and analyzed individually.

3.4 Selection and functional annotation of Llu_lncRNA_6595 and Llu_lncRNA_25730 targets

A careful selection of lncRNA targets was conducted using the methodology outlined in Section 2.6. Using this approach, 15 protein-coding genes were identified as targets that are strongly co-expressed with our lncRNAs and are associated with plant defense (Fig. 4B, Table 1). Notably, 12 of these targets belonged to Module 5, an exclusive module previously associated with the resistant response (Fig. 3B). It is important to note that *Llu_lncRNA_25730* exhibited stronger co-expression weights with its targets than *Llu_lncRNA_6595* (Table S3). Additionally, four targets were uniquely linked to *Llu_lncRNA_25730*: *Llu00152*, *Llu08368*, *Llu24029*, and *MSTRG.3105*.

According to individual functional annotations, the 15 selected genes are implicated in plant immune responses and can be classified into five principal functional categories: 1. Pathogen recognition, 2. Transcriptional regulation, 3. Transport, 4. Cell wall modification, and 5. Hydrolase activity. A comprehensive summary of the annotation for all these target genes is presented in Table 1.

These findings reveal a close relationship between upregulated lncRNAs and protein-coding genes involved in plant defense mechanisms against *C. lupini* infection in the resistant genotype, suggesting a potential role in resistance. Moreover, both lncRNAs exhibited similar expression patterns and a high degree of co-expression, indicating that immune responses are regulated not only through lncRNA-protein-coding gene interactions but also through potential lncRNA-lncRNA associations.

Table 1
Functional annotation of 15 targets co-expressed with Llu_lncRNA_25730 and Llu_lncRNA_6595 exclusively in the resistant genotype. Functional predictions based on *L. luteus* genome annotation and literature are shown. Genes are listed in ascending order.

Target	Annotation	Putative function	Reference
Llu00152	LRR receptor-like serine/threonine-protein kinase	Pathogen recognition	[59]
Llu00566	UDP-glucose 4,6-dehydratase	Cell wall modification	[60]
Llu08368	Caffeic acid 3-O-methyltransferase-like	Cell wall modification	[61]
Llu10106	Protein kinase RLK-LRR-XI-1 family	Pathogen recognition	[59]
Llu12288	GDSL esterase/lipase At5g14450-like	Hydrolase activity, defense-related enzyme	[62]
Llu12291	GDSL esterase/lipase At5g14450-like	Hydrolase activity, defense-related enzyme	[62]
Llu17446	Protein kinase RLK-RLCK-XII-1 family	Pathogen recognition	[59]
Llu21112	ABC transporter A	Transport	[63]
Llu24029	Peroxisomal 3-ketoacyl-CoA thiolase	Cell wall modification	[64]
Llu29589	UDP-glucose 4,6-dehydratase	Cell wall modification	[60])
Llu33929	LRR receptor-like serine/threonine-protein kinase isoform X3	Pathogen recognition	[59]
Llu33934	LRR receptor-like serine/threonine-protein kinase isoform X4	Pathogen recognition	[59]
Llu35227	Disease resistance protein RGA3	Pathogen recognition	[65]
MSTRG.16258	Cupredoxin	Transport	[66]
MSTRG.3105	Myb transcription factor	Transcriptional regulation	[67]

3.5 Expression levels and patterns of selected lncRNAs-targets in the resistant and susceptible genotype across C. lupini infection

To investigate how the expression patterns of the selected lncRNAs and their targets changed throughout the infection process in the resistant versus the susceptible genotype, *in silico* statistical analyses and visualization were performed across all post-infection times.

In general, both *Llu_lncRNA_25730* and *Llu_lncRNA_6595*, together with their 15 selected targets, exhibited markedly higher expression levels in the resistant genotype compared with the susceptible one across all post-infection times (Fig. 5). Both lncRNAs had been previously identified among the 20 DEGs that consistently responded to infection across all post-infection times in the resistant genotype (Fig. 2C,

Table S2). In our expression visualization, both lncRNAs appeared to increase gradually as the infection progressed, reaching their highest levels at 84 hpi, when the destructive necrotrophic phase becomes active (Fig. 5A, B). In particular, the *Llu_lncRNA_25730* (Fig. 5A) displayed the highest expression levels compared to *Llu_lncRNA_6595* (Fig. 5B) and even to its targets (Fig. 5C, Q), showing significant differences ($p < 0.01$) relative to the susceptible genotype, in which its expression was lower and appeared unchanged throughout disease progression.

Regarding the co-expressed targets, all were significantly upregulated in the resistant genotype, with considerably lower expression levels in the susceptible one (Fig. 5C, Q).

Most targets maintained stable high expression across all post-infection time points, suggesting their role in a basal defense mechanism within the resistant genotype, seemingly independent of the infection stimulus (Fig. 5). In contrast, four targets specific to the *Llu_lncRNA_25730* demonstrated progressive upregulation following *C. lupini* infection evidenced by differential expression at 60 hpi (Fig. 5K, *Llu24029*) and 84 hpi (Fig. 5A, *Llu00152*, Fig. 5E, *Llu08368*, and Fig. 5Q, *MSTRG.3105*).

These results confirm that the identified lncRNAs were co-expressed with targets putatively involved in defense responses, suggesting that they may play potential regulatory roles in basal defense barriers and post-infection responses against anthracnose disease in the resistant genotype.

3.6 Prediction of the regulation mechanism between lncRNAs and their targets

Surprisingly, the majority of our target genes were located on different chromosomes relative to the selected lncRNAs (Table S4), despite exhibiting high levels of co-expression (Table S3). Therefore, the interactions between both lncRNAs and most protein-coding targets were classified as *trans*-regulatory relationships (i.e., at distances greater than 100 kb). Nonetheless, only one interaction was classified as *cis*, corresponding to *Llu_lncRNA_25730-Llu12291* (co-expression weight 0.3).

To identify putative physical interactions between lncRNAs and their targets, the LncTar tool was used to evaluate hybridization stability in cases of potential direct regulation. Our results indicated that, in most cases, the regulation between lncRNAs and their targets was indirect or non-physical, even for the *cis*-regulation predicted between *Llu_lncRNA_25730* and *Llu12291*. Since LncTar only reports positive interaction results (ndG > -0.1), only one interaction exhibited a probable physical connection, corresponding to *Llu_lncRNA_6595–Llu12291*, with an ndG value of − 0.1065. However, − 0.1 represents the lowest cut-off threshold in this analysis, suggesting a slight interaction. Our results indicated that highly co-expressed interactions between targets and lncRNAs do not necessarily imply direct physical interactions, and other regulatory mechanisms may be involved.

4. Discussion

Long non-coding RNAs have recently emerged as crucial regulatory nodes in plant defense responses against pathogens and fungal infections [7, 9]. Here, we describe for the first time lncRNAs potentially associated with the immune response in the resistant *L. luteus* genotype during *C. lupini* infection.

Overall, we identified 2,785 lncRNA genes, most of which were intergenic. This pattern is consistent with previous reports indicating that intergenic lncRNAs represent the predominant lncRNA class in plants [3, 9]. Differential expression analysis showed that protein-coding genes and lncRNAs exhibited highly similar expression dynamics, with peaks of up- and downregulation occurring at the same time points. Notably, a predominant transcriptional response characterized by a higher number of DEGs that increased proportionally with disease progression was observed exclusively in the resistant genotype. Previous studies have reported similar patterns, in which plant resistant genotypes display more substantial and more sustained transcriptional responses than susceptible ones during anthracnose disease [10, 21, 23, 68], as well as in genotypes resistant to other fungal diseases [12, 69–71], bacterial pathogens [69, 72], and insects [73]. Collectively, these findings suggest that resistant genotypes maintain sustained transcriptional responses under biotic stress, including lncRNAs and protein-coding genes.

Among upregulated genes in the resistant *L. luteus* genotype under infection stimulus, our study found that the phenylpropanoid metabolic process (GO:0009698) was notably overrepresented and consistently enriched across all post-infection times. This is supported by evidence that the phenylpropanoid pathway (PPP) is widely associated with plant stress responses and resistance to fungal pathogens [19, 71, 74, 75]. Notably, experiments on *Juglans regia* (walnut) infected with anthracnose caused by *Colletotrichum gloeosporioides* also showed enrichment of PPP-related categories among protein-coding targets of lncRNAs, particularly in resistant genotypes [10].

In our study, we focused on two lncRNAs: *Llu_lncRNA_25730* and *Llu_lncRNA_6595*. These were chosen because they were consistently upregulated in the resistant genotype across all post-infection time points, and both showed strong co-expression with several protein-coding genes (targets) related to immune response. Significantly enriched categories observed for targets were related to rhamnose biosynthetic process (GO:0019300), JA and ET-dependent systemic resistance (GO:0090861), flavonol metabolic process (GO:0051554), cellular response to biotic stimulus (GO:0070497), and phenylpropanoid metabolic process (GO:0009698), similarly to the functions reported for lncRNA-target pairs associated with resistant genotypes in other plant species and pathogen-challenge experiments [10, 12, 71].

Among the selected protein-coding targets from *Llu_lncRNA_25730* and *Llu_lncRNA_6595*, six were related to pathogen recognition. The targets *Llu00152*, *Llu10106*, *Llu33929*, and *Llu33934*, putatively encode LRR receptor-like serine/threonine-protein kinases (LRR-RLKs). The LRR-RLK proteins recognize pathogen-associated molecular patterns (PAMPs), such as fungal chitin, in the Microbe-associated Molecular Pattern-triggered Immunity (MTI) [59]. Notably, these proteins have been reported as co-expressed with lncRNAs in the *J. regia* resistance response to anthracnose infection [10], consistent with

our findings. Curiously, the targets *Llu33929* and *Llu33934* were enriched to the JA and ET-dependent systemic resistance category (GO:0009861). In plant immunity, JA and ET are key hormones that regulate defense responses against hemibiotrophic and necrotrophic fungal pathogens [20, 76]. Curiously, these mechanisms can also be primed by rhizosphere-derived stimuli, leading to induced systemic resistance (ISR) [77, 78, 79]. Regarding *Llu17446* target, this was associated with an RLCK (receptor-like cytoplasmic kinases), a class of kinases that lack the extracellular and transmembrane domains of receptor-like kinases (RLKs), and are often associated with RLKs to downstream signal transduction in plant defense responses [80]. Finally, *Llu35227* putatively encodes a disease resistance protein, RGA3, which belongs to the resistance gene analog (RGA) family implicated in intracellular pathogen recognition [81]. Notably, a recent study of lncRNAs in *Oryza sativa* (rice) resistant to *Magnaporthe oryzae* reported strong co-expression between lncRNAs and the *RGA3* gene, exclusively in resistant plants [12]. All identified pathogen-receptor targets exhibited strong co-expression with our selected lncRNAs and were markedly upregulated in the resistant genotype at all post-infection times. Most of these targets showed increased expression as the disease progressed, which may reflect sustained activation during disease progression, particularly as severity intensifies during the necrotrophic phase.

The *MSTRG.3105* was an exclusive target of *Llu_lncRNA_25730*, and potentially encodes a MYB transcription factor (TF). MYB TFs are one of the largest families of TF in plants and perform a wide range of regulatory functions, particularly related to pathogen/pest resistance, abiotic resistance, flavonoid/terpenoid biosynthesis, nutrient absorption and development [82]. Interestingly, the interaction between lncRNAs and MYB TFs in response to the oomycete *Phytophthora infestans* has been characterized in tomato using knockout assays, revealing a strong positive correlation in which the simultaneous upregulation of both significantly enhances plant resistance [83]. In our study, *MSTRG.3105* expression increased with disease progression exclusively in the resistant genotype.

The targets *Llu21112* and *MSTRG.16258* were related to transport functions and showed a marked upregulation exclusively in the resistance genotype. However, they did not appear to fluctuate in response to the infection, suggesting a potential constitutive association with resistance genotype rather than an infection-induced response. The *Llu21112* encodes a putative ABC transporter A (ABCA), whereas *MSTRG.16258* encodes a cupredoxin. Plant ABC transporters have attracted considerable attention owing to their established roles in plant biological processes, including stress response [63, 84]. Furthermore, they have been associated with PPP and auxin transport [85]. Although *ABCA* genes have been reported as upregulated under abiotic stress [86, 87], their specific role in biotic stress remains incompletely characterized. Regarding cupredoxin-related proteins, recent research on Glycine max (soybean) infected with *Heterodera glycines* has demonstrated that resistance to the pathogen is enhanced by cupredoxin overexpression, with a probable involvement of the JA-mediated pathway [66].

Among the targets related to cell wall modification functions, *Llu00566* and *Llu29589*, putatively encode the enzyme UDP-glucose 4,6-dehydratase. This enzyme is crucial for rhamnose (Rha) biosynthesis, a component of the plant cell wall [60]. Since several plant defense strategies involve the reinforcement or

modification of cell wall components [88], the selected lncRNAs may be associated with this mechanism. Furthermore, Rha can conjugate with flavonoids (related to PPP) [89] and with other molecules to form steroidal glycoalkaloids, which have been previously associated with anthracnose resistance in tomato [90]. Both targets showed expression patterns unchanged upon infection. This pattern is consistent with a constitutive expression profile that may help maintain cell wall integrity in the resistant genotype. In contrast, the *Llu08368* and *Llu24029* targets exclusive of *Llu_lncRNA_25730*, showed increased expression as the disease progressed, suggesting a potential involvement in infection response.

Llu08368 and *Llu24029* encode putatively a caffeic acid 3-O-methyltransferase-like (COMT) enzyme and a peroxisomal 3-ketoacyl-CoA thiolase (KAT), respectively. COMT is a vital enzyme involved in the PPP and in the biosynthesis of lignin, a critical structural component of secondary plant cell walls [91]. In previous reports, COMT has been shown to respond to pathogen infection by strengthening or modifying plant cell wall components in *Avena sativa* (oat)[92]. Additionally, KAT enzymes are involved in the final stage of fatty acid β-oxidation, a crucial metabolic process for plant defense, responsible for synthesizing key stress response modulators, including JA, auxins, benzenoids, and hydrogen peroxide [64, 93].

Finally, two targets were related to hydrolase activity. The *Llu12288* and *Llu12291* encode a putative GDSL esterase/lipase protein. This protein family, known as GDSL-type esterase/lipase proteins (GELPs), plays key roles in plant development, secondary metabolite synthesis, and defense responses [94]. Notably, the overexpression of GDSL-related genes in *Arabidopsis thaliana* promotes resistance against *Sclerotinia sclerotiorum* [95], and in *Malus domestica* (apple) GELPs increase resistance against *C. gloeosporioides* [62]. Interestingly, a recent transcriptomic study in several *Vitis vinifera* (grapevine) libraries under multiple pathogen infections, reported lncRNAs co-expressed with GDSL genes only in resistant plants [69]. In our study, neither of these targets changed upon infection. It is also important to mention that the *Llu12288* exhibited the highest co-expression weights with both lncRNAs compared with the other targets.

Intriguingly, the identified lncRNA-target pairs were located on distinct chromosomes and exhibited strong co-expression patterns, consistent with a potential *trans*-regulatory relationship, and displaying low predicted probabilities of direct physical interaction. This observation raises the possibility of regulatory mechanisms involving transcriptional modulation through intermediate molecular interactions or competing endogenous RNA processes, in which lncRNAs may function as miRNA decoys [1]. Although such mechanisms have been widely investigated in plant resistance contexts [38, 71, 96], their contribution in lupins remains poorly understood and warrants further investigation.

In conclusion, we identified two lncRNAs: *Llu_lncRNA_25730* and *Llu_lncRNA_6595*, that were strongly associated with anthracnose resistance and co-expressed with target genes whose predicted functions are linked to basal defense barriers and infection-associated responses in the resistant genotype. These lncRNAs may represent promising candidates for future evaluation as molecular markers of resistant

genotypes, given their exclusive upregulation in resistant plants. Moreover, they could be explored as early molecular indicators during the biotrophic phase of infection. Nevertheless, further functional validation is required to clarify their regulatory roles in *L. luteus* resistance, particularly given the tissue-specificity of plant lncRNAs [2] and the fact that the present study was based on hypocotyl RNA libraries.

Overall, these findings provide strong evidence of an association between lncRNAs and the immune response of *L. luteus* against *C. lupini*, contributing to a broader understanding of resistance mechanisms from an lncRNA-centered perspective.

Abbreviations

ABCA
ABC transporter A
COMT
Caffeic acid 3-O-methyltransferase-like
DEGs
Differentially expressed genes
ET
Ethylene
FDR
False discovery rate
GELPs
GDSL-type esterase/lipase proteins
GO
Gene Ontology
GS
Gene significance
hpi
Hours post-infection
JA
Jasmonic acid
lncRNAs
Long non-coding RNAs
LRR-RLKs
LRR receptor-like serine/threonine-protein kinases
MTI
Microbe-associated Molecular Pattern-triggered Immunity
MM
Module membership
KAT
Peroxisomal 3-ketoacyl-CoA thiolase

PPP
Phenylpropanoid pathway
RGA
Resistance gene analog
Rha
Rhamnose
TOM
topological overlap matrix
TF
Transcription factor
TMM
Trimmed Mean of M-values

Declarations

This manuscript is original, has not been published previously, and is not under consideration by any other journal. All authors have approved the manuscript and agree with its submission to *BMC Plant Biology*.

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Author contributions:

J. E. M. Supervised the study and provided guidance throughout the research process. Designed, planned the scientific analysis and experiments, and critical revision of the manuscript, oriented and guided writing, results, and discussions of the paper.

A.H.C. performed all bioinformatic analyses and implemented the computational workflow. Also conducted data curation, developed the methodology, generated the visualizations, and wrote the manuscript.

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References

1. Chao H, Hu Y, Zhao L, Xin S, Ni Q, Zhang P, et al. Biogenesis, Functions, Interactions, and Resources of Non-Coding RNAs in Plants. Int J Mol Sci. 2022;23. https://doi.org/10.3390/ijms23073695.
2. Huang J, Zhou W, Zhang X, Li Y. Roles of long non-coding RNAs in plant immunity. PLoS Pathog. 2023;19. https://doi.org/10.1371/journal.ppat.1011340.
3. Li N, Wang Y, Zheng R, Song X. Research progress on biological functions of lncRNAs in major vegetable crops. Veg Res. 2022;2:1–14. https://doi.org/10.48130/VR-2022-0014.
4. Mattick JS, Amaral PP, Carninci P, Carpenter S, Chang HY, Chen LL, et al. Long non-coding RNAs: definitions, functions, challenges and recommendations. Nat Rev Mol Cell Biol. 2023;24:430–47. https://doi.org/10.1038/s41580-022-00566-8.
5. Zhang X, Wang W, Zhu W, Dong J, Cheng Y, Yin Z, et al. Mechanisms and functions of long non-coding RNAs at multiple regulatory levels. Int J Mol Sci. 2019;20. https://doi.org/10.3390/ijms20225573.
6. Bao X, Dai X, Chen J, Li R. Plant Long Non-Coding RNAs: Multilevel Regulators of Development, Stress Adaptation, and Crop Improvement. Agronomy. 2025;15:1950. https://doi.org/10.3390/agronomy15081950.
7. Kościelniak P, Walas Ł, Konecka A, Buraczyk W, Klupczyńska EA. Plant long noncoding RNAs: why do we not know more? Biol Res. 2025;58:37. https://doi.org/10.1186/s40659-025-00610-9.
8. Bhatia G, Upadhyay SK, Upadhyay A, Singh K. Investigation of long non-coding RNAs as regulatory players of grapevine response to powdery and downy mildew infection. BMC Plant Biol. 2021;21. https://doi.org/10.1186/s12870-021-03059-6.
9. Domínguez-Rosas E, Hernández-Oñate MÁ, Fernandez-Valverde S-L, Tiznado-Hernández ME. Plant long non-coding RNAs: identification and analysis to unveil their physiological functions. Front Plant Sci. 2023;14:1275399. https://doi.org/10.3389/fpls.2023.1275399.
10. Feng S, Fang H, Liu X, Dong Y, Wang Q, Yang KQ. Genome-wide identification and characterization of long non-coding RNAs conferring resistance to Colletotrichum gloeosporioides in walnut (Juglans regia). BMC Genomics. 2021;22. https://doi.org/10.1186/s12864-020-07310-6.
11. Gao C, Sun J, Dong Y, Wang C, Xiao S, Mo L, et al. Comparative transcriptome analysis uncovers regulatory roles of long non-coding RNAs involved in resistance to powdery mildew in melon. BMC Genomics. 2020;21. https://doi.org/10.1186/s12864-020-6546-8.
12. Shan X, Xia S, Peng L, Tang C, Tao S, Baig A, et al. Identification of Rice LncRNAs and Their Roles in the Rice Blast Resistance Network Using Transcriptome and Translatome. Plants. 2025;14:2752.

https://doi.org/10.3390/plants14172752.

13. an de Noort M, Lupin. An Important Protein and Nutrient Source. Sustain Protein Sources. 2024;219–39. https://doi.org/10.1016/B978-0-323-91652-3.00028-9.
14. Burgos-Díaz C, Opazo-Navarrete M, Wandersleben T, Soto-Añual M, Barahona T, Bustamante M. Chemical and Nutritional Evaluation of Protein-Rich Ingredients Obtained through a Technological Process from Yellow Lupin Seeds (Lupinus luteus). Plant Foods Hum Nutr. 2019;74:508–17. https://doi.org/10.1007/s11130-019-00768-0.
15. Dubrulle G, Pensec F, Picot A, Rigalma K, Pawtowski A, Nicolleau S, et al. Phylogenetic diversity and effect of temperature on pathogenicity of colletotrichum lupini. Plant Dis. 2020;104:938–50. https://doi.org/10.1094/PDIS-02-19-0273-RE.
16. Pandey AK, Kumar A, Mbeyagala EK, Barbetti MJ, Basandrai A, Basandrai D, et al. Anthracnose Resistance in Legumes for Cropping System Diversification. Crit Rev Plant Sci. 2023;42:177–216. https://doi.org/10.1080/07352689.2023.2228122.
17. Samad S, Gentzbittel L, Bondarenko E, Rickauer M, Volkova P, Ben C. Insights into the Pathogenic Mechanisms and the Control Strat-Egies of Anthracnose in Lupins – A Comprehensive Review. 2023. https://doi.org/10.20944/preprints202312.2132.v1
18. Guilengue N, Silva M do, Talhinhas C, Neves-Martins P, Loureiro J. Subcuticular–Intracellular Hemibiotrophy of Colletotrichum lupini in Lupinus mutabilis. Plants. 2022;11. https://doi.org/10.3390/plants11223028.
19. Armijo-Godoy G, Levicoy D, Martínez-Hernández JE, Rupayan A, Hernández S, Carrasco M et al. Defense mechanisms in Lupinus luteus against Colletotrichum lupini, involving TIR-NBS-LRR protein, hypersensitive response and phenylpropanoid pathways. 2024. https://doi.org/10.1101/2024.10.15.618496
20. Bentham AR, de la Concepcion JC, Mukhi N, Zdrzałek R, Draeger M, Gorenkin D, et al. A molecular roadmap to the plant immune system. J Biol Chem. 2020;295:14916–35. https://doi.org/10.1074/jbc.REV120.010852.
21. Yang M, Zhou C, Yang H, Kuang R, Liu K, Huang B, et al. Comparative transcriptomics and genomic analyses reveal differential gene expression related to Colletotrichum brevisporum resistance in papaya (Carica papaya L). Front Plant Sci. 2022;13. https://doi.org/10.3389/fpls.2022.1038598.
22. Yin H, Zhang X, Zhang B, Luo H, He C. Revealing the dominant long noncoding RNAs responding to the infection with Colletotrichum gloeosporioides in Hevea brasiliensis. Biol Direct. 2019;14. https://doi.org/10.1186/s13062-019-0235-z.
23. Zhou R, Dong Y, Wang C, Liu J, Liang Q, Meng X, et al. LncRNA109897-JrCCR4-JrTLP1b forms a positive feedback loop to regulate walnut resistance against anthracnose caused by Colletotrichum gloeosporioides. Hortic Res. 2023;10. https://doi.org/10.1093/hr/uhad086.
24. Aslam MM, Waseem M, Xu W, Ying L, Zhang J, Yuan W. Global Identification of White Lupin lncRNAs Reveals Their Role in Cluster Roots under Phosphorus Deficiency. Int J Mol Sci. 2022;23. https://doi.org/10.3390/ijms23169012.

25. Hidalgo M, Ramos C, Zolla G. Analysis of lncRNAs in Lupinus mutabilis (Tarwi) and Their Potential Role in Drought Response. Non-Coding RNA. 2023;9. https://doi.org/10.3390/ncrna9050048.
26. Lichtin N, Salvo-Garrido H, Till B, Caligari PDS, Rupayan A, Westermeyer F, et al. Genetic and comparative mapping of Lupinus luteus L. highlight syntenic regions with major orthologous genes controlling anthracnose resistance and flowering time. Sci Rep. 2020;10. https://doi.org/10.1038/s41598-020-76197-w.
27. Książkiewicz M, Rychel-Bielska S, Plewiński P, Nuc M, Irzykowski W, Jędryczka M, et al. The resistance of narrow-leafed lupin to diaporthe toxica is based on the rapid activation of defense response genes. Int J Mol Sci. 2021;22:1–30. https://doi.org/10.3390/ijms22020574.
28. Andrews S. FastQC: a quality control tool for high throughput sequence data. Available Online Httpwwwbioinformaticsbabrahamacukprojectsfastqc. 2010.
29. Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics. 2016;32:3047–8. https://doi.org/10.1093/bioinformatics/btw354.
30. Chen S. Ultrafast one-pass FASTQ data preprocessing, quality control, and deduplication using fastp. iMeta. 2023. https://doi.org/10.1002/imt2.107. 2.
31. Martinez-Hernandez JE, Aliaga-Tobar V, González-Rosales C, Monte-Neto R, Martin AJM, Maracaja-Coutinho V. Comparative and systems analyses of Leishmania spp. non-coding RNAs through developmental stages. PLoS Negl Trop Dis. 2025;19:e0013108. https://doi.org/10.1371/journal.pntd.0013108.
32. Martinez-Hernandez JE, Salvo-Garrido H, Levicoy D, Caligari PDS, Rupayán A, Moyano T, et al. Genomic structure of yellow lupin (Lupinus luteus): genome organization, evolution, gene family expansion, metabolites and protein synthesis. BMC Genomics. 2025;26:477. https://doi.org/10.1186/s12864-025-11678-8.
33. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nat Biotechnol. 2019;37:907–15. https://doi.org/10.1038/s41587-019-0201-4.
34. Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol. 2015;33:290–5. https://doi.org/10.1038/nbt.3122.
35. Pertea M, Pertea G. GFF Utilities: GffRead and GffCompare. F1000Research. 2020;9. https://doi.org/10.12688/f1000research.23297.1
36. Kang YJ, Yang DC, Kong L, Hou M, Meng YQ, Wei L, et al. CPC2: A fast and accurate coding potential calculator based on sequence intrinsic features. Nucleic Acids Res. 2017;45:W12–6. https://doi.org/10.1093/nar/gkx428.
37. Wucher V, Legeai F, Hédan B, Rizk G, Lagoutte L, Leeb T, et al. FEELnc: A tool for long non-coding RNA annotation and its application to the dog transcriptome. Nucleic Acids Res. 2017;45. https://doi.org/10.1093/nar/gkw1306.

38. Zhao X, Gan L, Yan C, Li C, Sun Q, Wang J, et al. Genome-wide identification and characterization of long non-coding RNAs in peanut. Genes. 2019;10. https://doi.org/10.3390/genes10070536.
39. Liao Y, Smyth GK, Shi W. FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics. 2014;30:923–30. https://doi.org/10.1093/bioinformatics/btt656.
40. Robinson MD, Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. 2010.
41. Robinson MD, McCarthy DJ, Smyth GK. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2009;26:139–40. https://doi.org/10.1093/bioinformatics/btp616.
42. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550. https://doi.org/10.1186/s13059-014-0550-8.
43. Maere S, Heymans K, Kuiper M. BiNGO: A Cytoscape plugin to assess overrepresentation of Gene Ontology categories in Biological Networks. Bioinformatics. 2005;21:3448–9. https://doi.org/10.1093/bioinformatics/bti551.
44. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A software Environment for integrated models of biomolecular interaction networks. Genome Res. 2003;13:2498–504. https://doi.org/10.1101/gr.1239303.
45. Klopfenstein DV, Zhang L, Pedersen BS, Ramírez F, Warwick Vesztrocy A, Naldi A, et al. GOATOOLS: A Python library for Gene Ontology analyses. Sci Rep. 2018;8:10872. https://doi.org/10.1038/s41598-018-28948-z.
46. Langfelder P, Horvath S. WGCNA: An R package for weighted correlation network analysis. BMC Bioinformatics. 2008. https://doi.org/10.1186/1471-2105-9-559. 9.
47. Han W, Zhang Z, He B, Xu Y, Zhang J, Cao W. Integrated analysis of long non-coding RNAs in human gastric cancer: An in silico study. PLoS ONE. 2017;12:e0183517. https://doi.org/10.1371/journal.pone.0183517.
48. Montenegro JD. Gene Co-expression Network Analysis. In: Edwards D, editor. Plant Bioinformatics: Methods and Protocols. New York, NY: Springer US; 2022. pp. 387–404. https://doi.org/10.1007/978-1-0716-2067-0_19.
49. Piro RM, Marsico A. Network-Based Methods and Other Approaches for Predicting lncRNA Functions and Disease Associations. In: Lai X, Gupta SK, Vera J, editors. Computational Biology of Non-Coding RNA: Methods and Protocols. New York, NY: Springer New York; 2019. pp. 301–21. https://doi.org/10.1007/978-1-4939-8982-9_12.
50. Quevillon E, Silventoinen V, Pillai S, Harte N, Mulder N, Apweiler R et al. InterProScan: Protein domains identifier. Nucleic Acids Res. 2005;33 SUPPL. 2. https://doi.org/10.1093/nar/gki442
51. Huerta-Cepas J, Szklarczyk D, Heller D, Hernández-Plaza A, Forslund SK, Cook H, et al. EggNOG 5.0: A hierarchical, functionally and phylogenetically annotated orthology resource based on 5090

organisms and 2502 viruses. Nucleic Acids Res. 2019;47:D309–14. https://doi.org/10.1093/nar/gky1085.

52. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10. https://doi.org/10.1016/S0022-2836(05)80360-2.
53. Bateman A, Martin MJ, Orchard S, Magrane M, Ahmad S, Alpi E, et al. UniProt: the Universal Protein Knowledgebase in 2023. Nucleic Acids Res. 2023;51:D523–31. https://doi.org/10.1093/nar/gkac1052.
54. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+: architecture and applications. BMC Bioinformatics. 2009;10:421. https://doi.org/10.1186/1471-2105-10-421.
55. Priyam A, Woodcroft BJ, Rai V, Moghul I, Munagala A, Ter F, et al. Sequenceserver: A Modern Graphical User Interface for Custom BLAST Databases. Mol Biol Evol. 2019;36:2922–4. https://doi.org/10.1093/molbev/msz185.
56. Kopp F, Mendell JT. Functional Classification and Experimental Dissection of Long Noncoding RNAs. Cell. 2018;172:393–407. https://doi.org/10.1016/j.cell.2018.01.011.
57. Yu L, Huang T, Qi X, Yu J, Wu T, Luo Z, et al. Genome-Wide Analysis of Long Non-coding RNAs Involved in Nodule Senescence in Medicago truncatula. Front Plant Sci. 2022;13:917840. https://doi.org/10.3389/fpls.2022.917840.
58. Li J, Ma W, Zeng P, Wang J, Geng B, Yang J, et al. LncTar: A tool for predicting the RNA targets of long noncoding RNAs. Brief Bioinform. 2014;16:806–12. https://doi.org/10.1093/bib/bbu048.
59. Reyes Zamora O, Troncoso-Rojas R, Báez-Flores ME, Tiznado-Hernández ME, Rascón-Chu A. Signaling of Plant Defense Mediated by Receptor-like Kinases, Receptor-like Cytoplasmic Protein Kinases and MAPKs Triggered by Fungal Chitin in Horticultural Crops. Horticulturae. 2024;10:361. https://doi.org/10.3390/horticulturae10040361.
60. Jiang N, Dillon FM, Silva A, Gomez-Cano L, Grotewold E. Rhamnose in plants - from biosynthesis to diverse functions. Plant Sci. 2021;302:110687. https://doi.org/10.1016/j.plantsci.2020.110687.
61. Chen S, Zhang S, Wang W, Zhou Y, Fan H, Meng X, et al. Characterization and functions of Caffeic acid O-methyltransferase genes in Cucumis sativus against Corynespora cassiicola infection. Plant Sci. 2026;362:112792. https://doi.org/10.1016/j.plantsci.2025.112792.
62. Ji Z, Wang M, Zhang S, Du Y, Cong J, Yan H, et al. GDSL Esterase/Lipase GELP1 Involved in the Defense of Apple Leaves against Colletotrichum gloeosporioides Infection. Int J Mol Sci. 2023;24:10343. https://doi.org/10.3390/ijms241210343.
63. Do THT, Martinoia E, Lee Y, Hwang J-U. 2021 update on ATP-binding cassette (ABC) transporters: how they meet the needs of plants. Plant Physiol. 2021;187:1876–92. https://doi.org/10.1093/plphys/kiab193
64. Pye VE, Christensen CE, Dyer JH, Arent S, Henriksen A. Peroxisomal Plant 3-Ketoacyl-CoA Thiolase Structure and Activity Are Regulated by a Sensitive Redox Switch. J Biol Chem. 2010;285:24078–88. https://doi.org/10.1074/jbc.M110.106013.

65. Sekhwal M, Li P, Lam I, Wang X, Cloutier S, You F. Disease Resistance Gene Analogs (RGAs) in Plants. Int J Mol Sci. 2015;16:19248–90. https://doi.org/10.3390/ijms160819248.
66. Zhang L, Zeng Q, Zhu Q, Tan Y, Guo X. Essential Roles of Cupredoxin Family Proteins in Soybean Cyst Nematode Resistance. Phytopathology®. 2022;112:1545–58. https://doi.org/10.1094/PHYTO-09-21-0391-R.
67. Biswas D, Gain H, Mandal A. MYB transcription factor: A new weapon for biotic stress tolerance in plants. Plant Stress. 2023;10:100252. https://doi.org/10.1016/j.stress.2023.100252.
68. Boufleur TR, Massola Júnior NS, Becerra S, Baraldi E, Bibiano LBJ, Sukno SA, et al. Comparative transcriptomic provides novel insights into the soybean response to Colletotrichum truncatum infection. Front Plant Sci. 2022;13:1046418. https://doi.org/10.3389/fpls.2022.1046418.
69. Cao W, Wang Y, Zhao X, Gan L, Sheng Y. Comprehensive analysis of lncRNAs and mRNAs in *Vitis vinifera* defense mechanisms against multiple pathogens. J Sci Food Agric. 2025;105:7417–28. https://doi.org/10.1002/jsfa.70005.
70. Zhang L, Liu J, Cheng J, Sun Q, Zhang Y, Liu J, et al. lncRNA7 and lncRNA2 modulate cell wall defense genes to regulate cotton resistance to Verticillium wilt. Plant Physiol. 2022;189:264–84. https://doi.org/10.1093/plphys/kiac041.
71. Zhang R, Chen J, Zhang Y, Lu X, Cheng C, Li J, et al. Long Noncoding RNA–Messenger RNA (lncRNA–mRNA) Network in Resistance to Gummy Stem Blight (Stagonosporopsis cucurbitacearum) of Melon Plant Introduction (PI) 420145. Horticulturae. 2025;11:31. https://doi.org/10.3390/horticulturae11010031.
72. Selvamani SB, Nallusamy S, Kamalanathan V, Ravichandran A, Singaravelu R, Selvapandian U, et al. Understanding the mechanisms of long noncoding RNAs in response to bacterial blight in rice. Physiol Mol Plant Pathol. 2025;135:102486. https://doi.org/10.1016/j.pmpp.2024.102486.
73. Zhang J, Li J, Saeed S, Batchelor WD, Alariqi M, Meng Q, et al. Identification and Functional Analysis of lncRNA by CRISPR/Cas9 During the Cotton Response to Sap-Sucking Insect Infestation. Front Plant Sci. 2022;13. https://doi.org/10.3389/fpls.2022.784511.
74. Kumar AP, Bhasker K, Nikhil BSK, Srinivas P. Role of Phenylpropanoids and Flavonoids in Plant Defense Mechanism. Int J Environ Clim Change. 2023;13:2951–60. https://doi.org/10.9734/ijecc/2023/v13i92534.
75. Ramaroson M-L, Koutouan C, Helesbeux J-J, Le Clerc V, Hamama L, Geoffriau E, et al. Role of Phenylpropanoids and Flavonoids in Plant Resistance to Pests and Diseases. Molecules. 2022;27:8371. https://doi.org/10.3390/molecules27238371.
76. Macioszek VK, Jęcz T, Ciereszko I, Kononowicz AK. Jasmonic Acid as a Mediator in Plant Response to Necrotrophic Fungi. Cells. 2023;12. https://doi.org/10.3390/cells12071027.
77. Jung J, Ahn S, Kim D-H, Riu M. Triple interactions for induced systemic resistance in plants. Front Plant Sci. 2024;15:1464710. https://doi.org/10.3389/fpls.2024.1464710.
78. Yadav M, Dubey MK, Upadhyay RS. Systemic Resistance in Chilli Pepper against Anthracnose (Caused by Colletotrichum truncatum) Induced by Trichoderma harzianum, Trichoderma asperellum

and Paenibacillus dendritiformis. J Fungi. 2021;7:307. https://doi.org/10.3390/jof7040307.

79. Yang F, Zhao D, Fan H, Zhu X, Wang Y, Liu X, et al. Functional Analysis of Long Non-Coding RNAs Reveal Their Novel Roles in Biocontrol of Bacteria-Induced Tomato Resistance to Meloidogyne incognita. Int J Mol Sci. 2020;21:911. https://doi.org/10.3390/ijms21030911.
80. Hailemariam S, Liao C-J, Mengiste T. Receptor-like cytoplasmic kinases: orchestrating plant cellular communication. Trends Plant Sci. 2024;29:1113–30. https://doi.org/10.1016/j.tplants.2024.04.006.
81. Dolatabadian A, Amas JC, Thomas WJW, Sayari M, Al-Mamun HA, Edwards D, et al. Exploring Cloned Disease Resistance Gene Homologues and Resistance Gene Analogues in Brassica nigra, Sinapis arvensis, and Sinapis alba: Identification, Characterisation, Distribution, and Evolution. Genes. 2025;16:849. https://doi.org/10.3390/genes16080849.
82. Wu X, Xia M, Su P, Zhang Y, Tu L, Zhao H, et al. MYB transcription factors in plants: A comprehensive review of their discovery, structure, classification, functional diversity and regulatory mechanism. Int J Biol Macromol. 2024;282:136652. https://doi.org/10.1016/j.ijbiomac.2024.136652.
83. Cui J, Jiang N, Hou X, Wu S, Zhang Q, Meng J, et al. Genome-Wide Identification of lncRNAs and Analysis of ceRNA Networks During Tomato Resistance to Phytophthora infestans. Phytopathology®. 2020;110:456–64. https://doi.org/10.1094/PHYTO-04-19-0137-R.
84. Li P, Luo T, Pu X, Zhou Y, Yu J, Liu L. Plant transporters: roles in stress responses and effects on growth and development. Plant Growth Regul. 2021;93:253–66. https://doi.org/10.1007/s10725-020-00684-3.
85. Kaneda M, Schuetz M, Lin BSP, Chanis C, Hamberger B, Western TL, et al. ABC transporters coordinately expressed during lignification of Arabidopsis stems include a set of ABCBs associated with auxin transport. J Exp Bot. 2011;62:2063–77. https://doi.org/10.1093/jxb/erq416.
86. Kou X, Zhao Z, Xu X, Li C, Wu J, Zhang S. Identification and expression analysis of ATP-binding cassette (ABC) transporters revealed its role in regulating stress response in pear (Pyrus bretchneideri). BMC Genomics. 2024;25:169. https://doi.org/10.1186/s12864-024-10063-1.
87. Ren H, Li X, Guo L, Wang L, Hao X, Zeng J. Integrative Transcriptome and Proteome Analysis Reveals the Absorption and Metabolism of Selenium in Tea Plants [Camellia sinensis (L.) O. Kuntze]. Front Plant Sci. 2022;13:848349. https://doi.org/10.3389/fpls.2022.848349.
88. Wan J, He M, Hou Q, Zou L, Yang Y, Wei Y, et al. Cell wall associated immunity in plants. Stress Biol. 2021;1:3. https://doi.org/10.1007/s44154-021-00003-4.
89. Kuhn BM, Errafi S, Bucher R, Dobrev P, Geisler M, Bigler L, et al. 7-Rhamnosylated Flavonols Modulate Homeostasis of the Plant Hormone Auxin and Affect Plant Development. J Biol Chem. 2016;291:5385–95. https://doi.org/10.1074/jbc.M115.701565.
90. Fabian ML, Zhang C, Sun J, Price NP, Chen P, Clarke CR, et al. Steroidal glycoalkaloids contribute to anthracnose resistance in Solanum lycopersicum. J Exp Bot. 2023;74:3700–13. https://doi.org/10.1093/jxb/erad108.
91. Eckardt NA. Probing the Mysteries of Lignin Biosynthesis: The Crystal Structure of Caffeic Acid/5-Hydroxyferulic Acid 3/5- O -Methyltransferase Provides New Insights. Plant Cell. 2002;14:1185–9.

https://doi.org/10.1105/tpc.140610.

92. Pan Y, Niu K, Shen F, Zhao G, Zhang Y, Chai J, et al. Genome-wide analysis of the COMT gene family in Avena sativa: insights into lignin biosynthesis and disease defense mechanisms. Front Plant Sci. 2025;16:1609698. https://doi.org/10.3389/fpls.2025.1609698.
93. Lackus ND, Schmidt A, Gershenzon J, Köllner TG. A peroxisomal β-oxidative pathway contributes to the formation of C6–C1 aromatic volatiles in poplar. Plant Physiol. 2021;186:891–909. https://doi.org/10.1093/plphys/kiab111.
94. Su H-G, Zhang X-H, Wang T-T, Wei W-L, Wang Y-X, Chen J, et al. Genome-Wide Identification, Evolution, and Expression of GDSL-Type Esterase/Lipase Gene Family in Soybean. Front Plant Sci. 2020;11:726. https://doi.org/10.3389/fpls.2020.00726.
95. Ding L, Li M, Guo X, Tang M, Cao J, Wang Z, et al. Arabidopsis GDSL1 overexpression enhances rapeseed Sclerotinia sclerotiorum resistance and the functional identification of its homolog in Brassica napus. Plant Biotechnol J. 2020;18:1255–70. https://doi.org/10.1111/pbi.13289.
96. Gareev I, de Jesus Encarnacion Ramirez M, Goncharov E, Ivliev D, Shumadalova A, Ilyasova T, et al. MiRNAs and lncRNAs in the regulation of innate immune signaling. Non-Coding RNA Res. 2023;8:534–41. https://doi.org/10.1016/j.ncrna.2023.07.002.
97. Kumar N. Molecular Marker Techniques: A Potential Approach of Crop Improvement. Springer Nature; 2023. https://doi.org/10.1007/978-981-99-1612-2.
98. Osorio CE, Udall JA, Salvo-Garrido H, Maureira-Butler IJ. Development and characterization of InDel markers for Lupinus luteus L. (Fabaceae) and cross-species amplification in other Lupin species. Electron J Biotechnol. 2018;31:44–7. https://doi.org/10.1016/j.ejbt.2017.11.002.

Figures

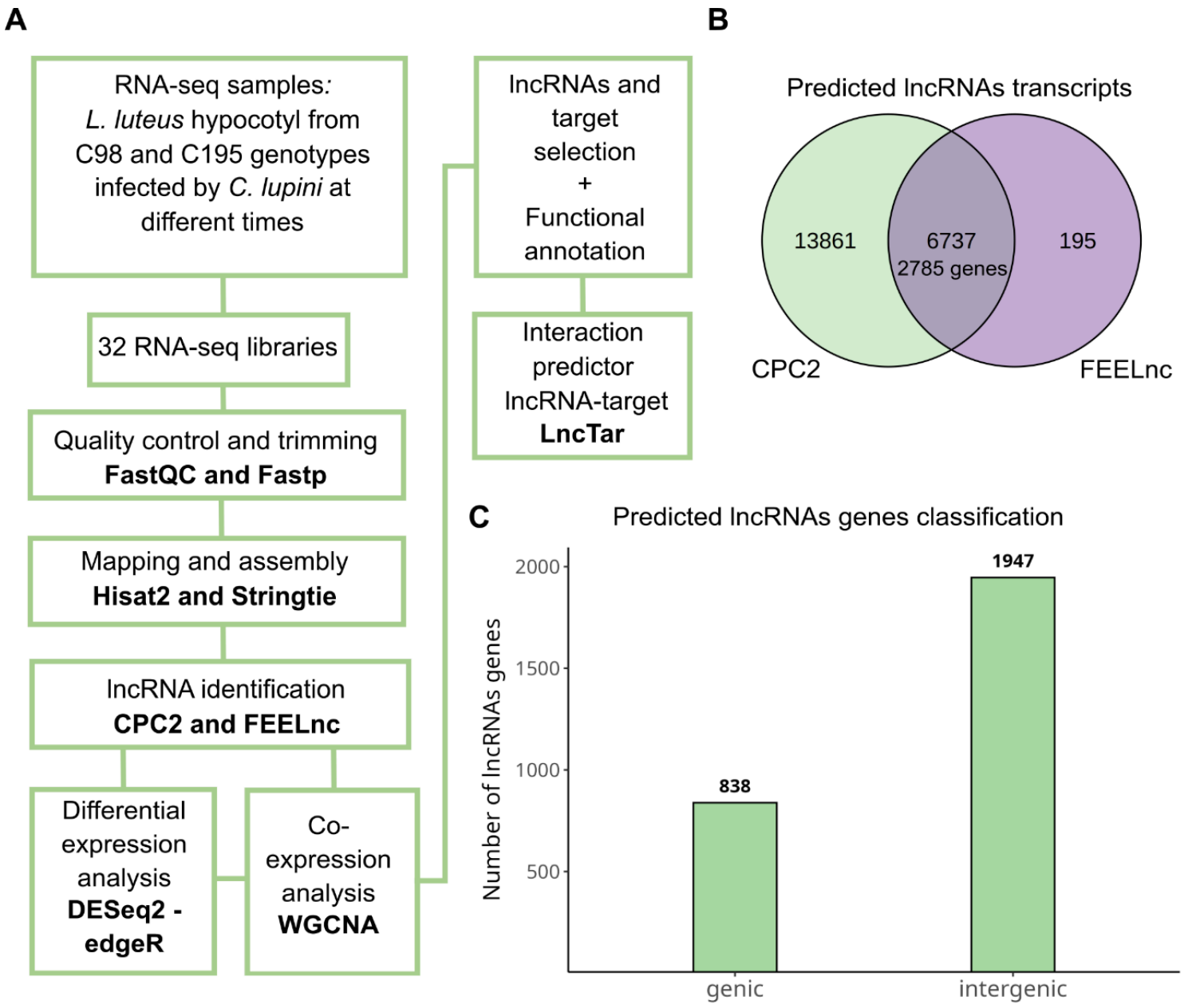

Figure 1

Identification of lncRNAs from *L. luteus* RNA-seq data. **(A)** Comprehensive analysis workflow: sequential steps to identify lncRNAs from RNA-seq data and predict their putative targets and functions. **(B)** Number of predicted transcripts of lncRNAs by CPC2 and FEELnc tools, noticing the 2,785 genes commonly predicted. **(C)** LncRNAs gene classification.

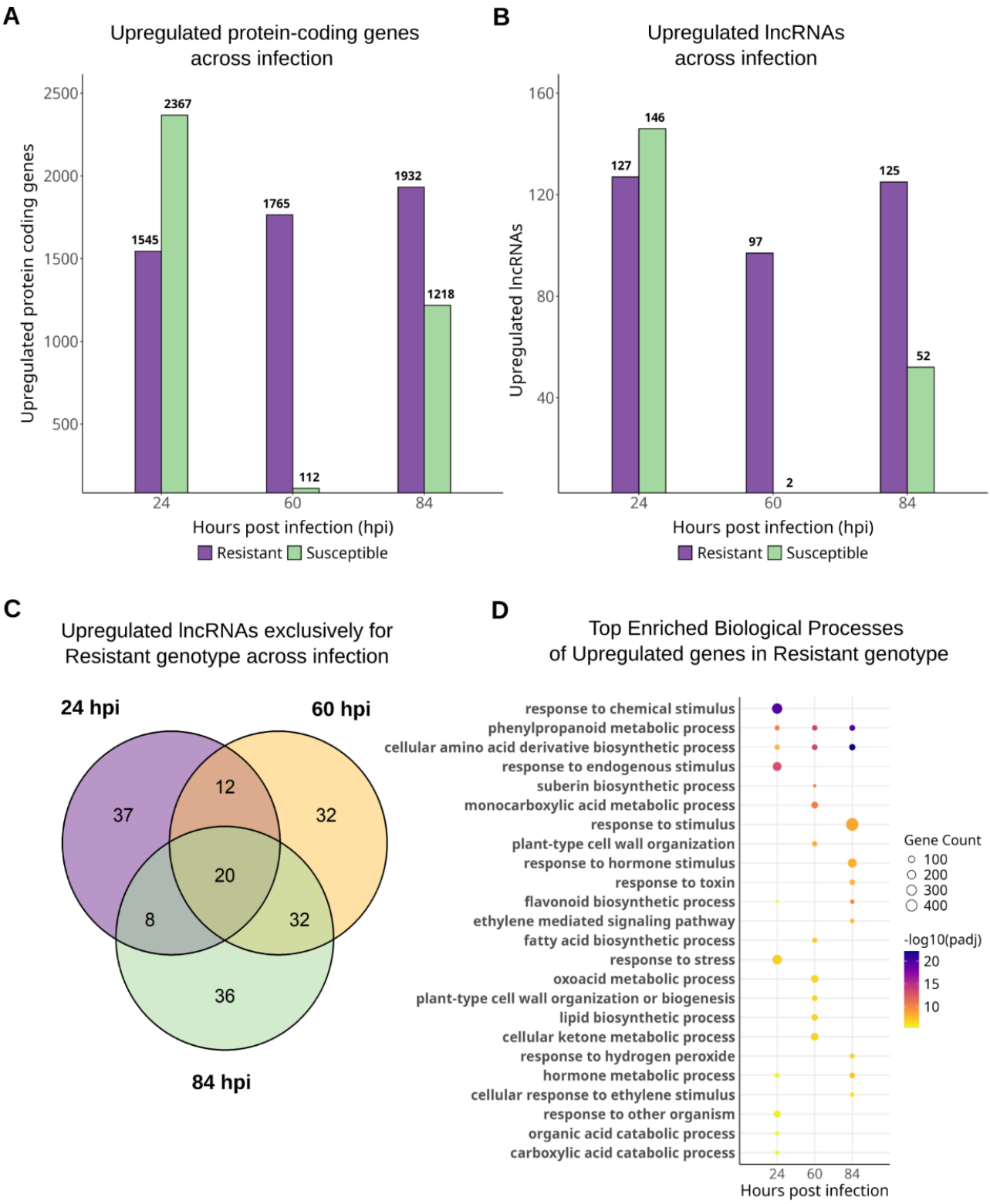

Figure 2

Differentially expressed protein-coding and lncRNA genes in *L. luteus* across infection with *C. lupini* in resistant and susceptible genotypes. **(A)** Upregulated protein-coding genes for both genotypes throughout different times post-infection. **(B)** Upregulated lncRNAs for both genotypes throughout different hours post-infection. **(C)**Venn diagram of exclusively upregulated lncRNAs in the resistant genotype across different infection stages; note the 20 shared lncRNAs. **(D)** Top enriched biological

processes according to upregulated protein-coding genes detected in the resistant genotype. padj: adjusted p-value.

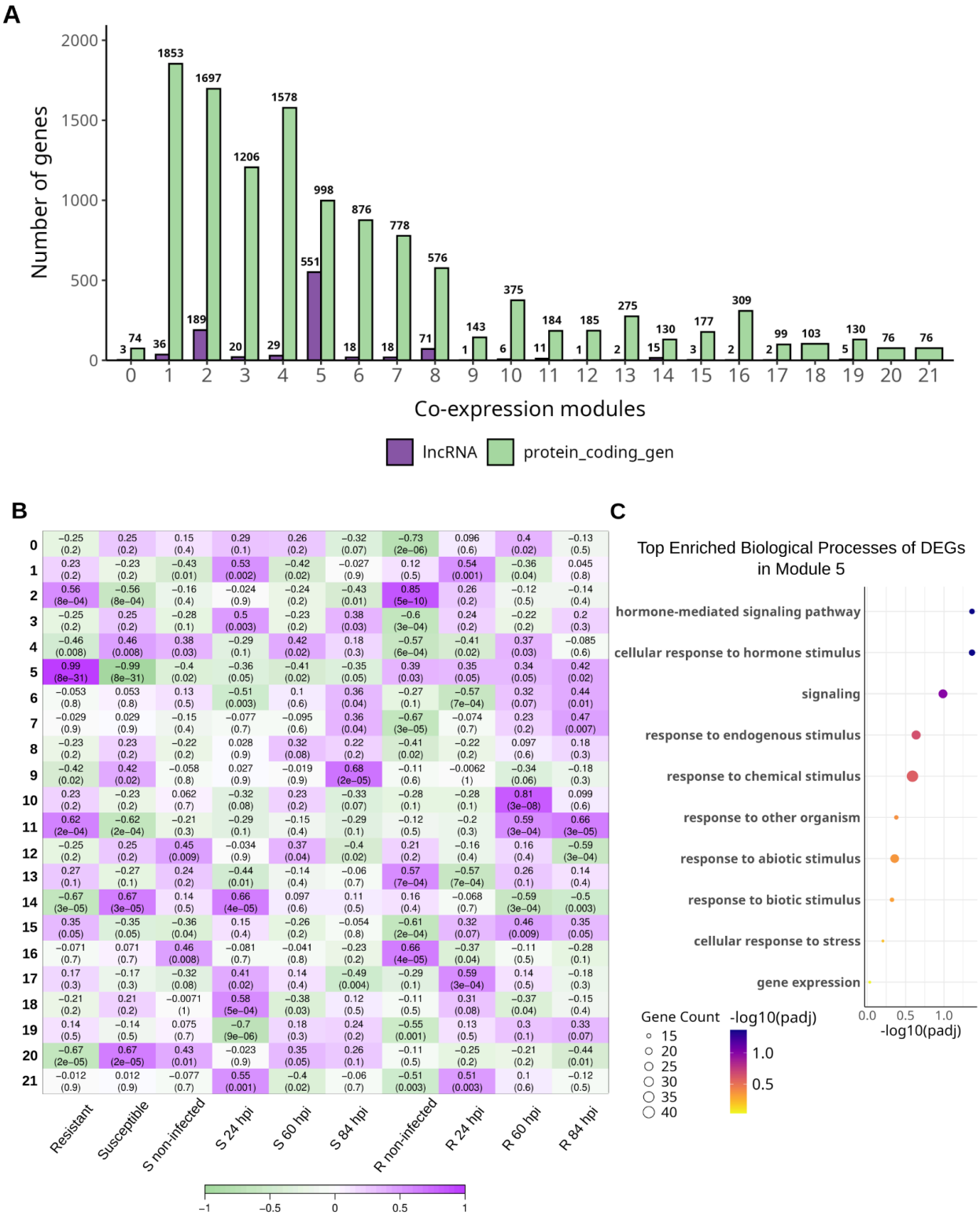

Figure 3

Co-expression modules in resistant and susceptible *L. luteus* genotypes and their associations with *C. lupini* infection. **(A)** Modules and their gene compositions. **(B)** Correlation heatmap of 21 modules

across various genotypes and hours post-infection. Each row indicates a module, and each column represents a condition. From left to right: Resistant (all resistant samples), Susceptible (all susceptible samples), followed by susceptible (S) and resistant (R) states at different hours post-infection (hpi). The heatmap displays correlation coefficients (−1 to 1) and significance levels for each comparison. **(C)** Top 10 GO biological processes assigned to differentially expressed genes (DEGs) within Module 5. padj: adjusted p-value.

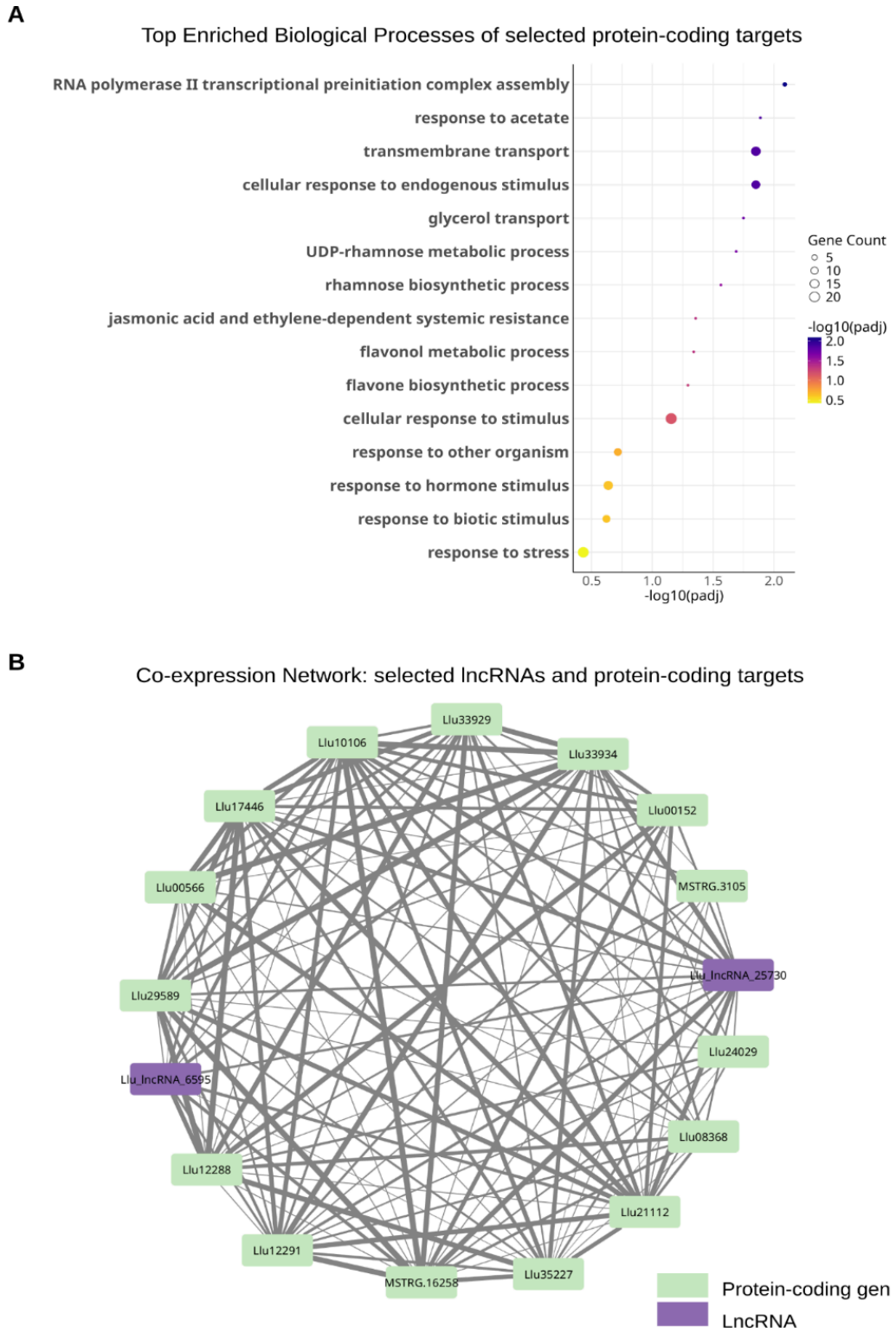

Figure 4

Co-expression network and functional associations linking the lncRNAs *Llu_lncRNA_25730* and *Llu_lncRNA_6595* with their respective protein-coding targets. **(A)** Top 15 GO biological processes assigned to the targets of *Llu_lncRNA_25730* and *Llu_lncRNA_6595*. **(B)** Co-expression network of selected lncRNAs-targets. This co-expression network was filtered to show the top 5% strongest gene-gene relationships. Edge thickness represents the correlation weight between gene pairs; thicker edges indicate stronger co-expression (0.14 to 0.53).

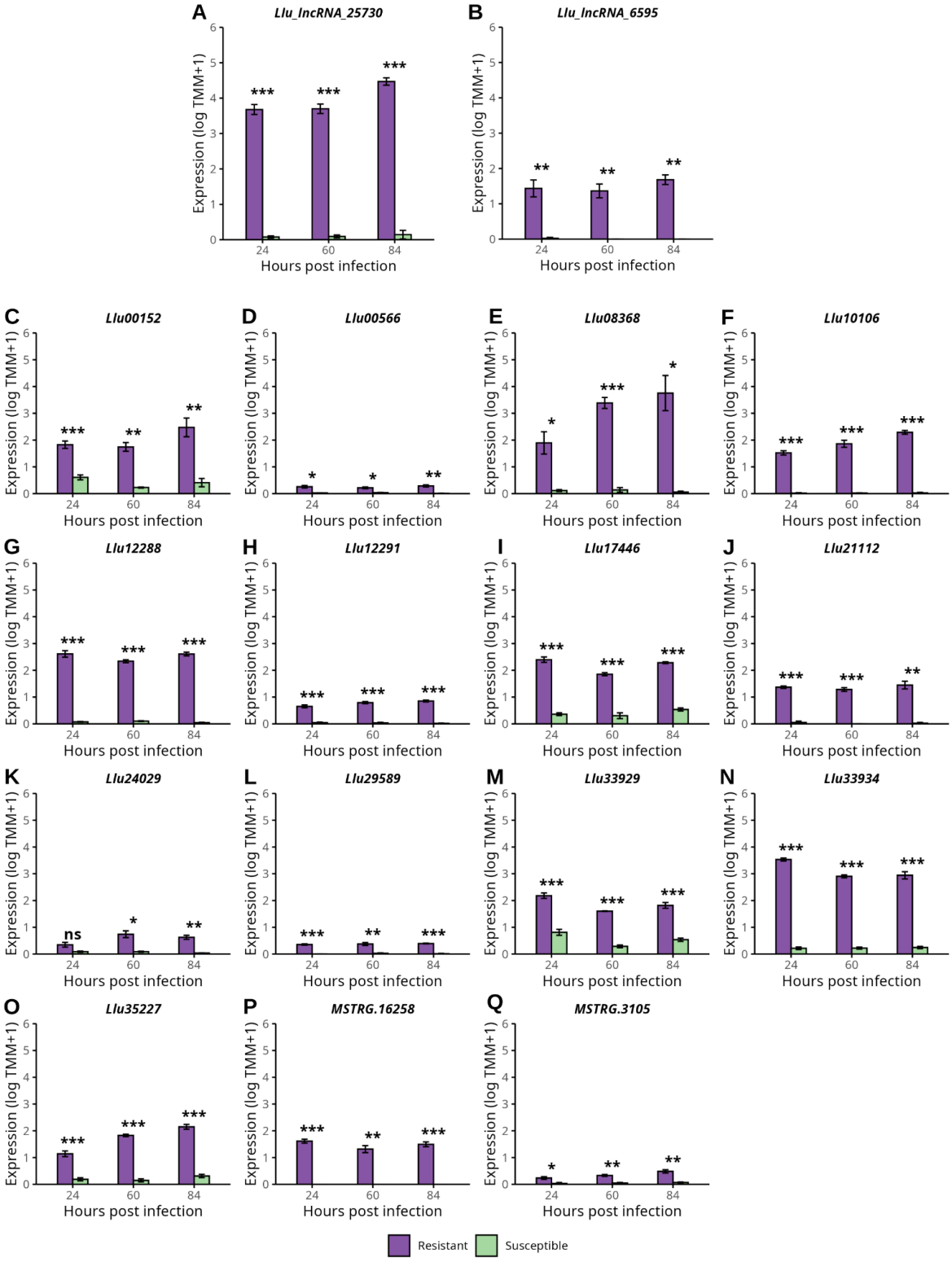

Figure 5

Gene expression of *Llu_lncRNA_25730*, *Llu_lncRNA_6595*, and their target genes related to immune response against *C. lupini* infection in both resistant and susceptible genotypes. Genes are listed in alphabetical order.

Supplementary Files

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