

Lignin, glutathione and kinases play important roles in *Paspalum regnellii* defense against spittlebug (*Mahanarva spectabilis*) attack

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Summary

- Spittlebugs cause large production losses that affect agribusiness worldwide. Understanding plant-herbivore interactions at molecular level may be the key to developing resistant cultivars.
- We assembled a *de novo* transcriptome for elucidate the roots response from two *Paspalum regnellii* genotypes (BGP 248 and 344) to spittlebug (*Mahanarva spectabilis*) nymph attack, integrating differential expression analysis and complex network modeling, supplemented by a resistance field experiment and root anatomical analysis.
- GO terms related to different stress responses were enriched in BGP 248, such as salicylic acid catabolic process, while some specific to spittlebugs like response to herbivores were enriched in BP 344. KEGG enriched pathways were related to structural differences between genotypes, for example, cutin, suberin and wax biosynthesis. BP 344 also presented pathways related to induced defense, such as glutathione metabolism. Metabolic networks highlighted kinases, and coexpression networks demonstrated a greater complexity in their response cascade, which includes lncRNAs.
- This study provides the first molecular insights into the defense mechanisms of *P. regnellii* against *M. spectabilis*. It was identified that the genotype with the highest nymph mortality (BGP 344) has constitutive barriers, such as lignin, which delay the attack. In addition to presenting a glutathione pathway enriched and greater presence of kinases.

Keywords: coexpression network, forage, herbivore, RNA-seq, root.

INTRODUCTION

Worldwide, livestock is responsible for ensuring the livelihood of approximately 1.3 billion people. In addition, pasture areas total more than half of the planet's land surface for cattle raising or animal feed cultivation (FAO). Brazil stands out as one of the largest producers, exporters and consumers of beef in the world. In 2022, approximately 194 million cattle were distributed in more than 160 million hectares and accounted for more than US\$ 180 billion in all businesses and movements, including inputs and animal nutrition, which basically consists of forage (Abiec Beef Report, 2022).

The large pasture areas started to cause environmental and market pressures, making the sector of tropical forage improvement spend on a refinement phase in recent years (Dias-Filho, 2014). The goal is to develop cultivars that increase quantity and quality to generate a significant impact on animal production (Simeão *et al.*, 2021) since the field usually consists of a few exotic species with clonal reproduction that causes a genetic vulnerability risk (Vigna *et al.*, 2016).

To cause a smaller biological imbalance, one option is to use a native genus with great genetic diversity and adaptability to different ecosystems of the country, such as *Paspalum* (Strapasson *et al.*, 2000). This genus originates from the tropical and subtropical regions of the Americas and is composed of species with high potential for pasture for livestock and forage production systems (Novo *et al.*, 2016; Acuña *et al.*, 2019). In South America, it is a native genetic resource for grasslands and a model for apomixis studies; however, since interest in the improvement of forage species is recent, some species remain undomesticated (Ortiz *et al.*, 2019).

Embrapa Pecuária Sudeste maintains a *Paspalum* germplasm bank that allowed breeding program development using *Paspalum regnellii* as a source of sexuality types in crosses (Matta *et al.*, 2022). In general, forage grasses have two usual cytotypes, diploid with sexual reproduction or polyploid with apomixis, a kind of procreation that generates an entire progeny of clones (Quarin, 1992). This can hamper genetic variability acquisition since it is necessary to cross a sexual parent with an apomictic parent of the same ploidy level (Gonçalves *et al.*, 2021), but fortunately, some natural tetraploid sexual accessions were found in the Virgata botanic group (Acuña *et al.*, 2019), such as those of *P. regnellii* species. Moreover, it is a perennial species with good biomass production, some resistance to shading and high forage potential (Bortolin *et al.*, 2019).

Recently, there has been interest in evaluating the resistance of *P. regnellii* to insect herbivory since some *Paspalum* species have proven to be more resistant than other forage genera to spittlebugs (Hemiptera: Cercopidae), the main agricultural pest of tropical pastures (Gusmão *et al.*, 2016). These insects cause global economic losses of more than US\$ 2 billion a year (Thompson, 2004) because of quantitative and qualitative damage that reduces the dry matter volume and decreases nutritional capacity (Congio *et al.*, 2012). In Brazil, the genus *Mahanarva* became highly reported as an agricultural pest of forage grasses and sugarcane in the 1990s (Congio *et al.*, 2020), with *Mahanarva spectabilis* (Distant, 1909) being responsible for limiting livestock production of beef and milk (Aua *et al.*, 2007).

The chemical control of insects, common in agriculture worldwide, is ecologically and economically unfeasible in the case of spittlebugs due to the large area of pasture. As a result, the best management involves the development of resistant forage cultivars (Silva *et al.*, 2019). This process traditionally involves crossing individuals to generate hybrids and fixing and establishing them in the field for testing, but it is time-consuming and does not guarantee the achievement of the desirable characteristic without other trait loss (Lenaerts *et al.*, 2019). In addition, herbivore resistance is a quantitative feature (Dinardo-Miranda *et al.*, 2014), a kind of trait that is difficult to completely fix in natural ways because it is controlled by many genes and affected by environmental pressures (Zhang *et al.*, 2020).

Currently, genomic information is essential in breeding programs to increase the gains and reduce the required time to release a new cultivar, but the application of these tools has been very limited until now (Pereira *et al.*, 2018). *P. regnellii*, for example, has only a few nucleotide sequences publicly available in the National Center for Biotechnology Information (NCBI) and no published genetic studies, so the usual techniques for locating biotic resistance, such as molecular marker-assisted selection and identifying quantitative trait loci (QTLs) for genetic maps (Dormatey *et al.*, 2020), can be laborious and produce results with information gaps (Wen *et al.*, 2019). Therefore, transcriptome analysis is an alternative that can provide the complete transcript expression profile in a specific response condition (Egan *et al.*, 2012), including new and rare transcripts even for species without prior genomic information (Wang *et al.*, 2009).

This analysis has been successfully applied in works that aimed to identify pest resistance genes in different plants, including species from the Poaceae family (Zhao *et al.*, 2017), and has generated significant differential expression results related to responses in infested plants (Becker *et al.*, 2017; Cui *et al.*, 2017; Wang *et al.*, 2018).

In the case of spittlebug infestation, the nymph form settles in the roots and feeds on the xylem, damaging the vessels and preventing full nutrient transport, while the adults attack the leaves, breaking the cellular wall and reducing the amount of chlorophyll until it leads to tissue death (Alvarenga *et al.*, 2019). Plant defense strategies to deal with herbivores generally consist of constitutive factors that are always present, such as morphological characteristics and chemically induced defenses (Walters, 2011). These secondary metabolites are important for recognizing and responding to environmental stressors, such as herbivory. They are not synthesized all the time due to high energy expenditure, but they start to be produced when the plant detects attack, allowing plant survival and preventing insects from developing (Singh *et al.*, 2021).

In addition to the lack of genomic studies in the species that are the subject of this study, no expression analysis of resistance to spittlebugs has been reported until now, representing an important information gap for such a complex process. In this context, this research aimed to characterize the roots of two *P. regnellii* genotypes, one susceptible and the other resistant to *M. spectabilis* spittlebug nymphs, in the scopes of nymph survival, root anatomy, transcriptomic analysis and coexpression and metabolic networks to elucidate the genetic and molecular mechanisms involved in plant defense.

MATERIALS AND METHODS

Resistance field experiment

Bioassays were conducted to determine the resistance level of *P. regnellii* accessions to *M. spectabilis* in a greenhouse at Embrapa Pecuária Sudeste, São Carlos, SP, Brazil. The effects on genotypes were evaluated using the nymphal survival parameter to test the antibiosis resistance hypothesis with methodology adapted from Lapointe *et al.* (1989), Valério *et al.* (1997) and Gusmão *et al.* (2016). Genotypes BGP-215, BGP-248, BGP-258, BGP-341, BGP-344, BGP-345 and BGP-397 were established by seeds from plants retained in the *Paspalum* Active Germplasm Bank at Embrapa Pecuária Sudeste and compared with the susceptible controls *Urochloa brizantha* (Hochst. ex A. Rich.) R. D. Webster cv. Marandu and *U. decumbens* (Stapf) R. D. Webster cv. Basilisk.

The seeds were sown in a 162-cell JKS® model tray with 50 cm³, filled with substrate based on sphagnum peat, expanded vermiculite, dolomitic limestone, agricultural plaster and NPK fertilizer (Carolina®). After 30 days, seedlings were transplanted to 480 ml Styrofoam cups (DART® 480J32) containing the substrate for Vivatto® Plus plants. Each one was

covered with J32 DART® Styrofoam cups containing a central hole for the plant aerial part to maintain the absence of light on the lap and allow superficial root growth to ensure local feed for nymphs hatched.

The experimental design was completely randomized, with ten replicates. Plants were infested with insect eggs 30 days after transplanting using five eggs obtained according to Auad *et al.* (2007). At the time of plant infestation, the eggs showed complete embryonic development, that is, the appearance of the operculum and reddish ocular and glandular spots (Valério, 2009). The nymphal survival evaluations occurred weekly, from plant infestation with eggs until the adult insects emerged.

Finally, the nymph survival percentage was calculated for each plant genotype and transformed into SQUARE ROOT ($X + 0.5$) and submitted to variance analysis, and the variable averages in the treatments were compared by Tukey's test ($p < 0.05$) based on the procedures of SAS, PROC-GLM (SAS Institute, 2010).

RNA-seq experimental design and plant material

The experiment was carried out in a greenhouse at Embrapa Pecuária Sudeste. Two sexual tetraploid genotypes (BGP 248 and BGP 344) of *P. regnellii* ($2n=4x=40$) were selected based on the contrast identified in previous results of spittlebug resistance evaluation in different species of the genus.

Seedlings of the two genotypes were obtained from tillers of the same adult plant already established to ensure genetic identity, and the plants were cultivated in Styrofoam cups for six weeks, so the root system was well developed. Adult insects of *M. spectabilis* were collected from pasture areas of *U. brizantha* cv. Marandu, and the eggs were obtained in the laboratory and incubated in the B.O.D chamber (T 25°C, RH 60% and photophase of 12 hours) until the complete development of the embryonic phase. Ten completely developed eggs were confined to the root system of the plant, starting from the opening made on the side of the styrofoam cup. After infestation, the system was closed with tape to guarantee nymph feeding. The same procedure was simulated in the control samples but without infestation with eggs.

Root samples were collected in three treatments, in triplicate, for each control and the two genotypes: without egg infestation (T0), after egg infestation and nymph eclosion (T1-48 h after T0) and with nymph colonization on the roots (T2-72 h after T0), totaling nine samples per genotype.

RNA isolation, library preparation, and sequencing

Total RNA (totRNA) from the roots was extracted with an RNeasy Plant Mini Kit (Qiagen Inc. Valencia, CA) according to the manufacturer's protocol. A NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to quantify the RNA. To assess the extraction efficiency and integrity, totRNA samples were evaluated using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, EUA).

The mRNA sequencing libraries were made for 18 samples and sequenced on Illumina HiSeq 2500 v4 with 100 bp paired-end reads.

Data quality and filtering

FastQC version 0.11.9 software (Babraham Bioinformatics) was used to assess the quality of the libraries sequenced. The reads were filtered with Trimmomatic version 0.39 (Bolger *et al.*, 2014) to remove the adapters with the Illumina Adapters parameter and low-quality reads with *phred* < 33 and SlidingWindow 4:20. In addition, the software SortMeRNA version 2.1b (Kopylova *et al.*, 2012) was used to eliminate the ribosomal RNA (rRNA) with default parameters.

Transcriptome assembly

Since *P. regnellii* does not have a publicly available reference genome, a *de novo* assembly was performed using Trinity version 2.5.1 (Grabherr *et al.*, 2012) with the target K-mer coverage set to 30 (normalize_max_read_cov 30) and other parameters in default. The longest isoform for each gene was selected with its own software.

An assembly using the genome sequence of diploid *Paspalum notatum* (available at NCBI Genome under accession ASM2253091v1) as a reference was also tested. STAR version 2.7.3a (Dobin *et al.*, 2013) was used to align the reads to the genome, and then StringTie version 2.1.6 (Kovaka *et al.*, 2019) was used to assemble the transcriptome.

Assembly quality was analyzed using the *Viridiplantae* dataset of BUSCO version 5.2.2 (Simão *et al.*, 2015), comparing the sequences of the transcripts with a set of orthologous genes conserved in plants, and Bowtie2 version 2.3.3.1 (Langmead & Salzberg, 2012), mapping the reads in the transcriptome to determine the transcript abundance with default parameters.

Differential gene expression analysis

Salmon software version 0.14.1 (Patro *et al.*, 2017) was used with default parameters to quantify the expression of transcripts. The differential gene expression (DGE) analysis was performed using the edgeR package version 3.38.4 (Robinson *et al.*, 2010) implemented in R (R Development Core Team, 2011). Raw count data were first normalized with at least 10 counts per million (CPM) in a minimum of three samples, and then we performed a differential gene expression analysis considering a false discovery rate (FDR) cutoff ≤ 0.05 and $\log_2\text{-fold change}$ (FC) ≥ 2 .

Treatment RNA-seq data samples were coded as resistant (R) or susceptible (S) followed by numbers 0 (control), 1 (48 h) and 2 (72 h). Comparisons for identifying the differentially expressed genes (DEGs) were made between each genotype at different time points, for example, the resistant in control treatment against resistant after 48 h of infestation (coded as R0 vs. R1), and between different genotypes at the same time points, such as resistant in control treatment against susceptible in control (coded as R0 vs. S0). A total of nine comparisons were performed, following the same codification cited before: R0 vs. R1, R0 vs. R2, R1 vs. R2, S0 vs. S1, S0 vs. S2, S1 vs. S2, R0 vs. S0, R1 vs. S1 and R2 vs. S2.

Functional annotation and enrichment

Trinotate software version 3.2.1 (Bryant *et al.*, 2017) was used to perform the functional annotation. Transcripts were annotated by searching for BLASTx homology against SwissProt databases (UniProt - <https://www.uniprot.org/>) with at least 60% similarity and Transdecoder coding regions predicted in protein databases by BLASTp. Moreover, protein domains were found using “hmmer” software, and finally, all results were integrated into the “sqlite” database to extract reports.

Gene Ontology (GO) (Ashburner *et al.*, 2000) terms were retrieved from the sequence that was functionally annotated in the database. We restricted the results for plants (*Arabidopsis thaliana*) and enriched the DEGs with the topGO R package (Alexa & Rahnenfuhrer, 2022) for biological processes using Fisher’s exact test (FDR < 0.01). The enriched terms were submitted to REVIGO (Supek *et al.*, 2011) with medium similarity allowed (0.7) for data summarization.

Additionally, the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 2000) database was used to map all DEGs with the *Panicum hallii* reference. Fisher’s test was performed to obtain enriched metabolic pathways.

Gene coexpression and metabolic network analysis

In our study, we made two distinct network models to infer biological relationships: (i) the construction of two gene coexpression networks based on RNA-seq data obtained from each genotype and (ii) the establishment of a metabolic network incorporating pathways associated with the DEGs.

For the creation of gene coexpression networks (i), we employed the highest reciprocal rank (HRR) methodology proposed by Mutwill *et al.* (2010). Initially, we computed pairwise correlations between genes using the R Pearson correlation coefficient. To ensure robust associations, we set a minimum absolute correlation coefficient threshold of 0.8 and considered a maximum of 30 strongest edges for the network connections. The network construction process was performed using R statistical software (R Development Core Team, 2011) and the igraph library version 1.3.5 (Csardi & Nepusz, 2006). Subsequently, we calculated the hub score for each gene using Kleinberg's hub centrality algorithm (Kleinberg, 1999).

For the metabolic network, we identified enzyme commission (EC) numbers associated with the annotations of the DEGs. Leveraging this information, we used the KEGG database (Kanehisa & Goto, 2000) to select the metabolic pathways in which these enzymes are involved. Subsequently, a unified metabolic network was constructed using BioPython version 1.78 (Cock *et al.*, 2009), and centrality measures were evaluated using Cytoscape version 3.91.1 (Shannon *et al.*, 2003).

Quantitative reverse transcription PCR (RT-qPCR)

The DEGs were validated by RT-qPCR. Ten target genes were selected from the DEG list by the fold-change value. In addition, five housekeeping genes were chosen based on the literature (Gimeno *et al.*, 2014; Andrade *et al.*, 2017; Liu *et al.*, 2017; Takamori *et al.*, 2017). Primer pairs (Supplementary Table 1) were designed using Primer3Input (Untergasser *et al.*, 2012) with usual parameters: size between 18 and 22 bases, amplification product size between 75 and 175 bp, melting temperature (T_m) between 57 and 59°C and GC content of approximately 50%. Dimers, heterodimers and hairpin were checked in NetPrimer (Premier Biosoft).

cDNA synthesis was performed with the GoScript™ Reverse Transcription System A5000 (Promega, Madison, WI, USA) according to the manufacturer's protocol. RT-qPCR

was performed on QuantStudio 6 Pro (Applied Biosystems, Waltham, MA, EUA) using GoTaq® qPCR Master MixA6001 (Promega, Madison, WI, USA) with the following protocol: 95°C for 120 s, followed by 40 cycles of 95°C for 15 s and 60 s at 60°C. Melting curves were obtained by heating from 60 to 95°C to verify the products after amplification. All qPCR experiments were performed using two technical and three biological replicates and analyzed with Design and Analysis Software 2.6.0 (Thermo Fisher Scientific Inc., Waltham, MA, EUA).

Anatomical analysis of the roots

As in the RNA-seq experiment, the seedlings were obtained from tillers of the same plant and cultivated for six weeks for the root system to develop. Roots of both genotypes, without spittlebug infestation, were fixed in BNF (phosphate buffer solution, formalin; 9:1 v/v) for 48 hours (Lillie, 1965) and stored in 70% ethanol. The material was dehydrated in an ethanol series including hydroxyethyl-methacrylate (Historesin® Leica) following the manufacturer's recommendations. Cross-sections 5 µm thick were made with the aid of a Microm HM 340E rotary microtome (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

The slides were stained with 0.05% toluidine blue in citrate buffer (pH = 4.5) (O'Brien *et al.*, 1964) and finally mounted on Entellan® synthetic resin (Merck KGaA, Darmstadt, Germany). Images were captured with a digital camera (Olympus DP71) coupled to an Olympus BX51 optical microscope (Olympus Optical Co., LTD, Japan). To detect the main chemical compounds present in the cell walls of root cells, we used the following histochemical tests: acidified phloroglucinol for lignin detection (Johansen, 1940) and Nile red fluorochrome for lipid substances under a DAPI-LP filter (ex377/50 nm; em447/60 nm) (Greenspan *et al.*, 1985).

RESULTS

1. Spittlebug resistance experiments

The nymph survival average, percentage and standard error were calculated from the seventh day of plant infestation with *M. spectabilis* eggs until the emergence of the last adult insects at 56 days after infestation (Fig. 1A). The percentages in the controls, *U. brizantha* and *U. decumbens*, were higher than those verified in *P. regnellii* genotypes. Between *P.*

regnellii accessions, there were significant differences in nymph survival until 21 days after infestation, with mortality being more intensive in BGP 344 (Fig. 1A).

Significant differences were observed from the average nymph survival percentage for each different treatment between the *P. regnellii* genotypes, highlighting nymph mortality between BGP 344 and BGP 248, mainly young nymphs of the 1st and 2nd instars until the 21st day after infestation (Fig. 1B).

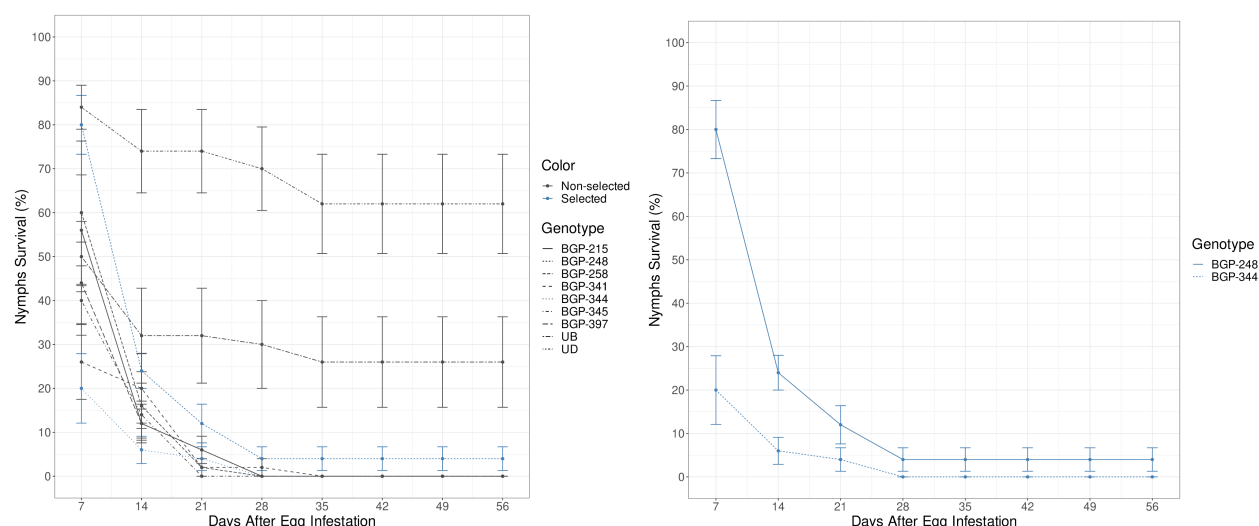


Figure 1. Survival percentage (average $\pm$ standard error) of *M. spectabilis* nymphs **(A)** in seven *Paspalum regnellii* genotypes in comparison with susceptible controls *Urochloa brizantha* and *U. decumbens*. **(B)** The two genotypes (BGP-248 and BGP-344) selected for transcriptome analysis based on their contrasting behavior to *M. spectabilis* nymph survival.

2. Transcriptome assembly and annotation

The sequencing from eighteen samples generated a total of 806,942,117 paired-end reads from the *P. regnellii* root transcriptome. Sequence quality analysis performed with FastQC software revealed that all 18 samples presented a similar quality profile. With the filtering procedures, ~19.97% of the reads were discarded as low-quality sequences (remaining 645,827,820 reads) and ~4.95% as ribosomal RNA residues (remaining 613,888,166 reads).

Both assemblies, *de novo* and with the *P. notatum* genome reference, were performed, and their statistics were compared (Table 1). The second method generated good transcript results, but in the initial stage of aligning the reads with the reference, we obtained only 55.96% success, losing a large amount of data. In addition, it was possible to observe with

BUSCO that the assembly generated many duplicate transcripts (44.9%), probably because of the different species ploidy levels.

Table 1. Comparison between the *de novo* assembly (Trinity) and the genome reference (StringTie) followed by their quality statistics.

Software	Alignment (STAR)	Transcripts	N50	Mean size (pb)	BUSCO	Bowtie2
Trinity	-	575,219	788	568.39	Complete: 90.4% Duplicate: 8%	80.89%
StringTie	55.96%	73,887	2003	1335.87	Complete: 95.2% Duplicate: 44.9%	65.51%

Despite the large transcript number and lower values of N50 and mean size, transcriptome *de novo* assembly in polyploid plants is naturally complex because of the homeologous presence that creates many transcripts with structural abnormalities (Payá-Milans *et al.*, 2018). Trinity software lost less data and generated successful results in other *Paspalum* species (de Oliveira *et al.*, 2020), so this assembly was selected for the following analyses.

Almost 16% (92,035) of the transcripts were annotated against the SwissProt database, and 7.3% (42,040) of the proteins were predicted with Transdecoder.

3. Differential Expression Analysis and Enrichment

After CPM normalization and expression filtering of the raw count data, we obtained a total of 21,508 genes. As there is a biotic stressor that was inserted in the samples, to confirm the similarity between the replicates, a Principal component analysis (PCA) (Fig. 2A) was performed, and a hierarchical cluster (Fig. 2B) from the weighted pair group method with arithmetic mean (WPGMA) was organized. In both, it was possible to observe a clear genotype separation in addition to a proximity between the samples of the control treatment and 48 h after plant infestation, while the last treatment was more distant.

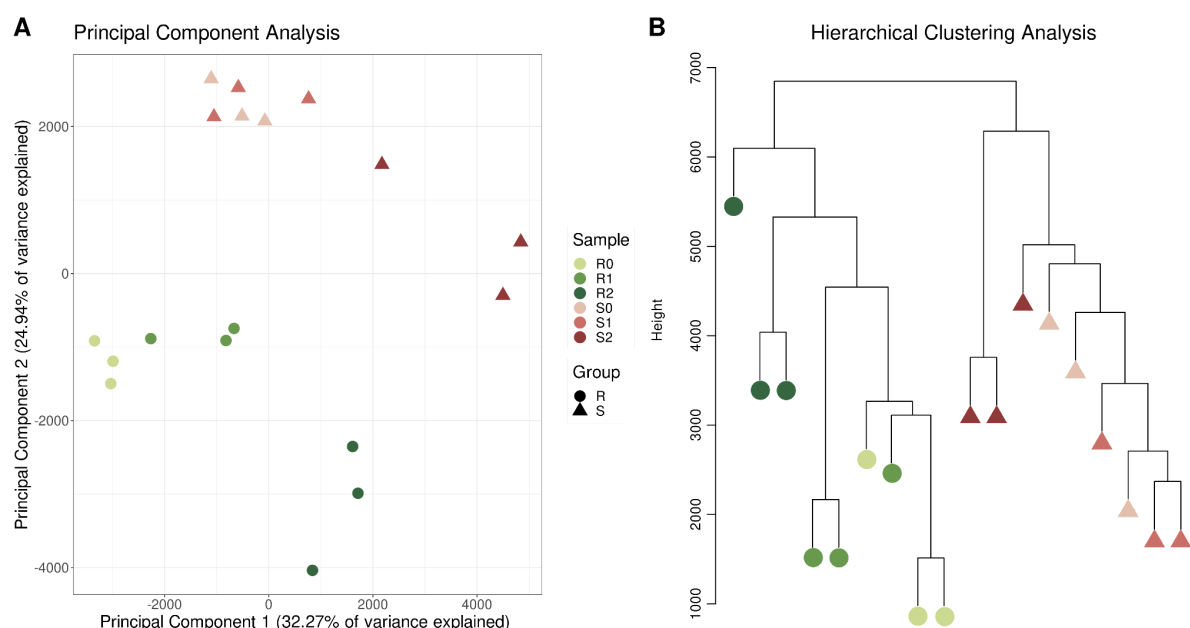


Figure 2. Sample distribution by (A) principal component analysis. (B) Hierarchical clustering analysis performed with Euclidean distance and WPGMA. Abbreviations: R - BGP 344 and S - BGP 248.

The genes were submitted to differential expression analysis (Supplementary Table 2), and contrasts between genotypes at the same treatment time obtained many DEGs even in the control, showing a considerable basal difference (Table 2). In the treatment comparison, neither genotype showed DEGs between the control and after 48 h, but many genes changed their expression after 72 h (T2), mostly in the BGP 344 genotype.

Table 2. Number of total, up- and downregulated DEGs obtained in each treatment comparison.

DEGs	R0 vs. R1	R0 vs. R2	R1 vs. R2	S0 vs. S1	S0 vs. S2	S1 vs. S2	R0 vs. S0	R1 vs. S1	R2 vs. S2
Total	0	408	30	0	65	41	865	783	1039
Up-regulated	0	1	0	0	14	13	533	469	500
Down-regulated	0	407	30	0	51	28	332	314	539

Venn diagrams were created to analyze transcripts that were considered DEGs in more than one comparison (Fig. 3). The contrasts in the same genotype showed that only some genes participated in more than one treatment, and most were only after 72 hours (Fig. 3A-B). On the other hand, in genotype contrasts, 616 genes were shared between all three treatments, demonstrating the existence of an important natural difference in expression among plants even with no infestation (Fig. 3C).

Aiming to exclusively select genes related to resistance, an analysis of the intersection between the genotypes was performed in R software. Some transcripts had increased expression over time in both genotypes, but even in the control, they were still more highly expressed in BGP 344 (Table 3), and their annotations are possibly related to resistance: suppressor cell death (LSD1), resistance gene analogs (RGA5R) and one domain-containing protein (DUF4220). Other transcripts expand their expression in BGP 344 and are naturally more abundant in BGP 248: transcription factors that regulate secondary cell wall (NST1) and response inducers to ozone and pathogens (AtOZI1). Last, those that increased the expression in the BGP 248 genotype were mostly related to abiotic stress (PLA7).

Table 3. Main hub genes and their relation to resistance in both genotypes.

Gene name	Annotation	Genotype with more expression	After BGP 344 treatments	After BGP 248 treatments
TRINITY_DN200773_c0_g1	LSD1		-	
TRINITY_DN221780_c5_g1	DUF4220	BGP 344	Upregulated	Upregulated
TRINITY_DN219172_c0_g1	RGA5R		-	
TRINITY_DN212267_c2_g2	PLA7			
TRINITY_DN189406_c0_g1	PLA7	BGP 248	-	Upregulated
TRINITY_DN190702_c3_g1	NST1			
TRINITY_DN205618_c0_g1	AtOZI1	BGP 248	Upregulated	-

a) GOs

The functional classes of all DEGs were enriched in 89 different GO terms of biological processes in topGO (Supplementary Table 3). The BGP 344 genotype after spittlebug infestation showed terms closely related to the resistance process (Fig. 3A), such as

the chitin catabolic process (GO:0006032), response to oxidative stress (GO:0006979), response to other organisms (GO:0051707) and response to herbivores (GO:0080027).

In contrast, in BGP 248, there were some terms related to resistance (Fig. 3B), including salicylic acid catabolic process (GO:0046244) and regulation of anthocyanin biosynthetic process (GO:0031540), but they were not very specific. Furthermore, some terms revealed that the plant ignores infestation and continues its normal growth, such as seed maturation (GO:0010431) and positive regulation of lateral root development (GO:1901333).

When we compared the genotypes in the last infestation treatment (Fig. 3C), we obtained some interesting terms that had different expression levels between them, such as defense response (GO:0006952), positive regulation of circadian rhythm (GO:0042753), retrotransposition (GO:0032197) and protein phosphorylation (GO:0006468).

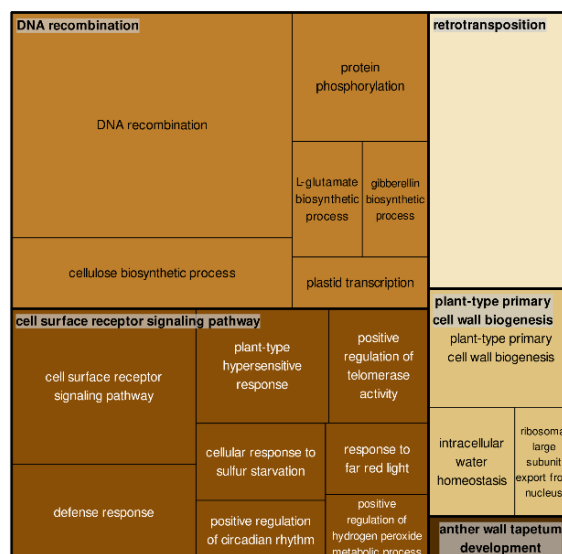
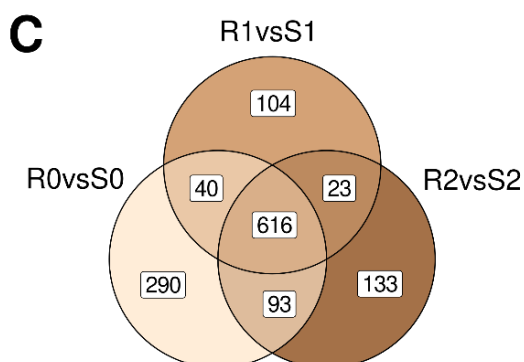
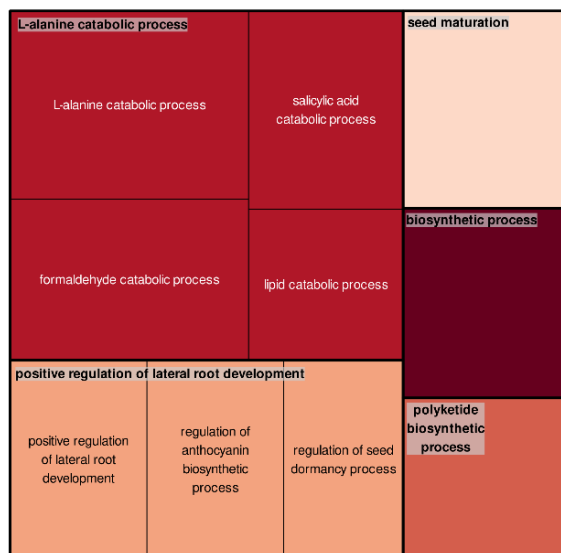
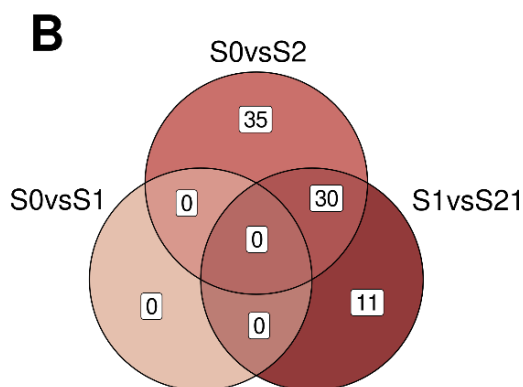
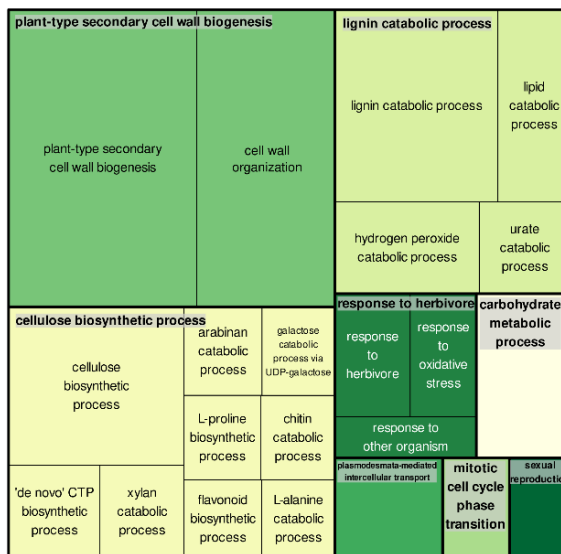


Figure 3. Venn Diagram of DEGs among all treatment treatments for BGP 344 (A) and BGP 248 (B) and summarized GOs TreeMap (A) for BGP 344 between control and second treatment. (B) BGP 248 between the control and second treatment. (C) Venn Diagram between BGP 344 and BGP 248 genotypes and GOs TreeMap for R2 vs. S2 after 72 h of infestation.

b) KEGG

Furthermore, the DEGs were enriched in 13 metabolic pathways by the KEGG database (Table 3). Of these, some were related to basal cellular mechanisms and enriched in the comparisons of both genotypes: glycolysis/gluconeogenesis, fatty acid degradation, tyrosine metabolism, alpha-linolenic acid metabolism and pyruvate metabolism (Table 3). Enrichment of the DNA replication pathway, as it is a more structural part, was not selected for further study.

The other seven pathways that occurred in just one treatment comparison and are possibly related to resistance were chosen to make a unique metabolism network: purine metabolism, caffeine metabolism, glutathione metabolism, galactose metabolism, cutin, suberin and wax biosynthesis, and circadian rhythm - plant, starch and sucrose metabolism.

Table 3. KEGG enriched pathways arranged in decreasing *p* value order and their respective related genotype contrast comparison.

ID	Pathway	Contrast	<i>p</i> value
map00500	Starch and sucrose metabolism	R0 vs. R2	0.044433
map00480	Glutathione metabolism	R1 vs. S1	0.041352
map00071	Fatty acid degradation	S0 vs. S2	0.038649
		R1 vs. R2	0.025122
map00620	Pyruvate metabolism	S0 vs. S2	0.038649
		R1 vs. R2	0.025122
map04712	Circadian rhythm - plant	R0 vs. S0	0.027134
map00592	alpha-Linolenic acid metabolism	S0 vs. S2	0.026678
		R1 vs. R2	0.017209

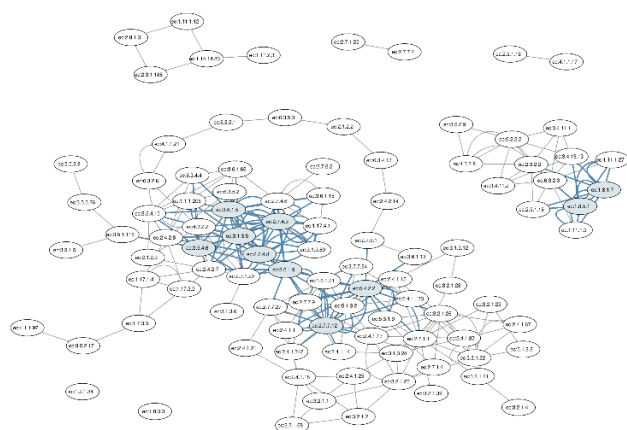
map00073	Cutin, suberin and wax biosynthesis	R0 vs. S0	0.026091
		S1 vs. S2	0.023988
map00010	Glycolysis/Gluconeogenesis	S0 vs. S2	0.018660
		R1 vs. R2	0.009861
map00230	Purine metabolism	R1 vs. R2	0.017209
		S0 vs. S2	0.016575
map00350	Tyrosine metabolism	R1 vs. R2	0.010610
map00232	Caffeine metabolism	R1 vs. R2	0.010610
map03030	DNA replication	R0 vs. S0	0.010392
map00052	Galactose metabolism	R0 vs. R2	0.000021

4. Network Analysis

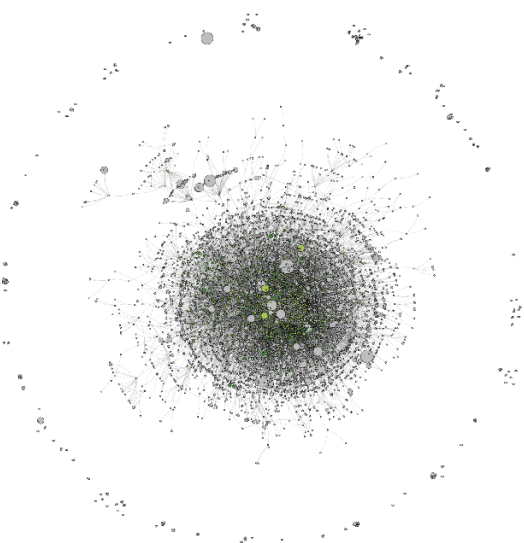
A metabolic pathway was prepared from the KEGG enriched pathways and resulted in 109 enzymes related to the resistance process (Supplementary Table 4). The enzymes with greater influence on the network (“EdgeCount”) and in relationship with other enzymes (In and Outdegree) are highlighted in blue in Figure 4A and listed here: adenylate kinase (ec:2.7.4.3), glutathione reductase (ec:1.8.1.7), guanylate kinase (ec:2.7.4.8), AMP deaminase (ec:3.5.4.6), apyrase (ec:3.6.1.5), nucleotide diphosphatase (ec:3.6.1.9), AMP phosphatase (ec:3.1.3.5) phosphoglucomutase (ec:5.4.2.2), glutathione dehydrogenase (ec:1.8.5.1) and uridyl transferase (ec:2.7.7.12).

Gene coexpression networks were constructed for each genotype separately. The BGP 344 network (Fig. 4B) had 7,410 nodes (Supplementary Table 5), so we observed many more hubs and connections in its network compared to that of BGP 248 (Fig. 4C), which had 5,275 nodes (Supplementary Table 6). This suggests a large difference in transcript number expression between genotypes as well as in DEG analyses, showing the complexity of the genetic response. Furthermore, some of the transcripts with the greatest impact on the networks because of the higher “Degree” number were not characterized or not annotated.

A



B



C

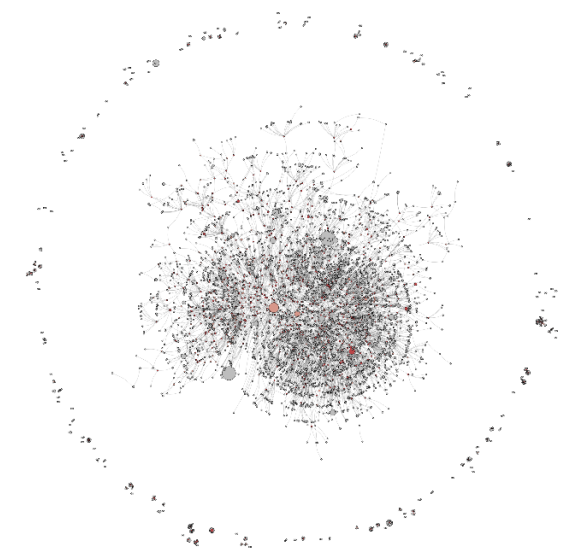


Figure 4. Network representations. **(A)** Metabolic network, with the most significant enzymes and their connections in blue. **(B)** BGP-344 coexpression network. The green nodes represent hub genes. **(C)** BGP-248 coexpression network. The pink nodes represent hub genes.

5. RT-qPCR validation

A total of ten transcripts were selected to validate the differential gene expression, and in two of them, flavonol 3-sulfotransferase (F3) and IQ domain-containing protein (IQ), primers did not score properly, so they were discarded. Validation was performed with two unknown transcripts (D1 and D2) and six annotated genes, phospholipase D alpha 2 (PL), enhancer of mRNA-decapping protein 4 (VC), acyclic sesquiterpene synthase (AC), lipoxygenase 2 (LO), protein reveille 2 (RE) and protein iron-related transcription (IR).

In addition, two of five normalizers were selected as endogenous controls in the experiment based on lowest quantification cycle (Cq) variation values using five methods collected by RefFinder (Xie *et al.*, 2012): NADH dehydrogenase ubiquinone 1 alpha (NDAU1) and splicing factor U2af small subunit B (U2AFB) (data not shown). The expression patterns generated by RT-qPCR analysis agree with the transcriptome data, proving the accuracy of the bioinformatic results (Figure 5A), in addition to the data showing a good correlation (Figure 5B).

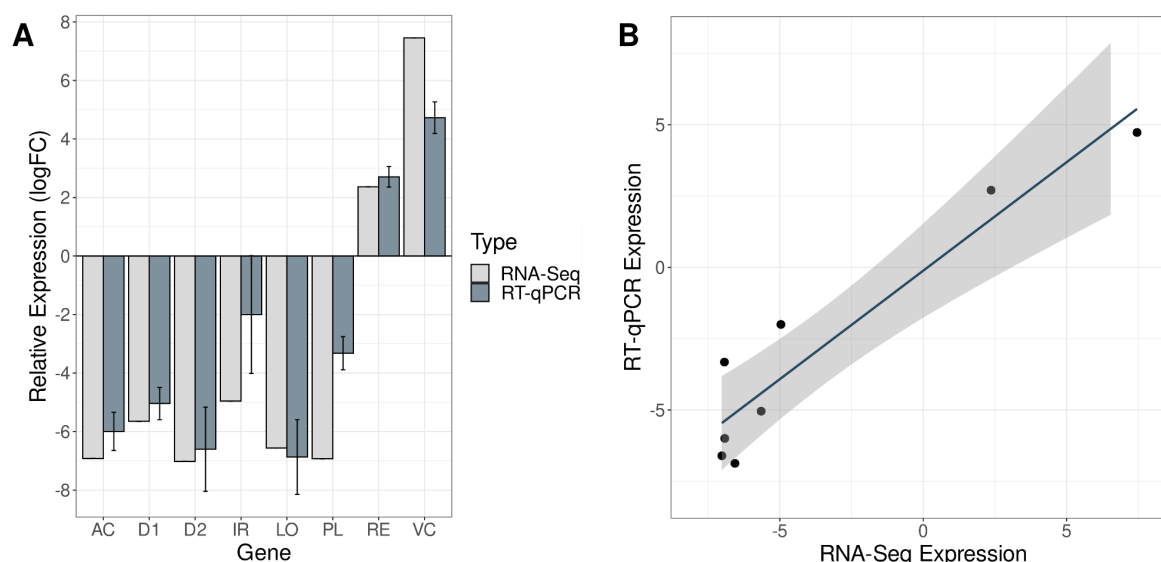


Figure 5. RT-qPCR transcriptome validation results. **(A)** Relative expression (log2-fold change) of RT-qPCR and transcriptome data represented by the mean expression value $\pm$ SEM (n = 3) relative to BGP 344 without infestation (R0). **(B)** Correlation between RNA-seq

and RT-qPCR analysis. Abbreviations - AC: acyclic sesquiterpene synthase; D1: unknown transcript 1; D2: unknown transcript 2; IR: protein iron-related transcription; LO: lipoxygenase 2; PL: phospholipase D alpha 2; RE: protein reveille 2; VC: enhancer of mRNA-decapping protein 4.

6. Anatomical analysis of the roots

We performed a comparative anatomical analysis between roots of the two *P. regnellii* genotypes without biotic stress to investigate whether transcriptomic variations are evidenced in the fine root structure, where spittlebugs prefer to feed.

Both genotypes present a uniseriate epidermis, with a slightly thickened external periclinal cell wall and the presence of secretion (Fig. 6A-B). Regarding the cortex, we observed the presence of an exodermis in both genotypes, characterized by a layer of cells with differences in the cell wall composition (Fig. 6A-B). In BGP 248, the exodermis has a thickened cell wall consisting of lipid material (Fig. 6C-D), and in BGP 344, the cells show lignified secondary cell wall deposition, as confirmed by the acidified phloroglucinol test (Fig. 6E). Additionally, we observed that the exodermis cells of this genotype collapse and acquire irregular shapes, a process that begins close to the root apex (Fig. 6B).

The cortical parenchyma in both genotypes has wide intercellular spaces and forms an aerenchyma, which is more developed in BGP 344, contributing to the formation of irregular cells of the exodermis (Fig. 6A-B). In the BGP 344 genotype, the internal cellular layers of the cortical parenchyma present globular cells arranged in a spiral pattern around the endodermis (Fig. 6B). The endodermis has Casparian strips, and the cells form “U”-shaped lignified wall thickenings (Fig. 6A-B). The pericycle is uniseriate, and the vascular system is composed of few protoxylem poles alternating with phloem strands and few central metaxylem elements (Fig. 6A-B).

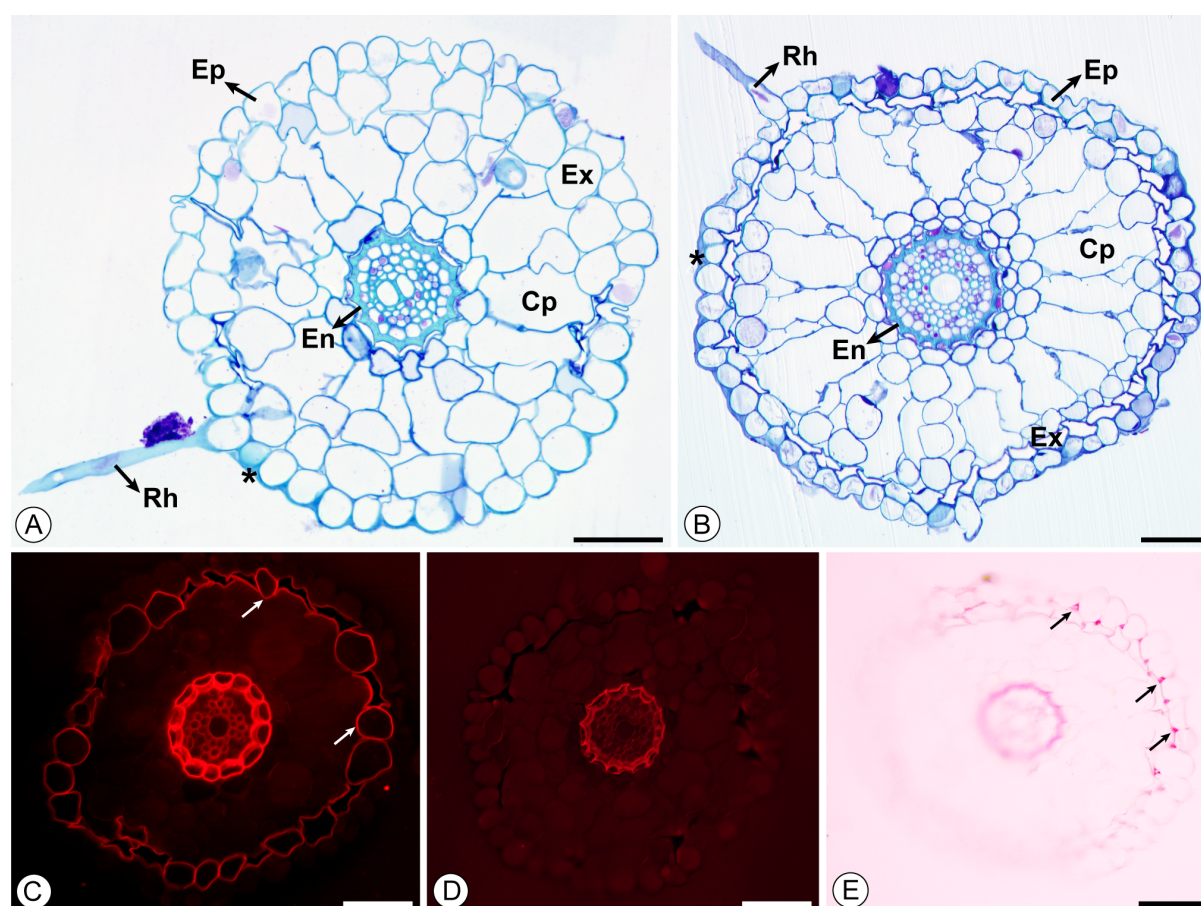


Figure 6. Anatomical structure and histochemical tests in root cross-sections of different *Paspalum regnellii* genotypes. (A) BGP 248 and (B) BGP 344, with asterisks indicating secretion covering the epidermis. (C) Positive reaction to Nile red fluorochrome, with arrows showing wall thickenings with lipid composition in BGP 248. (D) Negative reaction to Nile red fluorochrome on the cell walls of BGP 344 cells. (E) Positive reaction to acidified phloroglucinol showing lignin deposition in the cell walls of BGP 344. Abbreviations - En: endoderm; Ep: epiderm; Ex: exoderm; Rh: root hairs; Cp: cortical parenchyma. Scale bars: 50 μm (A-E).

DISCUSSION

Desirable characteristics for a superior forage plant consist of, in addition to resistance to biotic and abiotic stresses, high nutritional value and biomass production, and good palatability, regrowth ability and adaptation to infertile soils (Matias *et al.*, 2018). However, complex genetic architectures are obtained by combining these traits because of the correlation from close linkages between genes or when they are responsible for multiple

characteristics. Therefore, the main focus of forage breeding is still biomass production, which is the trait that is present in practically all cultivar studies (Casler & Santen, 2010), and for that reason, the largest pasture area in South America is composed of *Urochloa* species with African origin, primarily *U. decumbens* and *U. brizantha* (Matias *et al.*, 2021). Despite their productivity qualities, these forage grasses do not survive well under biotic stress and are more susceptible to many spittlebug species than other grass species, such as those from the genus *Paspalum* (Gusmão *et al.*, 2016).

Gaining resistance through interspecific crossings may not be so simple and may lead to the loss of other good traits. In addition, current biotechnologies such as gene editing enable specific gene transference into plant varieties and accelerate the breeding cycle for improving genetic transformation in grass species (Ramstein *et al.*, 2019). An interesting approach is the transference of resistance genes (Jacob *et al.*, 2018), such as identifying targets related to herbivore resistance present in *Paspalum* and inserting them in cultivars of interest that do not have natural resistance to spittlebugs, such as the highly productive *Urochloa* grasses.

Despite the importance of some species of the genus *Paspalum* as forage, turf and grain (Acuña *et al.*, 2019), knowledge of their molecular genetics is still lacking, with low genomic resources available. Therefore, access to the transcriptome can be very useful in the beginning, serving as a pseudoreference for further studies (Cardoso-Silva *et al.*, 2014). The RNA-seq approach allows us to understand the expression profile and complex trait interaction mechanisms between plants and their pathogens at the molecular level, revealing transcription features and RNA editing possibilities by identifying genes involved in resistance (Pan *et al.*, 2019; Saidi & Hajibarat, 2020; Campos *et al.*, 2021).

In this study, the *de novo* transcriptome assembly represents the first large-scale molecular resource for *P. regnellii* and generates reliable novel information on gene expression, confirmed through RT-qPCR data validation. Furthermore, the low alignment with another species of the same genus (*P. notatum*) suggests genomic variation even within the genus itself, showing a large difference in genome composition, which was also confirmed by the low number of annotations. This was expected because of the great complexity and diversity present in *Paspalum* that resulted in a separation of the two species into informal morphological groups primarily by Chase (1929), a phylogenetic segregation in distinct clades (Delfini *et al.*, 2023) and the different genomic composition by comparing a diploid

genome from the Notata group (NN) and an allotetraploid from the Virgata group (IIJJ) (Cidade *et al.*, 2013).

Tropical grasses tend, similar to plants in general, to have well-established defense mechanisms against insect pests and pathogens, highlighting secondary metabolites, but first, the physical barriers delay infestation with, for example, cell wall adaptations (Zhang *et al.*, 2020). Among the cell wall components that can cause mechanical difficulties in infestation is lignin, which reduces the palatability of plants for herbivores (Xiao *et al.*, 2023). It increases cellular rigidity, and its higher concentration has already been described as a resistance factor (Lynch *et al.*, 2021; Wu *et al.*, 2022), avoiding colonization of the plant by insects, which characterizes the antixenosis resistance mechanism (Kogan and Ortman, 1978). In our root histochemical analyses, it was possible to observe that lignification occurs only in the walls of the resistant genotype, confirming that there is already a natural anatomical predisposition that makes it difficult to choose these plants as hosts. Moreover, in this same genotype, we can observe in the histological images a differentiation of the sclerenchymatous tissue into aerenchyma that can contribute to the resistance process, since it has already been reported as responsible for improving the gas displacement in the cellular environment when there is an oxygen shortage, helping to avoid hypoxia (Yamauchi & Nakazono, 2022; Teixeira *et al.*, 2022).

Secondary metabolites reduce insect survival and development, preventing the insect from feeding and characterizing resistance by antibiosis (Valério *et al.*, 2001). However, it is necessary that the plant has a certain tolerance to damage since spittlebug resistance is a quantitative trait, with more than one gene controlling it (Dinardo-Miranda *et al.*, 2014). For this reason, through DEGs analysis, we observed that in the treatment after 48 h of infestation, the anatomical tolerance and the time for nymphal adaptation to then infest the tissue were influential, since they can live without feeding for some time. In the roots collected at the second treatment, after 72 h, a greater amount of nymphal foam was observed, which guaranteed that the insect was feeding.

With the beginning of insect feeding on plant tissues, changes first occur in the cell membrane conformation and then begin the production of reactive oxygen species (ROS) (Walters, 2011). The second defense line then begins, with secondary metabolites and herbivore-induced plant volatiles (HIPVs), which stimulate defense in neighboring plants and bring predators, both controlled by phytohormones with emphasis on jasmonic acid (Lin *et al.*, 2022). This is consistent with the GO enrichment results obtained in BGP 344 since we

found cell configuration terms, such as cell wall organization and plant-type secondary cell wall biogenesis, together with some terms with a regenerative character, such as cellulose biosynthetic process and lignin catabolic process, both related to the root anatomic findings. ROS-related terms were also identified as hydrogen peroxide catabolic process, response to oxidative stress and response to herbivores.

An interesting GO found in BGP 248 is the salicylic acid catabolic process. It is usually linked to the stress response, but in this case, it alone does not seem to play a large role in spittlebug defense due to compounds in *M. spectabilis* saliva that may impede its signaling, although in some species (such as *U. decumbens*), it has been identified after infection (Barros *et al.*, 2021). In addition, it is common for plants to focus their energy expenditure on resisting herbivores, increasing the production of resistance mechanisms to the detriment of their development (Divekar *et al.*, 2022); this does not occur in the BGP 248 genotype, which continues to produce root growth and seed ripening, for example. With this, it is possible to understand that forages susceptible to this pest have a defense mechanism, but it is not very effective.

This difference in response level becomes more evident with the pathway analysis. The metabolism of starch and sucrose, purine and caffeine were more active over time only in BGP 344 and, for that reason, are possibly related to the plant response to herbivores. Starch is an energy reserve that releases it in the form of sucrose (Du *et al.*, 2020). Under environmental stress, starch provides plant resources to recover and alleviates the problems generated by carbon consumption (Qiu *et al.*, 2021; Zhang *et al.*, 2021), consequently being mainly related to the response and tolerance to abiotic factors (Thalmann & Santelia, 2017; Chen *et al.*, 2023). In addition, purine metabolism generates secondary metabolites called purine alkaloids, and caffeine is one of them (Ashihara *et al.*, 2008). This compound plays a role in biotic stress plant defense, inducing the production of hormones such as salicylic and abscisic acid (Wang *et al.*, 2016). Thus, the increased activity of these three metabolisms in the resistant genotype when infected by spittlebugs suggests involvement in the plant's defense response, generating energy and releasing secondary metabolites.

The pathways enriched in the contrasts between the genotypes in the control sample (circadian rhythm - plant and cutin, suberin and wax biosynthesis) are related to morphological characteristics intrinsic to each genotype that confer plant tolerance. The circadian rhythm regulates plant physiological processes according to environmental differences, providing survival advantages (Xu *et al.*, 2022). By regulating carbon metabolism

and hormone signaling pathways, it is closely linked to stress responses for stabilizing plant development (Bhattacharya *et al.*, 2017), including metabolite liberation against herbivore attack (Hua, 2013); that is, the plant seems to be naturally more prepared to deal with spittlebugs. In the second, cutin, suberin and wax constitute the cuticle structure, which is responsible for stiffening the cell wall and forming the first layer of plant immunity (Mahatma *et al.*, 2021). Studies suggest that cutin is involved in plant defense (Fich *et al.*, 2016) and that suberin plays an important role as a plant root cell barrier against adverse environmental situations (Xiao *et al.*, 2020) and pathogens (Guo *et al.*, 2022). In treatments with infestation, an elicited response commanded by the glutathione pathway is enriched. It is one of the major contributors to antioxidant defense, controlling ROS under biotic and abiotic stress conditions (Gong *et al.*, 2018; Huang *et al.*, 2019). That is, in the BGP 344 genotype, in addition to structural differences such as wall constitution and a greater release of compounds from the purine and caffeine pathways, there is an induction of the glutathione pathway after infestation, unlike in BGP 248, in which it is not present.

Herbivore resistance, in addition to secondary metabolites and phytohormones, generally includes important proteins such as kinases and transcription factors (Wang *et al.*, 2020). To find this specific information, network analyzes can bring pontual inferences. Metabolic networks highlight two enzymes from the ascorbate-glutathione cycle, dehydroascorbate reductase (DHAR) and glutathione reductase (GR), that handle oxidative stress conferring plant tolerance but also regenerate AsA and GSH to maintain the redox state (Hasanuzzaman *et al.*, 2019; Dorion *et al.*, 2021). Furthermore, some important enzymes from the kinase family responsible for protein phosphorylation are constantly associated with stress tolerance (Patil & Senthil-Kumar, 2020). Some of these enzymes were identified in our data and can be good resistance targets: adenylate kinase and guanylate kinase. Both enzymes participate in the cellular homeostasis process, converting adenine and guanine nucleotides (Dzeja & Terzic, 2009; Sekulic *et al.*, 2002). The first is reported in maize with a regulatory role against salt stress, maintaining normal growth (Chen *et al.*, 2022), and in forage grass (*U. decumbens*) roots, high enzyme expression is related to a constant supply of necessary substrates to deal with aluminum resistance (Arroyave *et al.*, 2018). Second, guanylate kinase genes change their expression when sugarcane is under armyworm herbivory attack, assisting the resistance process (Wang *et al.*, 2021). With this, the importance of glutathione pathways and the relation between the kinase family and herbivore defense are demonstrated, and the metabolic network provides new interesting targets of the resistance process.

Coexpression network construction highlighted hub genes that were not identified in the other analyses but are essential to the complex process of spittlebug resistance, influencing several others by connections. The first hub in the BGP 344 network is a *Setaria viridis* hypothetical gene of ncRNA; that is, it does not encode proteins but acts post-transcriptionally to modify gene expression in plants according to abiotic and biotic stress (Hou *et al.*, 2019; Song *et al.*, 2021). Furthermore, we obtained a retrotransposon gag protein and a retrovirus-related Pol polyprotein from transposon TNT 1-94 (RPPT) that regulate the transcription levels of adjacent genes when exposed to environmental changes (Yang *et al.*, 2020; Hao & Zhang, 2022). Finalizing the cytosol changes, putative ubiquitin-like-specific protease 1 (ULP1B) is a post-translational gene modification responsible for stress defense in eukaryotes (Pedley *et al.*, 2018; Morrell & Sadanandom, 2019). To complete the main hubs, we annotated the trehalose 6-phosphate synthase gene, which is related to abiotic response (Sarkar & Sadhukhan, 2022), and two others are described as uncharacterized because we had no correspondence in the literature. This indicates how networks can bring new discoveries and indicates that not only do the transcribed genes influence the defense process against herbivores but RNAs also affect several expression genes linked to resistance, since they are central in the networks and have many connections. Therefore, specific studies of these ncRNAs can provide important information for unveiling the resistance mechanism for spittlebug resistance.

With this work, it was possible to conclude that the process of *P. regnellii* resistance to *M. spectabilis* spittlebugs is extremely complex, being governed by morphological characteristics that make infection difficult and secondary metabolites, enzymes and noncoding RNAs that are produced for plant defense. These findings will be useful to direct future genomic studies and for breeders to select new insect-resistant tropical forage cultivars.

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Competing interests

None declared.

Author contributions

MRG, WMJ and BBZV conceived the work. ISB, DSG, MRG, WMJ and BBZV obtained the data. ISB, AHA and RCUF analyzed the transcriptome and network data, ISB and WMJ analyzed the RT-qPCR results, and DSG and SMC-G analyzed the anatomy. MRG, BBZV and APS acquired funding and oversighted the work. ISB led the writing of the manuscript, and all authors contributed to the writing. All authors revised the work critically and approved the submitted version.

Data availability

The data that support the findings of this study are openly available in Sequence Read Archive (SRA) at <https://www.ncbi.nlm.nih.gov/sra>, reference number PRJNA999588.

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