Categorisation of lowly expressed cassava (*Manihot esculenta*) genes activated by mealybug (*Phenacoccus manihoti*) infestation

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Phân nhóm các gene biểu hiện yếu ở cây sắn (*Manihot esculenta*) được kích hoạt bởi sự tấn công của rệp sáp bột hồng (*Phenacoccus manihoti*)

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ABSTRACT

Cassava (Manihot esculenta) plays a crucial role in food security and industrial use in tropical and subtropical regions. However, its productivity is severely affected by Phenacoccus manihoti (mealybug) infestation. While several studies have examined cassava's responses to abiotic stress, the molecular mechanisms driving cassava's defence against mealybugs remain poorly understood. This study aimed to uncover the transcriptional dynamics of cassava leaves in the early stages of P. manihoti infestation by reanalysing public RNA-Seq datasets. The authors focused on genes that are lowly expressed under normal conditions, hypothesising that they may play inducible roles in stress responses. Our analysis revealed 546 such genes that became differentially expressed one day post-inoculation, including 263 up-regulated and 283 down-regulated genes. Functional enrichment indicated significant involvement in RNA biosynthesis and transcriptional regulation, phytohormone signaling (notably auxin-related pathways), lipid metabolism (especially glycerolipid biosynthesis), and secondary metabolism (such as phenolic compound production). Key transcription factors (e.g., WRKY, ERF, and MYB) and signaling modules (e.g., MAPK cascades) were also activated, pointing to a rapid reprogramming of the cassava transcriptome to mount an effective defence. Additionally, genes related to redox regulation, cell wall modification, and solute transport were enriched, suggesting a coordinated physiological response. This study provides new insights into the early inducible gene networks that contribute to cassava's defence against mealybug attack. These findings highlight novel genetic targets that can inform future molecular breeding and genome-editing strategies to enhance cassava's resilience against mealybug infestation.

Từ khóa:
Cây sắn, gen biểu hiện khác biệt, gen biểu hiện thấp, rệp sáp, RNA-Seq.

TÓM TẮT

Cây sắn (Manihot esculenta) đóng vai trò quan trọng trong an ninh lương thực và công nghiệp tại các khu vực nhiệt đới và cận nhiệt đới. Tuy nhiên, năng suất sắn bị ảnh hưởng nghiêm trọng bởi sự gây hại của rệp sáp Phenacoccus manihoti. Trong khi nhiều nghiên cứu đã khảo sát phản ứng của sắn đối với các loại stress phi sinh học, cơ chế phân tử chi phối phản ứng phòng vệ của sắn trước rệp sáp vẫn chưa được hiểu rõ. Nghiên cứu này nhằm làm sáng tỏ động học phiên mã ở lá sắn trong giai đoạn đầu bị rệp sáp gây hại thông qua việc phân tích lại dữ liệu RNA-Seq công khai. Nhóm tác giả tập trung vào các gen có mức biểu hiện thấp trong điều kiện bình thường, với giả thuyết rằng chúng có thể đóng vai trò cảm ứng trong phản ứng với stress. Phân tích của nhóm nghiên cứu xác định được 546 gen như vậy thay đổi biểu hiện chỉ sau một ngày sau khi cấy rệp, bao gồm 263 gen được điều hòa tăng và 283 gen bị điều hòa giảm. Phân tích làm giàu chức năng cho thấy sự tham gia đáng kể của các quá trình sinh tổng hợp RNA và điều hòa phiên mã, tín hiệu phytohormone (đặc biệt là các con đường liên quan đến auxin), chuyển hóa lipid (đặc biệt là sinh tổng hợp glycerolipid) và chuyển hóa thứ cấp (như sản xuất hợp chất phenolic). Các yếu tố phiên mã chủ chốt (như WRKY, ERF và MYB) và các mô-đun tín hiệu (như chuỗi MAPK) cũng được kích hoạt, cho thấy sự tái lập trình nhanh chóng của bộ gen sắn để khởi động cơ chế phòng vệ hiệu quả. Ngoài ra, các gen liên quan đến điều hòa cân bằng ôxi hóa-khử, biến đổi thành tế bào và vận chuyển chất hòa tan cũng được làm giàu, gợi ý một phản ứng sinh lý phối hợp. Nghiên cứu này cung cấp những hiểu biết mới về mạng lưới gen cảm ứng sớm góp phần vào phản ứng phòng vệ của sắn trước rệp sáp. Việc xác định các gen trước đây có biểu hiện thấp nhưng phản ứng với stress là cơ sở quan trọng cho các nghiên cứu chức năng tiếp theo và là mục tiêu tiềm năng trong việc chọn tạo giống sắn kháng sâu hại bằng phương pháp lai tạo phân tử hoặc kỹ thuật di truyền.

1. INTRODUCTION

Cassava (*Manihot esculenta*) is a vital food and industrial crop cultivated extensively in tropical and subtropical regions, particularly in Southeast Asia, Africa and Latin America [1, 2]. In Vietnam, cassava has emerged as one of the key industrial crops, grown on approximately half a million hectares and contributing significantly to food security, livestock feed, and the production of starch and bioethanol [2, 3]. Its adaptability to marginal soils and tolerance to drought make cassava an attractive crop for smallholder farmers in rural and upland areas [4, 5]. Despite its resilience to abiotic stress, cassava is highly vulnerable to a wide range of biotic stressors [5], including viral, bacterial, fungal pathogens, and insect pests [6]. Among these, the invasive mealybug (*Phenacoccus manihoti*, Hemiptera, Pseudococcidae) poses a serious threat to cassava productivity through leaf chlorosis, leaf deformation, and photosynthetic impairment [7]. The interaction between cassava and this pest remains underexplored at the molecular level. Thus, gaining insight into the molecular mechanisms underlying cassava's defence responses is essential for developing sustainable pest management strategies.

Recent advances in transcriptomics have provided valuable insights into cassava's defence against biotic stress. Earlier transcriptomic studies on cassava mosaic disease [8], cassava brown streak disease [9], and mealybug [10] showed that biotic stress infection triggers extensive shifts in hormone signalling, phenylpropanoid biosynthesis, and redox regulation. However, transcriptomic information on cassava's interaction with insect pests remains limited. Only a few studies have investigated early molecular responses to chewing or sucking insects, and the defence mechanisms specific to mealybug infestation are still poorly understood. Under normal growth conditions, many cassava genes remain expressed at very low levels, but they can be quickly activated when the plant experiences

stress [11]. These inducible genes likely have specialized roles in regulating defence and adaptation [12]. In the case of cassava subjected to mealybug infestation, it is hypothesised that certain lowly expressed genes may be activated as part of the plant's molecular response to biotic stress. This hypothesis underpins a functional genomics pipeline aimed at identifying and characterising such genes, which may otherwise be overlooked under non-stress conditions. Recent advances in high-throughput RNA sequencing (RNA-Seq) have enabled comprehensive and quantitative profiling of gene expression across different physiological states [13]. By comparing transcriptomic data between untreated and mealybug-infested cassava leaves, it is now possible to gain valuable insights into the complex regulatory networks and defence pathways that are activated in response to infestation.

This study aimed to investigate the early transcriptional responses of cassava leaves to mealybug infestation by reanalysing publicly available RNA-Seq datasets. Specifically, the focus was on identifying genes that are typically expressed at low levels under normal, unstressed conditions but become responsive upon biotic challenge. By integrating transcriptomic comparisons with functional annotation and pathway analysis, the study sought to identify key genes and regulatory networks involved in cassava's inducible defence mechanisms, characterise the biological processes and metabolic pathways affected by mealybug attack, and highlight novel or uncharacterized genes that may contribute to pest resistance. The findings aim to support future efforts in functional genomics and cassava improvement through targeted breeding or biotechnology.

2. RESEARCH METHODS

2.1. Data collection

Newest assembly (genome and proteome) of cassava (NCBI RefSeq assembly: GCF_001659605.2, submitted GenBank assembly: GCA_001659605.2) [14] was obtained from the Phytozome [15] and NCBI portals. Two RNA-Seq datasets, including GSE82279 (normal condition) [16] and GSE234712 (mealybug infestation), were explored in the GEO NCBI databases.

2.2. Reanalysis of transcriptomic datasets

To investigate the transcriptional response of cassava to mealybug infestation, a reanalysis of two publicly available RNA-Seq datasets was conducted. For the normal condition dataset (GSE82279), transcript abundance was estimated using FPKM (Fragments Per Kilobase of transcript per Million mapped reads) [16]. Genes with FPKM values < 10 were classified as lowly expressed under non-stress conditions [16]. For the inoculation dataset (GSE234712), differential expression analysis was performed using fold-change values. Genes with a fold change ≥ 2 were considered up-regulated, while those with a fold change ≤ -2 were considered down-regulated in response to mealybug infestation as previously described **[17].**

2.3. Venn analysis

A Venn analysis was performed to identify stress-responsive genes that are low in expression but activated upon mealybug infestation. The study involved two gene sets, including genes with FPKM < 10 from the normal condition dataset (GSE82279), representing lowly expressed genes, and differentially expressed genes from the inoculation dataset (GSE234712), defined by a fold change ≥ 2 (up-regulated) or ≤ -2 (down-regulated). The two gene sets were compared using R and the VennDiagram package to identify overlapping genes.

2.4. Investigation of biological pathways

To gain functional insight into the overlapping genes identified from the Venn analysis, MapMan was used to visualise and categorise these genes into biological pathways [18]. The list of overlapping genes was annotated with BIN codes using the Mercator tool or available cassava genome annotations. These BIN codes were then imported into the MapMan software and visualised gene expression changes across

different biological processes.

2.5. Functional enrichment analysis

To assess the statistical significance of functional enrichment among the overlapping genes, a BIN enrichment analysis was performed using MapMan's integrated statistical tools [18]. Each gene was assigned a BIN code representing a specific biological function. The observed frequency of genes in each BIN was compared against their background frequency in the entire cassava transcriptome. Particularly, *p*-values were calculated using Fisher's exact test, and significance was determined at $p < 0.05$. To account for multiple testing, p-values were adjusted using the Benjamini-Hochberg false discovery rate correction.

3. RESULTS AND DISCUSSION

3.1. Reanalysis of transcriptomic datasets of mealybug-infested cassava leaves

The reanalysis of the RNA-Seq dataset corresponding to cassava leaves inoculated with mealybug for one day revealed significant transcriptional changes. Out of the total genes analysed, 1,131 were up-regulated, 283 were down-regulated, and 25,887 showed no significant change in expression (Figure 1A). Among the differentially expressed genes, *Manes.13G132300* showed the strongest activation, with a 16.95-fold increase after mealybug infestation. Conversely, *Manes.11G028100* was the most reduced gene, with a log2 fold change of -7.58-fold.

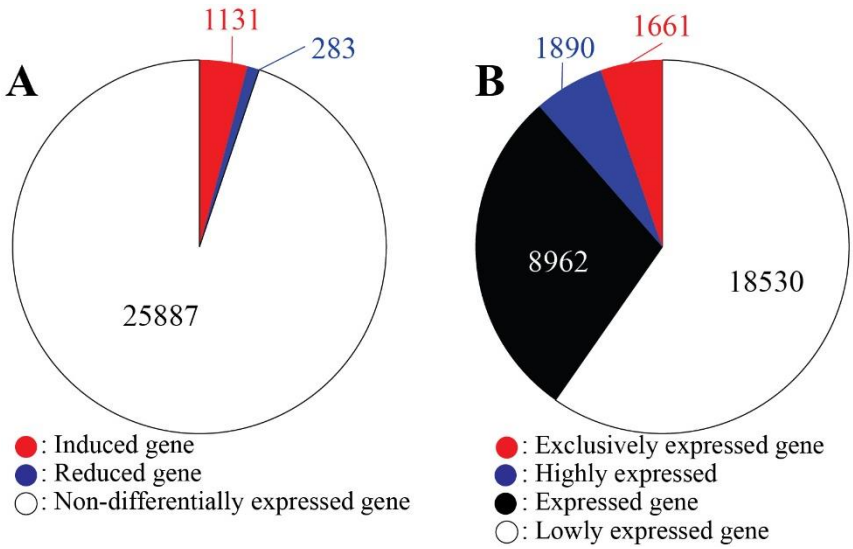

Figure 1. Transcriptomic distribution of cassava leaf genes under mealybug inoculation and normal conditions

(A) Pie chart showing the proportion of differentially expressed genes in cassava leaves one day after mealybug inoculation; (B) Pie chart representing the expression status of genes under normal conditions.

Under normal conditions, the tissue-specific expression analysis of cassava leaf transcriptomes revealed a broad distribution of gene activity levels (Figure 1B). Of the total genes analysed, 18,530 were classified as lowly expressed. These genes are often transcriptionally silent or expressed at minimal levels in the absence of stress. Additionally, 8,962 genes were moderately expressed, and 1,890 genes were highly expressed, indicating active roles in fundamental cellular processes required for normal physiological function. A smaller subset of 1,661 genes was exclusively expressed in leaf tissue, which suggests that they have strong tissue-specific functions. For downstream functional analysis, particularly the Venn analysis with differentially expressed genes under mealybug inoculation, we focused on the 18,530 lowly expressed genes to uncover defence-related genes that are activated specifically in response to biotic stress.

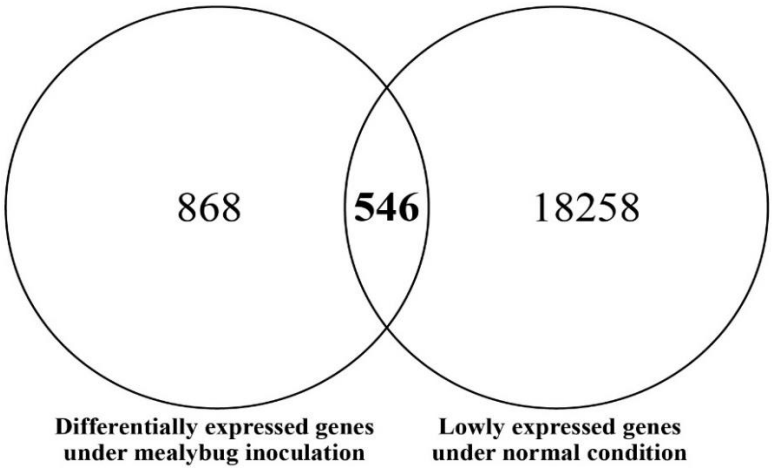

Figure 2. Venn diagram showing the overlap between differentially expressed genes under mealybug inoculation and lowly expressed genes in cassava leaves under normal conditions

Next, the Venn analysis presented in Figure 2 reveals a subset of 546 genes that are lowly expressed under normal conditions yet become differentially expressed following mealybug inoculation. Among these, 263 genes showed increased expression after pest attack. They may play roles in immune signalling, stress detection, or the production of defensive compounds. *Manes.13G132300* was the most strongly up-regulated gene, with a fold change of 16.95-fold. This suggests a potential role as a key component in cassava's early defence signalling or resistance mechanism. In contrast, 283 genes showed decreased expression. Their suppression may represent a strategic shift in the transcriptome to prioritise defence processes and limit energy use for non-essential functions. Particularly, *Manes.11G028100* was the most strongly down-regulated gene, with a fold change of -7.58-fold, which showed a marked reduction in activity after mealybug attack. The repression of this gene may indicate its involvement in growth or metabolic processes that are deprioritised during biotic stress. Overall, the nearly balanced distribution between up- and down-regulated genes highlights the complexity of cassava's transcriptional adjustment during the early phase of mealybug infestation.

3.2. Functional categorisation of mealybug-responsive genes with low basal expression in cassava leaves

The functional categorisation of the 546 lowly expressed genes that were differentially expressed one day after mealybug infestation reveals a complex and multi-layered transcriptional response in cassava. Among the 37 functional BINs annotated, several categories exhibited strong representation and biological relevance, although only a few reached statistical significance (p-value < 0.05).

The most significantly enriched category was RNA biosynthesis (BIN 15), which included 71 genes (p-value = 0.0183). This result shows a major adjustment in the transcriptional machinery in response to pest stress. This suggests that transcription factors and RNA polymerases are rapidly activated or regulated to initiate downstream defence responses. The transcriptional regulation subcategory (BIN 15.5) is closely linked, which shares the exact gene count and p-value. This finding supports the idea of coordinated control over gene expression. Phytohormone action (BIN 11) was also significantly enriched (p-value = 0.0279), with 24 genes involved. This indicates that hormone signalling pathways, likely including auxin, jasmonic acid, and salicylic acid, play a vital role in modulating cassava's defence mechanisms. Hormonal crosstalk may orchestrate early signalling and gene activation patterns needed for an effective stress response.

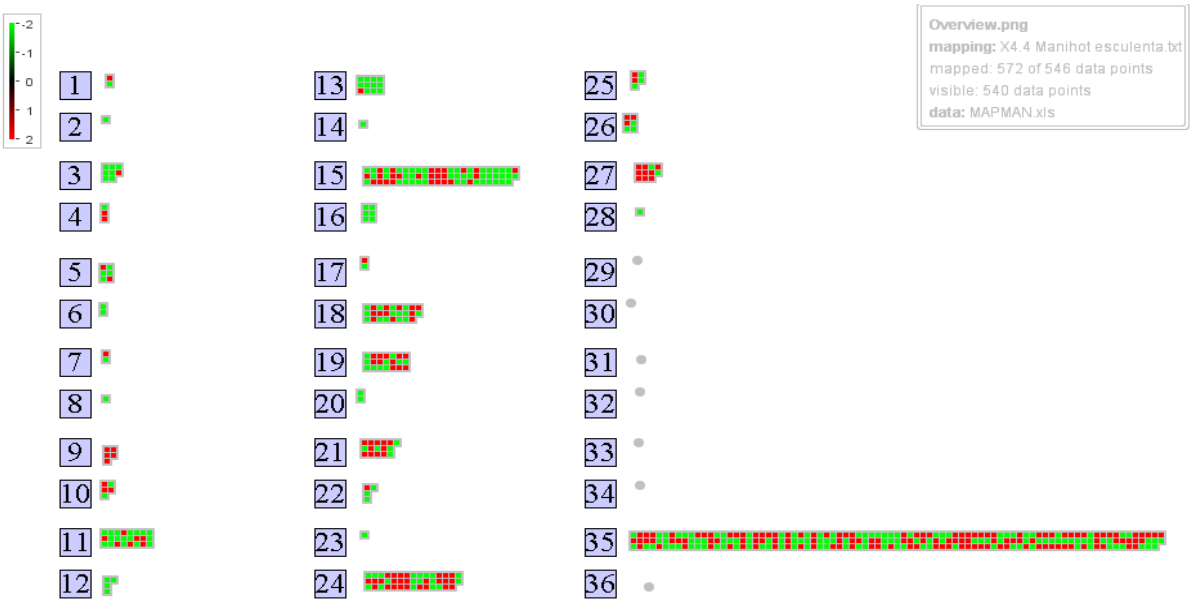

Figure 3. Functional overview of 546 lowly expressed and differentially regulated genes in cassava leaves one day after mealybug inoculation

Each colored square represents a gene mapped to a specific BIN (biological process). Red indicates up-regulation (fold change ≥ 2), green indicates down-regulation (fold change ≤ -2)

Additionally, two metabolic categories were significantly enriched. The enzyme classification group (BIN 50) contained 32 genes (*p*-value = 0.0480) and indicated the activation of catalytic proteins that are essential for biochemical defence. Secondary metabolism (BIN 9), with five genes (p = 0.0483), suggests the upregulation of pathways that produce antimicrobial compounds or signalling molecules that strengthen cassava's resistance. While other BINs did not reach statistical significance, they are still functionally relevant. Categories such as solute transport (BIN 24, 43 genes), protein modification (BIN 18, 26 genes), protein homeostasis (BIN 19, 21 genes), and cell wall organisation (BIN 21, 16 genes) represent critical support systems involved in cellular remodelling, resource redistribution, and reinforcement of physical barriers during stress. Chromatin organisation (BIN 12) and DNA damage response (BIN 14) may also contribute to epigenetic regulation and genome stability under biotic pressure. Interestingly, a notable finding of this study is the large group of 248 genes that were not assigned to any known functional category. These uncharacterized genes may include cassava-specific regulators of stress signalling and defence. Their activation in the early phase of mealybug infestation suggests potential roles in fine-tuning physiological or metabolic adjustments that are not yet represented in existing gene databases. Exploring these genes through functional validation, gene knockout, or overexpression studies could reveal new molecular mechanisms underlying pest resistance. Such investigations would not only expand current knowledge of cassava defence biology but also uncover novel genetic resources for breeding pest-resilient varieties.

Phytohormone-related pathways, especially those involving auxin, jasmonic acid, and salicylic acid, were strongly represented. These hormones likely interact to fine-tune the plant's early defence response by reducing growth and redirecting energy toward protection [19]. Such hormonal coordination agrees with the inducible resistance patterns observed in other plant-insect interactions. Additionally, metabolic changes were also apparent. Lipid-associated pathways such as glycerolipid metabolism, suggest that the plant adjusts its membranes and may use lipid-derived molecules as signals. The enrichment of secondary metabolic routes, particularly those that produce phenolic compounds, points to greater synthesis of antimicrobial and antioxidant substances. Genes involved in cell wall structure and modification support the

idea that cassava strengthens its outer barriers soon after infestation. At the same time, solute transporters and redox-regulating enzymes likely help redistribute nutrients and maintain internal balance under stress [20].

3.3. Investigation of metabolic pathways related to mealybug-responsive genes with low basal expression in cassava leaves

The investigation of metabolic pathways related to mealybug-responsive genes with low basal expression in cassava leaves revealed significant transcriptional shifts in multiple biological categories. RNA biosynthesis, particularly transcriptional regulation (BIN 15 and 15.5), was significantly enriched with 71 genes (*p*-value = 0.0183). This result suggests that cassava undergoes broad changes in gene expression after pest infestation. The phytohormone action pathway (BIN 11) showed significant enrichment with 24 genes (*p*-value = 0.0279). Within this group, the auxin-related subcategory (BIN 11.2) contained 8 genes (*p*-value = 0.0242). These results highlight the key role of auxin signalling in cassava's defence against biotic stress. Lipid metabolism pathways also displayed significant enrichment, particularly glycerolipid metabolism (BIN 5.2, 4 genes, *p*-value = 0.0144), potentially indicating alterations in membrane lipids for signalling or defence responses. The enzyme classification group (BIN 50) was also significantly enriched, with 32 genes (*p*-value = 0.0480). This finding indicates that many enzymatic processes become active to support metabolic adjustment under stress. Secondary metabolism (BIN 9) showed enrichment as well, with 5 genes (*p*-value = 0.0483), particularly those involved in phenolic compound production (BIN 9.2, 4 genes, *p*-value = 0.0375). These results suggest an increase in antimicrobial and other defence-related secondary metabolites.

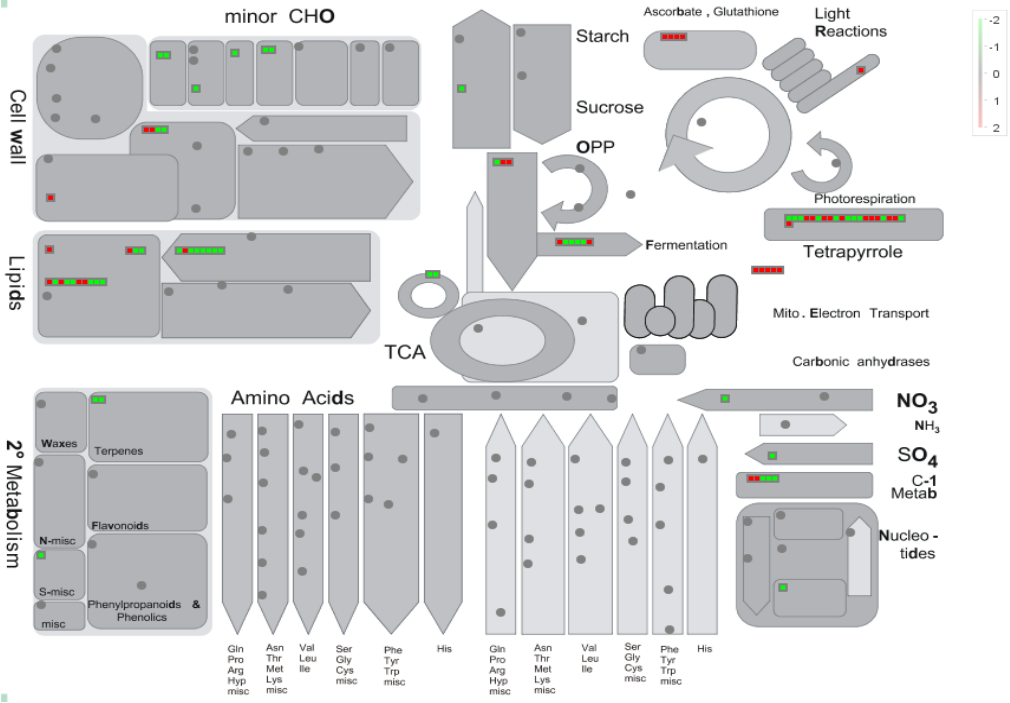

Figure 4. Overview of metabolic pathways affected in cassava leaves one day after mealybug inoculation, based on 546 differentially expressed genes with low basal expression under normal conditions

The figure was generated using MapMan, showing functional categories of primary and secondary metabolism. Colored squares represent individual genes: red indicates up-regulation (fold change ≥ 2), green indicates down-regulation (fold change ≤ -2)

Other important pathways, though not statistically significant, included cell wall organization (BIN 21, 16 genes), solute transport (BIN 24, 43 genes), protein modification (BIN 18, 26 genes), protein homeostasis (BIN 19, 21 genes), carbohydrate metabolism (BIN 3, 8 genes), and nutrient uptake, particularly transition metal

homeostasis (BIN 25.4, 3 genes, *p*-value = 0.0314). These categories suggest broader cellular remodelling, metabolic resource reallocation, and reinforcement of physical barriers during infestation. A large proportion of genes (248) were not assigned to any category (BIN 35). This suggests that many genes responsive to mealybug infestation have unknown or cassava-specific functions in defence, which marks an important direction for future research. Overall, the results show that cassava's early response to mealybug attack involves coordinated regulatory, hormonal, metabolic, and structural changes. These insights provide a strong basis for future functional studies and the development of improved cassava varieties.

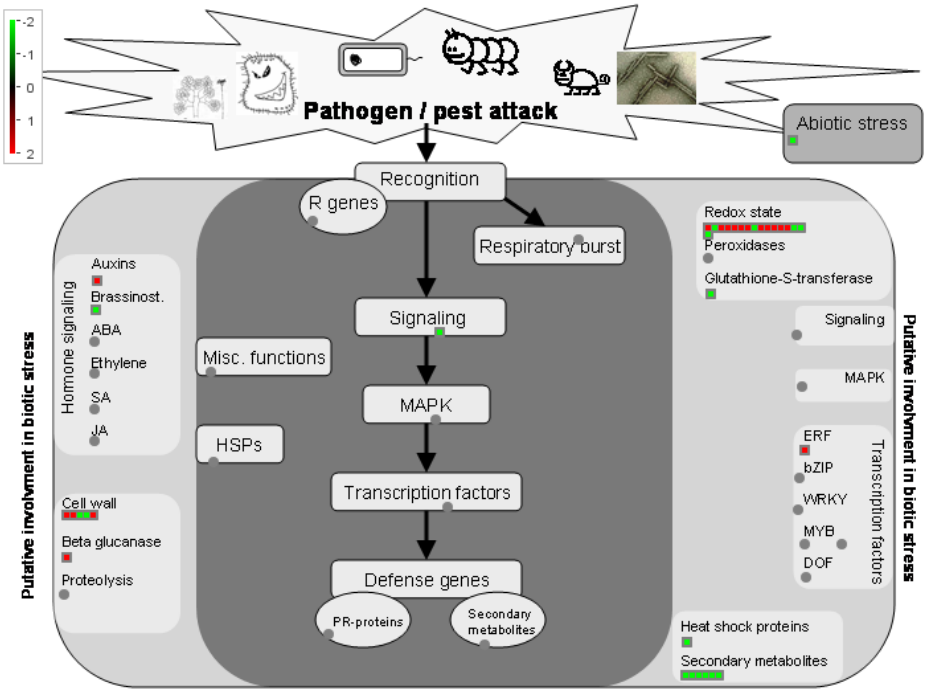

Figure 5. Functional overview of cassava defence-related pathways in response to mealybug infestation, based on differentially expressed genes with low basal expression

This schematic, generated using MapMan, illustrates the putative involvement of these genes in biotic stress signalling cascades. Colored squares indicate gene regulation: red for up-regulated (fold change ≥ 2), green for down-regulated (fold change ≤ -2)

The transcriptional activation of genes that are typically lowly expressed under normal conditions but become responsive upon mealybug inoculation reveals a tightly regulated, inducible defence mechanism in cassava. These genes are often maintained in a silent or repressed state to conserve energy and avoid unnecessary metabolic costs during unstressed growth. Upon mealybug inoculation, cassava leaves activate a highly coordinated defence response involving recognition, signal transduction, transcriptional reprogramming, and metabolic reconfiguration [21]. Based on the transcriptomic and pathway analyses of 546 lowly expressed but inducible genes, the defence mechanism begins with pest recognition through receptor-like proteins (R genes), which lead to the initiation of a respiratory burst and generation of reactive oxygen species. These reactive oxygen species serve as secondary messengers that activate downstream mitogen-activated protein kinase cascades, as shown by the up-regulation of genes in the mitogen-activated protein kinase signalling pathway [22]. This signalling is accompanied by changes in phytohormone signalling, especially auxin, as well as jasmonic acid and salicylic acid pathways, key hormonal regulators of biotic stress. These hormone signals lead to the induction of various transcription factors, including WRKY, bZIP,

ERF, and MYB, which in turn activate the expression of defence-related genes. These include pathogenesis-related proteins, enzymes involved in secondary metabolism (such as phenolics and flavonoids), and proteins regulating cell wall remodeling, which fortify physical barriers against insect penetration. Metabolically, cassava shows a shift in lipid metabolism, particularly glycerolipid metabolism, and carbohydrate pathways such as starch degradation and sucrose metabolism, possibly to redirect energy towards defence [10]. Enzymatic responses also include the activation of peroxidases and glutathione-*S*-transferases, reflecting adjustments in the redox state to counter oxidative stress. Meanwhile, heat shock proteins and protein degradation machinery (e.g., ubiquitin-proteasome components) are mobilized to maintain protein homeostasis during stress. Previous transcriptomic studies in other crops under insect attack revealed similar patterns of complex defence reprogramming [23]. For example, in cotton (*Gossypium hirsutum*) infested with the whitefly (*Bemisia tabaci*), it has been identified extensive transcriptional changes involving protein kinases, transcription factors, secondary metabolism, and phytohormone signalling, particularly jasmonic acid and ethylene pathways, have been identified as key mediators of insect resistance [23]. Comparable regulatory and metabolic shifts were observed in the current cassava - mealybug system, though with distinct enrichment in auxin-related genes and stress-specific enzyme categories. These differences suggest that while cassava shares core components of insect defence signalling with other crops, its early transcriptional response involves unique, possibly species-specific mechanisms, which exhibit evolutionary adaptation to piercing - sucking herbivores. Together, these responses demonstrate a dynamic and systemic shift from a quiescent transcriptional state to an active defence mode in cassava leaves. The rapid and selective induction of previously silent genes allows cassava to mount an efficient and resource-conscious response to mealybug attack.

4. CONCLUSION

This study provides a comprehensive reanalysis of transcriptomic data to uncover the molecular responses of cassava leaves to mealybug infestation. By focusing on genes that are lowly expressed under normal conditions, we identified 546 genes that become significantly regulated upon biotic stress, 263 up-regulated and 283 down-regulated. This pattern suggests a tightly controlled defence mechanism where gene activation is triggered only upon threat, thereby conserving energy during non-stress conditions. Functional categorisation and pathway analyses revealed that these inducible genes participate in key biological processes, including RNA biosynthesis, hormone signalling (notably auxin, jasmonic acid, and salicylic acid), enzyme regulation, and secondary metabolism. Importantly, pathways such as glycerolipid metabolism, redox balance, transcriptional regulation, and cell wall remodeling were also activated, highlighting a multifaceted defence strategy. Overall, our findings emphasize that the inducible expression of otherwise silent genes is central to cassava's early defence against mealybug infestation. This work lays a foundation for the functional characterization of these candidate genes and supports their potential utility in breeding or engineering mealybug-resistant cassava varieties.

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