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Gene Set Enrichment Analysis Highlights ATP Hydrolysis and Membrane-Associated Pathways in Drought-Tolerant Potato Cultivars

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Abstract

This study investigated the molecular responses of drought-tolerant and drought-sensitive potato cultivars using Gene Set Enrichment Analysis (GSEA) on RNA-seq data. The main objective was to identify activated or suppressed biological pathways and functional gene groups under drought stress, providing insights into drought tolerance mechanisms. Principal Component Analysis (PCA) and heatmap analysis revealed distinct gene expression profiles between the drought-tolerant cultivar FB and the drought-sensitive cultivar Cardinal. GSEA identified statistically significant enrichment of the ATP hydrolysis activity (GO:0016887) term in the FB cultivar, suggesting enhanced energy metabolism under drought conditions. While other GO terms—such as those related to the plasma membrane (GO:0005886), transmembrane transport (GO:0055085), and intracellular membrane-bounded organelles (GO:0043231)—did not reach statistical significance, their positive normalized enrichment scores (NES) indicated a trend toward higher expression in FB. The observed upregulation of ATP hydrolysis activity suggests the critical role of energy-dependent processes, such as protein repair and ion transport, in drought tolerance. Additionally, the results showed the importance of maintaining plasma membrane integrity, regulating transmembrane transport for water conservation, and the involvement of intracellular organelles in adapting to drought stress.

Keywords: GSEA, Enrichment analysis, Potato, RNA-seq, Drought tolerance

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INTRODUCTION

Drought significantly reduces crop yield by affecting the relationships between nutrients, decreasing water use efficiency, leaf area, and the rates of cell division and growth (Movahedi et al., 2023). Drought stress causes the loss of more than 40% of crops cultivated worldwide and continues to place increasing pressure on agricultural production due to climate change (P. Wang et al., 2025) . Potato (*Solanum tuberosum*) is the third most consumed crop in the world and is sensitive to drought, making it prone to yield losses under drought conditions (Yi et al., 2024). However, the increasing effect of climate change necessitates rapid drought tolerant potato cultivars adapting and enduring to exacerbating environmental conditions and increasing global temperatures (Kawamoto et al., 2024). This situation necessitates the development of drought-tolerant potato cultivars in terms of sustainable agricultural practices and food security. In this context, understanding the molecular mechanisms developed by the potato plant in response to drought stress is of great importance for guiding breeding efforts (Jian et al., 2024).

Traditionally, methods such as differential gene expression analysis are used to identify genes involved in drought response. However, since these approaches focus on individual genes, they may overlook complex biological processes driven by subtle but coordinated changes in gene expression (Subramanian et al., 2005). Therefore, there is a need for more comprehensive and integrative analytical approaches to uncover meaningful or biologically relevant information from gene expression data (Maleki et al., 2020).

Gene Set Enrichment Analysis (GSEA) is a computational method used to determine whether predefined sets of genes (e.g., those involved in a specific biological pathway or function) show statistically significant, coordinated differences in expression between two conditions (Han et al., 2019). This approach focuses on the collective changes of gene groups rather than individual genes. In doing so, it can detect pathways with subtle but coordinated expression changes that might be

overlooked when only highly differentially expressed genes are considered. As a result, GSEA facilitates the biological interpretation of large RNA-seq datasets by revealing which cellular processes, pathways, or functional gene groups are activated or repressed under specific stress conditions, such as drought (P. Wang et al., 2025). GSEA is used to identify which functional categories (such as Gene Ontology terms or KEGG metabolic/pathway information) are enriched among differentially expressed genes under a specific condition (e.g., drought stress), to test whether certain biochemical pathways are generally activated or suppressed (P. Wang et al., 2025), and to compare responses between different genotypes or conditions. In doing so, it reveals gene sets that are more significantly affected in one condition compared to another (Qin et al., 2024)

The main objective of this study is to identify which biological pathways and functional gene groups are activated or suppressed under drought stress through Gene Set Enrichment Analysis (GSEA), using RNA sequencing (RNA-seq) data obtained from drought-tolerant and drought-sensitive potato cultivars. In doing so, the study aims to provide a deeper understanding of the molecular mechanisms involved in drought tolerance and to lay a foundation for future potato breeding programs. The results obtained will play a key role in shaping strategies for the development of drought-tolerant potato varieties.

MATERIALS AND METHODS

In this study, RNA-seq analysis was performed to identify transcripts expressed under drought stress. This study used publicly available RNA-seq data (accession number GSE140083) from the Gene Expression Omnibus (GEO). The dataset was originally generated by Barra et al. (2019) and includes two potato cultivars: Clone 37 FB (highly drought-tolerant) and Cardinal (highly drought-sensitive). The dataset includes three biological replicates each for control (well-watered) and drought-stressed conditions in both cultivars. The analysis was carried out on the Galaxy platform (Afgan et al., 2016) following methods outlined in training materials by Batut et al. (2016), Doyle et al. (2020), and Hiltemann et al. (2023).

Statistical Analysis, GSEA, and Visualization

Gene Set Enrichment Analysis (GSEA) developed by Subramanian et al. (2005) was applied to transcriptomic data from *Solanum tuberosum* to identify biologically relevant gene sets and explore their functional relationships based on shared leading edge genes. The analysis was conducted using the fgsea package in R, which implements a computationally efficient GSEA algorithm developed by Korotkevich et al. (2016) .

All statistical analyses and visualizations were performed in the R programming language (Team, 2023). To visualize gene expression patterns, a heatmap was generated from DESeq2-normalized expression values (log2-transformed) using the “pheatmap package” in R (Kolde, 2019). Principal Component Analysis (PCA) was performed on variance-stabilized expression data using the “prcomp() function” in R to visualize clustering patterns among samples (Hotelling, 1933; Pearson, 1901). The hierarchical clustering was carried out with the “hclust() function” (Johnson, 1967).

RESULTS

According to the data obtained from the Principal Component Analysis (PCA) (Fig. 1) , the normalized samples of the drought-sensitive cultivar Cardinal and the drought-tolerant cultivar FB are clearly clustered separately. This clustering is due to an expression-based differentiation between the Cardinal and FB cultivars. The clustering observed in the PCA plot indicates that the RNA expression profiles of Cardinal (sensitive) and FB (tolerant) cultivars are distinct from each other. This difference likely results from the activation or repression of different genes in response to drought stress.

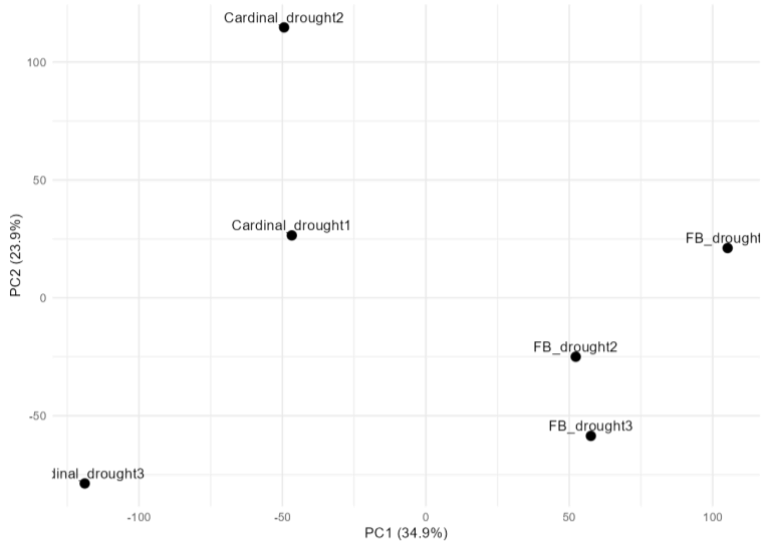

Figure 1. Results of Principal Component Analysis (PCA) showing the distribution of samples based on gene expression profiles. FB_drought1–3 and Cardinal_drought1–3 represent three biological replicates of the drought-stressed FB and Cardinal cultivars, respectively

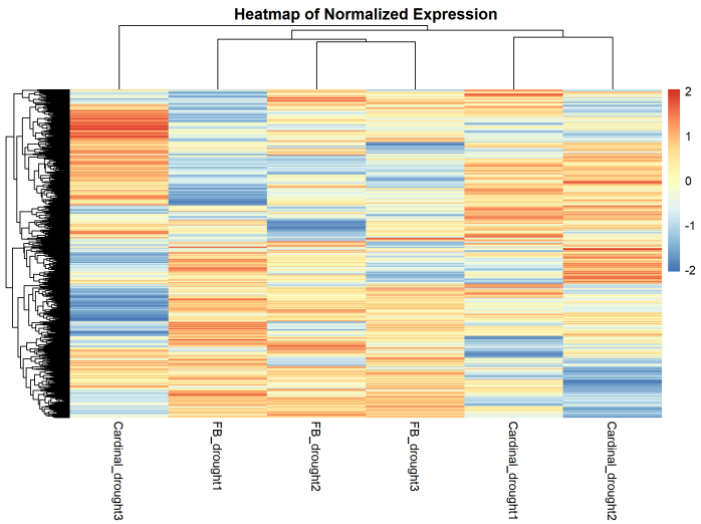

Figure 2. Heatmap analysis of normalized expression data for FB and Cardinal varieties.

In Fig. 2, the dendrogram at the top shows how the samples are grouped based on their similarity. In this context, the FB drought samples (FB_drought1, FB_drought2, and FB_drought3) are located on the same branch, indicating that they exhibit very similar expression patterns and that the normalized count values for FB are consistent within the group. In contrast, the Cardinal drought samples (Cardinal_drought1, Cardinal_drought2, and Cardinal_drought3) are located on different branches, suggesting that there may be greater variation among the Cardinal samples. Since this variation could stem from either technical factors (e.g., differences in library preparation, sequencing depth, or read quality) or biological factors (e.g., natural genetic variability or physiological differences among plants), the exact cause cannot be determined from the current dataset. In conclusion, the clustering of FB samples into a single group indicates that this genotype exhibits a similar gene expression response under drought stress. However, the scattered clustering of the Cardinal samples suggests either individual differences among the replicates or that this genotype displays a more irregular expression pattern in response to drought.

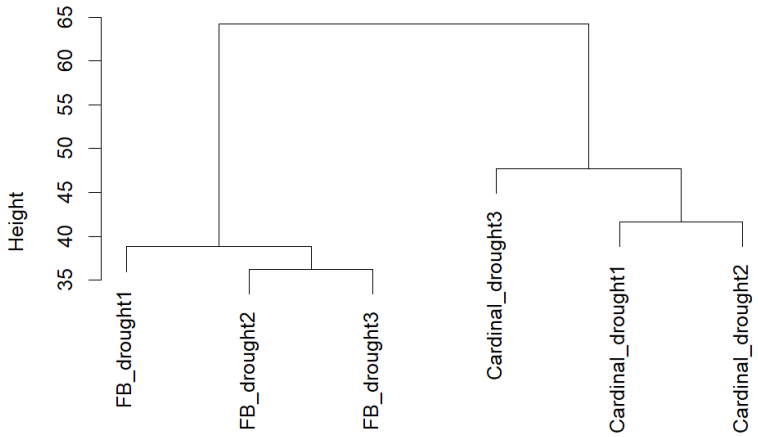

Figure 3. Results of the hierarchical clustering analysis of the cultivars used in the study.

In Fig. 3, it is observed that the FB genotype exhibits a more consistent and organized gene expression response under drought stress. This supports the idea that this cultivar responds to stress in a more resilient and controlled manner. In contrast, the Cardinal genotype displays a more scattered and variable gene expression pattern, indicating a weaker or more inconsistent response to drought stress. Although variation is observed in the normalized count data for both cultivars, this is particularly evident in the Cardinal samples. A plausible explanation for this outcome is that the FB genotype may possess a more tightly regulated transcriptional network under stress, potentially due to the presence of key regulatory genes (e.g., drought-responsive transcription factors, ABA-related signaling components) that ensure coordinated expression changes across replicates. Conversely, the higher variability in Cardinal could reflect a less robust stress-regulation system, where gene expression responses are more stochastic, possibly due to differences in signal transduction efficiency, transcription factor activity, or stress perception mechanisms. It is also possible that Cardinal's physiological state under drought stress is more unstable, leading to greater heterogeneity among biological replicates.Investigating the source of this variation—whether it stems from technical error or biological diversity—could be beneficial; however, since this falls outside the scope of the current study, it is not further explored here.

Table 1. ATP hydrolysis activity-associated GO terms and their parameters

Name	NES	padj	size
GO:0005886	1.023143	0.873134	477
GO:0016887	1.760746	0.008025*	139
GO:0055085	0.984171	0.919667	264
GO:0070588	1.442477	0.440154	20
GO: 0043231	0.901799	1	94

* significant term

Table 1 presents the statistically significant (p.adj < 0.05) drought-tolerance-related GO term, along with its associated GO terms and parameters in Figure 4. Among the analyzed GO terms, only ATP hydrolysis activity (GO:0016887) was significantly enriched (NES: 1.760746, p.adj: 0.008025). This high positive NES value indicates that genes related to ATP hydrolysis activity were significantly upregulated in the drought-tolerant FB cultivar. This finding suggests that energy metabolism and intracellular processes, such as protein folding and ion pump activities, are supported under drought stress conditions. The significant enrichment of ATP hydrolysis activity in the drought-tolerant FB cultivar underscores the pivotal role of energy-dependent processes in stress adaptation. Consistent with this, Liu et al. (2018) demonstrated that overexpression of the vacuolar H^+-ATPase subunit A (GhVHA-A) in cotton and tobacco enhanced drought tolerance through improved osmotic adjustment, increased antioxidant enzyme activities, and reduced oxidative damage. These findings support the notion that ATP hydrolysis-driven proton pumping is a key mechanism for maintaining cellular homeostasis and water balance under water-deficit conditions . Overall, the results suggest that energy-dependent processes are central to effective drought stress tolerance (Liu et al., 2018).

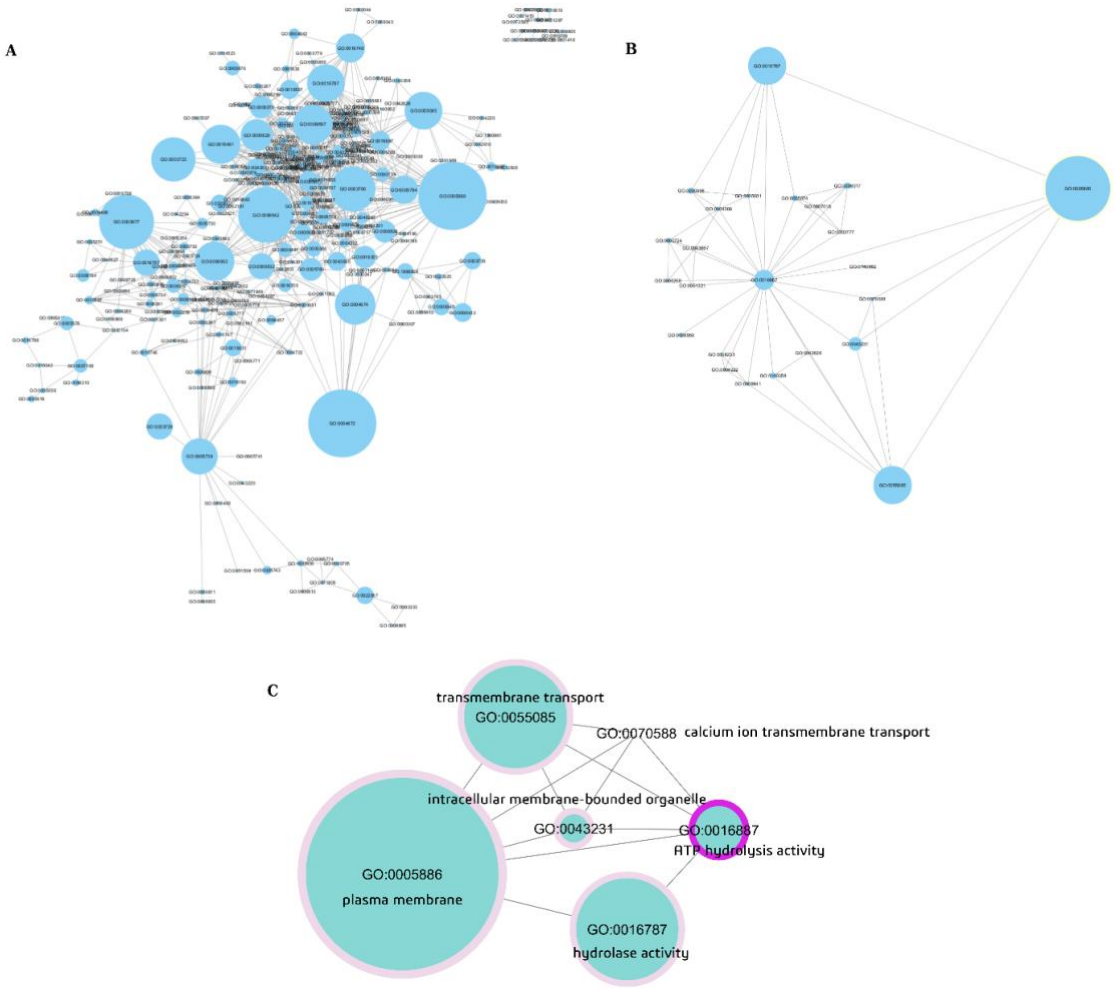

Figure 4. Relationships between biological pathways based on the results of the Gene Set Enrichment Analysis (GSEA) comparing FB and Cardinal. *ATP hydrolysis activity* is the only significantly represented term (padj < 0.0081) in the analysis and is indicated by a purple-circled sphere. The size of the spheres represents the number of genes classified under the corresponding annotation. Panel A shows an overall view of the ontological analysis; Panel B displays the pathways associated with the significantly enriched term; Panel C highlights the enriched pathway in relation to other significantly associated pathways according to the Normalized Enrichment Score (NES).

The other GO terms were not found to be statistically significant. For example, gene sets associated with *plasma membrane* (GO:0005886, NES: 1.023143, padj: 0.873134), *transmembrane transport* (GO:0055085, NES: 0.984171, padj: 0.919667), and *intracellular membrane-bounded organelle* (GO:0043231, NES: 0.901799, padj: 1) did not show significant enrichment in the FB cultivar. Similarly, the terms *hydrolase activity* (GO:0016787, NES: 0.838967, padj: 1) and *nuclear organization* (GO:0070588, NES: 1.442477, padj: 0.440154) were also not statistically significant. However, GO terms with positive NES values, despite not reaching statistical significance, indicate a trend toward higher expression in the FB cultivar.

In conclusion, the statistically significant enrichment of *ATP hydrolysis activity* supports the critical role of energy metabolism in drought tolerance. On the other hand, the lack of significance in other gene sets suggests that their roles in drought tolerance may be more indirect, complex, or less pronounced. It is therefore concluded that the potential involvement of these processes in drought tolerance should be investigated further using more detailed and diverse approaches.

DISCUSSION

The strong expression of genes associated with the plasma membrane (GO:0005886 – Plasma membrane) in the drought-tolerant plant indicates that this plant is adapted to maintain membrane integrity and regulate water/ion flow and signaling mechanisms under drought conditions. The plasma membrane is a fundamental structure that separates the cell from its external environment and controls the movement of water and ions. Drought stress can directly affect the plasma membrane, disrupting its selective permeability; in such cases, it becomes more difficult for the cell to maintain internal homeostasis (ElBasyoni et al., 2017). The expression of plasma membrane-related genes suggests activation of mechanisms that preserve membrane integrity and enable effective signal transduction under drought. Proteins that stabilize the plasma membrane play a crucial role during drought stress. For instance, overexpression of COR413, a protein localized to the plasma membrane, has been shown to reduce water loss in leaf cells, lower malondialdehyde (MDA) accumulation, and minimize membrane damage, thereby enhancing plant vitality under water stress (M. Wang et al., 2023). Moreover, the plasma membrane contains receptor and transporter proteins essential for sensing and responding to drought signals. For example, a mutation in an ABC transporter protein (AtMRP5) found in stomatal guard cells leads to irregular stomatal movement and increased drought sensitivity, highlighting the importance of membrane transporters in drought tolerance(Jarzyniak & Jasiński, 2014).

Hydrolases (GO:0016787 – Hydrolase Activity) are enzymes that break down large biomolecules using water. Under drought stress, the production of many hydrolase enzymes increases in plants; this is important both for the degradation of damaged macromolecules and for the mobilization of alternative resources. Indeed, drought triggers the upregulation of various protease enzymes (protein-degrading hydrolases) at the gene level; increased expression of protease genes has been observed in various plants, especially in leaves and roots (Luciński & Adamiec, 2023). The enrichment of genes showing hydrolase activity in drought-tolerant potato varieties indicates that these plants effectively utilize cellular reorganization and resource recycling mechanisms under drought. For example, drought-activated phospholipase D (PLD) enzymes break down membrane phospholipids to produce second messenger molecules such as phosphatidic acid (PA); PA plays a key role in drought signaling and accelerates stomatal closure, thereby reducing water loss (Hong et al., 2010). Similarly, drought stress activates the mobilization of stored carbohydrates: starch is broken down by hydrolase enzymes into soluble sugars within the cell. These accumulated sugars act as osmoprotectants, protecting the cell during drought while also providing metabolic energy. Additionally, drought stress stimulates the autophagy pathway in plants; autophagy recycles damaged organelles and proteins with the help of vacuolar hydrolases, maintaining cellular homeostasis (Guo et al., 2021). The prominence of hydrolase activity in the tolerant variety suggests that this plant facilitates the controlled degradation of intracellular macromolecules under drought (e.g., breaking down proteins into amino acids, converting starch into sugars) to enable their reuse. It also indicates that the plant accelerates defense responses by producing stress-related signaling molecules (e.g., PA). This catabolic adaptation provides the plant with a survival advantage under drought conditions by efficiently utilizing both energy and building blocks.

The activation of genes with ATP hydrolysis activity (GO:0016887 – ATP hydrolysis activity) in the drought-tolerant potato variety indicates that the plant invests energy to protect itself under stress. In drought tolerance mechanisms, proteins are safeguarded by chaperones such as HSPs, and cellular ion/pH homeostasis is maintained through proton pumps, allowing vital cellular functions to continue under drought conditions. ATP hydrolysis-based activities represent proteins that perform essential cellular functions at an energy cost. During drought stress, plant cells enhance certain energy-intensive responses to survive. The differential expression of genes in this category in the tolerant variety reflects an adaptive strategy to resist stress through energy utilization. Notably, molecular chaperones such as heat shock proteins (HSPs) function via ATP hydrolysis and play a crucial role in repairing protein damage caused by drought. For instance, the HSP70 family is highly expressed under various stress conditions, including drought, and helps protect the plant by preventing protein misfolding and assisting in proper folding. HSP70 has indeed been identified as a key component of the drought stress response (Movahedi et al., 2023). Furthermore, many promoters of P-type ATPase genes in potato contain cis-acting elements responsive to drought and low temperature, suggesting that specific isoforms may be selectively activated under stress to enhance both ion transport and proteostasis(Zhang et al., 2024). This specific function is defined in Gene Ontology as proton-transporting ATPase activity (GO:0046961) and is hierarchically a subcategory of the broader ATP hydrolysis activity (GO:0016887). In addition, ATPase pump proteins play a critical role in drought tolerance. Proton pumps (H^+-ATPases) located in cell membranes transport ions through ATP hydrolysis and help maintain intracellular ion balance. In particular, H^+ pumps found on the vacuolar membrane (tonoplast), such as vacuolar H^+-ATPases and H^+-PPases (GO annotation: vacuolar proton-transporting V-type ATPase complex, GO:0016469), are involved in osmotic regulation under drought conditions. By increasing the activity of these proton pumps and transporting more H^+ ions into the vacuole, the accumulation of solutes (ions and metabolites) within the vacuole rises. This reduces the water potential of the cell, thereby facilitating

water uptake from the soil (Liu et al., 2018). Therefore, increased activity of the tonoplast H^+ pump helps the plant maintain cell turgor even under water-limited conditions. Indeed, overexpression of genes encoding these pumps in various plant species has been shown to significantly enhance tolerance to drought and salt stress (Liu et al., 2018)

In drought-tolerant plants, the strong expression of transmembrane transport-related genes (GO:0055085 – Transmembrane transport), including ion channels, pumps, and carrier proteins, contributes to efficient water conservation by enabling rapid and controlled adjustment of stomatal opening. Under drought conditions, plants tightly regulate the movement of substances across cell membranes to retain water and prevent the accumulation of toxic ions. The potentially highly expressed genes associated with transmembrane transport in the tolerant variety suggests that these plants effectively utilize transport proteins to regulate water and ion flows as part of its adaptation to drought stress. In particular, transmembrane transport in guard cells plays a critical role in controlling water loss. When the level of the abiotic stress hormone ABA increases, various ion channels and transporters in the guard cell membranes are activated. Anion channels (such as S-type and R-type) export Cl^- and other anions from the cell, leading to a change in membrane potential. This triggers the efflux of potassium ions through K^+ channels (e.g., GORK). Simultaneously, water exits the cell via aquaporins, specialized water channels (Jarzyniak & Jasiński, 2014). This coordinated efflux of ions and water reduces the turgor pressure in guard cells, leading to stomatal closure. As a result, the plant effectively limits water loss under drought conditions (Jarzyniak & Jasiński, 2014). Transmembrane transport not only regulates water loss in leaves but also plays a vital role in water and nutrient uptake from the roots and in maintaining intracellular ion homeostasis. Aquaporin water channels, located in both the plasma membrane and the tonoplast, regulate water movement between cells and within organelles. Under drought conditions, the expression levels and activity of certain aquaporins change. Drought-tolerant plants strategically regulate specific aquaporins to use water more efficiently. For example, the precise opening and closing of aquaporins help the plant maintain water uptake even in dry environments (Movahedi et al., 2023) . This is consistent with the observed changes in aquaporin gene expression in the drought-tolerant potato variety. Additionally, transporter proteins play a crucial role in establishing the plant's osmotic balance. Under saline and drought conditions, membrane-associated proteins such as sodium/hydrogen antiporters, potassium channels, and ATP-binding cassette (ABC) transporters help protect the cell by directing ions to appropriate compartments—for example, sequestering excess ions into the vacuole or removing them from the leaves (Jarzyniak & Jasiński, 2014; Liu et al., 2018). The identification of such transmembrane transporters in the enrichment analysis of the drought-tolerant variety indicates that the plant actively reprograms its internal transport regulation under drought conditions. In summary, transmembrane transport processes are vital for retaining water within the cell and for maintaining ion and metabolite balance during drought stress. Tolerant plants enhance these processes at the gene expression level, allowing them to mount a more effective response to environmental stress.

The term Intracellular membrane-bounded organelle (GO:0043231) encompasses all intracellular organelles enclosed by membranes, including the nucleus, chloroplast, mitochondria, vacuole, endoplasmic reticulum, and peroxisomes. The plant's response to drought stress also involves functional changes and adaptations within these organelles. In the drought-tolerant potato variety, a significant portion of the differentially expressed genes is associated with organelles, suggesting that the plant responds to stress through extensive reorganization of its internal structures. Chloroplasts are among the primary organelles affected by drought stress. When water is limited, stomata close, restricting the entry of carbon dioxide. This slows down photosynthesis and leads to the accumulation of excess light energy, increasing the production of reactive oxygen species (ROS). Drought-tolerant plants activate protective mechanisms in their chloroplasts to prevent damage to the photosynthetic machinery. For example, under drought conditions, the expression of antioxidant enzymes such as 2-Cys peroxiredoxin in the chloroplast is upregulated. These enzymes reduce elevated peroxide levels during water stress, thereby protecting photosystems from oxidative damage (Movahedi et al., 2023). Similarly, mitochondria—the central organelles for aerobic respiration—can also become sites of reactive oxygen species (ROS) production under drought conditions. In drought-tolerant plants, mitochondrial antioxidant enzymes (e.g., superoxide dismutase and ascorbate peroxidase) and alternative respiratory pathway proteins are upregulated to protect the cell from oxidative damage. Furthermore, the regulation of the mitochondrial electron transport chain is modified, and in some cases, plants upregulate alternative oxidase (AOX) to reduce ROS formation (Yang et al., 2021). Dehydrins, a sub family of LEA (Late Embryogenesis Abundant), and other LEA proteins can localize to various organelles and play protective roles. For instance, in maize, the dehydrin protein DHN1 has been shown to bind to vesicle membranes containing phospholipids, and structural changes in the protein during this interaction are thought to help maintain vesicle (vacuole-like) membrane stability. This finding demonstrates that proteins like dehydrins can directly stabilize organelle membranes under drought conditions. Indeed, in many plant species, dehydrin genes are induced by drought and accumulate in regions such as the cytoplasm and nucleus, helping to minimize cellular damage caused by water loss.

The vacuole is also a critical organelle during drought stress, as plant cells maintain osmotic balance by storing ions and metabolites within their vacuoles. Drought-tolerant plants accumulate higher levels of osmotic regulators in their vacuoles during drought conditions. For example, amino acid derivatives such as proline and soluble sugars are transported into vacuoles to increase the cell's water retention capacity. As previously mentioned, the activity of proton pumps on the vacuolar membrane is crucial in this process. In the drought-tolerant potato variety, differential expression of tonoplast H^+-ATPase and H^+-PPase genes reflects the importance of intracellular osmotic adjustment. Experimental studies have shown that overexpression of these pumps enhances drought tolerance by promoting proline accumulation and increasing antioxidant enzyme activity in plants (Liu et al., 2018).

CONCLUSION

The results of this study indicate that drought-tolerant potato cultivars develop adaptations to drought stress through various molecular mechanisms. In particular, maintaining plasma membrane integrity, protecting proteins in an energy-dependent manner, regulating water and ion balance, and adapting at the organelle level are key components of these strategies. These findings provide valuable insights for the development of drought-resistant potato varieties and lay an important foundation for sustainable agriculture.

Compliance with Ethical Standards

Peer Review

This article has been peer-reviewed by independent experts in the field using a double-blind review process.

Conflict of Interest

The author declares that there is no conflict of interest.

Author Contribution

The author solely conceived, designed, and conducted the study, analyzed the data, and wrote the manuscript.

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