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Identification and Functional Characterization of Glutamate Decarboxylase (*GAD*) Genes in Potato (*Solanum tuberosum* L.) and Analysis of Their Expression under Drought and Salt Stress Conditions

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Abstract

Gamma-aminobutyric acid (GABA) plays a critical role in plant stress responses and development by regulating osmotic balance, mitigating oxidative stress, and maintaining cellular homeostasis. Glutamate decarboxylase (GAD) is the first and key enzyme in the GABA biosynthesis pathway. In this study, three distinct *GAD* genes in potato—*StGAD1*, *StGAD2*, and *StGAD3*—were identified, each distributed on separate chromosomes, indicating non-redundant functional roles. Expression profiling revealed that *StGAD1* is the primary stress-responsive gene, with significant upregulation in both roots and leaves under stress, promoting GABA accumulation to enhance water-use efficiency and reduce oxidative damage. Protein-protein interaction analysis highlights functional relationships among the *GAD* proteins, with *StGAD1* and *StGAD2* sharing significant homology. The findings suggest that GABA metabolism, primarily driven by *StGAD1*, plays a more prominent role in drought tolerance than salt stress adaptation, where other regulatory mechanisms such as ion homeostasis may be more critical. This study provides foundational insights into the molecular mechanisms of *GAD*-mediated stress

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responses in potatoes, offering potential avenues for enhancing crop resilience through targeted genetic strategies.

Key Words: Potato; Glutamate decarboxylase (*GAD*); Gamma-aminobutyric acid (GABA); Drought stress; Salt stress.

Patateste (*Solanum tuberosum* L.) Glutamat Dekarboksilaz (GAD) Genlerinin Tanımlanması ve Fonksiyonel Karakterizasyonu ile Kuraklık ve Tuz Stresi Koşullarında İfade Analizleri

Öz

Gamma-aminobutirik asit (GABA), bitkilerin stres tepkileri ve gelişiminde osmotik dengeyi düzenleyerek, oksidatif stresi azaltarak ve hücresel homeostazı koruyarak kritik bir rol oynar. Glutamat dekarboksilaz (GAD), GABA biyosentez yolunda yer alan ilk ve temel enzimdir. Bu çalışmayla üç farklı patates *GAD* geni—*StGAD1*, *StGAD2* ve *StGAD3*—belirlenmiştir. İfade profili analizi, *StGAD1*'in stres yanıtı veren ana gen olduğunu göstermiştir. *StGAD1* hem kök hem de yapraklarda stres altında önemli ölçüde yukarı yönlü düzenlenerek, GABA birikimini artırmakta ve su kullanım verimliliğini iyileştirmekte ve oksidatif hasarı azaltmaktadır. Protein-protein etkileşim analizleri, *GAD* proteinleri arasında işlevsel ilişkiler olduğunu ve *StGAD1* ile *StGAD2* arasında önemli ölçüde benzerlik bulunduğunu ortaya koymaktadır. Bulgular, GABA metabolizmasının, özellikle *StGAD1* tarafından yönlendirilen süreçlerin, kuraklık toleransında daha belirgin bir rol oynadığını, tuz stresi adaptasyonunda ise iyon homeostazı gibi diğer düzenleyici mekanizmaların daha kritik olabileceğini düşündürmektedir. Bu çalışma, *GAD* aracılı stres tepkilerinin moleküler mekanizmalarına ilişkin temel bilgiler sunmakta ve hedeflenmiş genetik stratejilerle bitki dayanıklılığının artırılmasına yönelik potansiyel yollar önermektedir.

Anahtar Kelimeler: Patates, Glutamat dekarboksilaz (*GAD*), Gamma-aminobutirik asit (GABA), Kuraklık stresi, Tuz stresi.

1. Introduction

Gamma-aminobutyric acid (GABA) is widely recognized as a crucial neurotransmitter in the animal nervous system, where it plays a significant role in inhibiting nerve transmission and promoting relaxation [1]. However, GABA's importance extends beyond the animal kingdom, as it is also a vital component in plants, contributing to a myriad of physiological and biochemical

processes [2]. The study of GABA in plants has garnered increasing attention in recent years, revealing its multifaceted roles in plant growth, development, and stress response mechanisms [3].

In plants, GABA is synthesized from the amino acid glutamate through the action of the enzyme glutamate decarboxylase (GAD) [4]. GAD is encoded by a family of GAD genes, which are highly conserved and widely distributed across different plant species [5]. These genes are crucial for the regulation of GABA synthesis, and their expression is tightly controlled by various developmental cues and environmental stimuli [6]. The GAD enzyme catalyzes the irreversible decarboxylation of glutamate to GABA, a key step in the GABA shunt pathway [7]. This pathway serves as an alternative metabolic route that bypasses two steps of the tricarboxylic acid (TCA) cycle, aiding in maintaining the balance of carbon and nitrogen within plant cells and playing a pivotal role in regulating the cytosolic pH [3]. This pH regulation is crucial for various cellular processes, including enzyme activities and metabolic reactions [8]. Glutamate decarboxylase (*GAD*) is an essential enzyme in plants, responsible for catalyzing the conversion of glutamate to γ-aminobutyric acid (GABA), a non-proteinogenic amino acid with key roles in both metabolism and signaling. The GABA shunt, comprising *GAD*, GABA transaminase (*GABA-T*), and succinic semialdehyde dehydrogenase (*SSADH*), links glutamate metabolism to the tricarboxylic acid (TCA) cycle, enabling plants to manage stress and maintain cellular homeostasis [9-10]. The GAD gene family is highly conserved across plant species, with Arabidopsis having five *GAD* isoforms (*GAD1*–*GAD5*). These isoforms are differentially expressed, with GAD1 playing a crucial role in roots, where it helps maintain GABA levels under normal and stressful conditions. Studies have shown that GAD1 is particularly important during heat stress, where its activation ensures a rapid increase in GABA levels, protecting roots from damage caused by high temperatures [11]. Meanwhile, *GAD2* is more ubiquitously expressed and plays a broader role in responding to stresses in multiple tissues [10-11].

Regulation of GAD enzymes involve calcium/calmodulin (Ca^{2+}/CaM) signaling, a critical pathway that allows plants to sense and respond to environmental stimuli. GAD activity is enhanced in the presence of Ca^{2+}, which binds to the CaM-binding domain in GAD, triggering GABA synthesis in response to stress [12-13]. This regulation is particularly important under salt stress, where increased GABA levels help maintain ion homeostasis and protect against oxidative damage caused by reactive oxygen species (ROS) [14-15].

Drought is one of the most critical environmental stressors affecting plants. GABA accumulation during drought stress helps regulate stomatal closure, reducing water loss through transpiration. In Arabidopsis, *GAD1* and *GAD2* mutants show increased susceptibility to drought

due to their inability to produce sufficient GABA, which is essential for maintaining ion homeostasis and activating antioxidant defense mechanisms [15]. Moreover, exogenous GABA application has been shown to improve drought tolerance in several plant species, enhancing photosynthesis and reducing oxidative damage by increasing the activity of enzymes such as superoxide dismutase (SOD) and catalase (CAT) [13].

GABA also plays a key role in salt stress tolerance. High salinity disrupts cellular ion balance, leading to toxic levels of sodium ions (Na^+) in plant tissues. GABA helps mitigate this by modulating ion transport through aluminum-activated malate transporters (ALMTs), reducing Na^+ uptake and preventing ion toxicity [14-15]. In Arabidopsis, GAD4 is specifically upregulated during salt stress, further underscoring the role of GAD genes in stress adaptation [15]. GABA also contributes to the stabilization of cellular osmotic pressure, which is crucial for plant survival in saline environments [13]. Heat stress induces a rapid accumulation of GABA, particularly in roots, where GAD1 plays a dominant role [11]. GABA production under heat stress helps mitigate the effects of high temperatures by maintaining cellular pH and reducing ROS levels. Additionally, GABA functions as an osmoprotectant, stabilizing proteins and membranes, thus enabling plants to survive prolonged heat exposure [13]. This is evident in crops like rice, where exogenous GABA application enhances heat tolerance by improving leaf turgor, reducing oxidative damage, and activating heat shock proteins [15].

In addition to abiotic stress, GABA is involved in biotic stress resistance. During pathogen infection, such as that caused by *Pseudomonas syringae*, GABA enhances plant immunity by modulating defense-related signaling pathways [16]. In Agrobacterium tumefaciens-mediated gene transformation, GABA inhibits the conjugation of the Ti plasmid, reducing the severity of crown gall disease in plants [13].

GABA also has a significant role in plant development. It regulates pollen tube growth by forming concentration gradients that guide the pollen tube toward the ovule during fertilization [13]. Furthermore, GABA modulates root growth by influencing hormone signaling pathways, including those involving abscisic acid (ABA) and ethylene, which are key regulators of seed germination and root elongation [13-15]. This multifaceted role of GABA in development underscores its importance beyond stress tolerance.

Heavy metal pollution poses a significant threat to plant growth and crop yield. GABA plays a role in mitigating heavy metal toxicity by activating antioxidant enzymes and modulating metal transport. For example, under cadmium (Cd) stress, GABA accumulation helps protect plants by enhancing Cd sequestration and reducing oxidative stress [15]. Studies on soybean and

rice have shown that GABA levels rise in response to zinc (Zn) and copper (Cu) stress, further highlighting its role in heavy metal detoxification [13]. Interestingly, while GABA typically increases under heavy metal stress, its accumulation must be tightly regulated. Excessive GABA levels can sometimes lead to toxicity, as seen in some plants subjected to extreme concentrations of metals like arsenic (As), where high GABA levels may exacerbate stress [13].

The expression of *GAD* genes is regulated by various transcription factors in response to environmental stimuli. For instance, under salt stress, WRKY and MYB transcription factors bind to the promoter regions of *GAD4*, enhancing its expression [15]. Similarly, STOP1 and WRKY40, which are known repressors of *GAD* expression, modulate GABA levels under stress, ensuring that GABA accumulation is appropriately regulated during abiotic stress [15]. This intricate transcriptional regulation allows plants to fine-tune GABA production in response to fluctuating environmental conditions.

Overall, GAD-mediated GABA production is a critical mechanism for stress adaptation in plants. By modulating ion fluxes, scavenging ROS, and maintaining cytosolic pH, GABA plays a multifaceted role in enhancing plant tolerance to abiotic stresses such as drought, salinity, and heat [13-15]. Additionally, GABA is involved in biotic stress responses and developmental processes such as pollen tube guidance and root elongation [13]. As a versatile metabolite and signaling molecule, GABA enables plants to survive and thrive in challenging environments. Continued research into the regulatory networks controlling GABA synthesis and signaling could offer new avenues for improving crop resilience in the face of climate change and environmental stressors [15].

2. Material and Methods

2.1. Chromosomal distribution and phylogenetic analysis

The locations of the *StGAD* genes on each chromosome were obtained from the potato genome database (Ensembl Plants; https://plants.ensembl.org/index.html), and their chromosomal distribution was illustrated using the Mapgene2chrom 2.1 (MG2C v2.1) online tool (http://mg2c.iask.in/mg2c_v2.1/) [17-18]. The protein sequences were obtained from Phytozome (https://phytozome-next.jgi.doe.gov) and analyzed to construct a phylogram using the Clustal Omega web tool (https://www.ebi.ac.uk/jdispatcher/msa/clustalo).

2.2. 3D modeling, subcellular localization prediction and protein–protein interactions

3D modeling of StGAD proteins was conducted using previously identified sequences, with support provided by the UniProt database [19]. Predictions of subcellular localization were generated through the WoLF PSORT database [20]. To investigate both the physical and functional interactions among StGAD proteins, the STRING database was utilized [21].

2.3. Plant materials and stress treatments

In this study, the potato (*Solanum tuberosum*) cultivar 'Agria' was utilized. Plantlets were initially sub-cultured on MS-0 medium, which contained 4.4 g/L MS salts, 3% sucrose, and 0.7% agar, with the pH adjusted to 5.7. The cultures were maintained in a growth chamber set at 22°C with 70% relative humidity, under a 16-hour light / 8-hour dark photoperiod. After five weeks, the plantlets were transferred to hydroponic systems filled with Hoagland's solution (pH 5.8). Following a two-day acclimation period, the nutrient solution was replaced with a fresh Hoagland's solution containing either 25% polyethylene glycol (PEG-6000) to induce drought stress, or 200 mM sodium chloride (NaCl) for salt stress. Control plants received only refreshed Hoagland's solution. After 24 hours of exposure to drought or salt stress, leaves and roots were harvested for RNA extraction.

2.4. RNA isolation and gene expression analysis

RNA was extracted from leaf and root tissues using the RNA Plant Mini Kit (Qiagen, USA) according to the manufacturer's protocol. To eliminate potential genomic DNA contamination, the RNA samples were treated with RQ1 RNase-Free DNase (Promega, USA). The integrity of the RNA and the absence of DNA contamination were confirmed by gel electrophoresis. RNA concentrations were quantified using a Qubit fluorometer (Invitrogen, USA). Subsequent RT-qPCR analyses were conducted on a CFX384 Real-Time PCR System (Bio-Rad, USA). For each sample, 10 ng of RNA was used in the reaction, employing the Luna Universal One-Step RT-qPCR Kit (NEB, USA). Gene sequences of *StGAD* genes were retrieved from the Phytozome database (Phytozome v13, https://phytozome-next.jgi.doe.gov/), and primers were designed using the Primer3 Input web tool (https://primer3.ut.ee/). Gene expression was quantified using specific forward and reverse primers (Table 1). The *StSec3* gene served as the internal reference control for normalization [22].

Table 1: Primers used for RT-qPCR analysis of *StGAD* genes

Genes	Phytozome ID	Forward primer sequences	Reverse primer sequences
StGAD1	PGSC0003DMG400031042	ATGGGATTTCCGTTTGCCT	ATATCACCCAACCGACACCAG
StGAD2	PGSC0003DMG400013331	TAGCGTCGGAAAGTGACATG	CAAGTGGTGCATTAAATAAGT
StGAD3	PGSC0003DMG400022764	CTTCCTCTGAGTCTGATGAT	AAGATCATGCAAGAGTTTG
StSec3	PGSC0003DMG402015451	GCTTGCACACGCCATATCAAT	TGGATTTTACCACCTTCCGCA

3. Results and discussion

3.1. Chromosomal distribution of *StGAD* genes

Queries performed in the Phytozome and Ensembl Plants databases identified three distinct members of GAD gene family in *S. tuberosum*. The chromosomal distribution of GAD genes in the potato genome reveals that each gene is located on a distinct chromosome (Fig. 1). This non-redundant, dispersed distribution suggests that each *GAD* gene is positioned to function independently, potentially reducing overlaps in their roles and regulatory mechanisms.

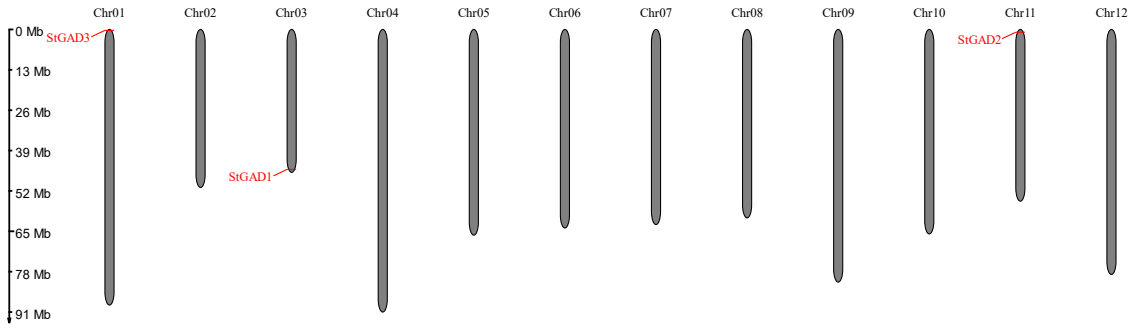

Figure 1: Chromosomal locations of *StGAD* genes in the potato genome, visualized using the MG2C tool. Chromosome numbers are indicated at the top of each chromosome, and the genomic scale in megabases (Mb) is provided on the left.

The GAD genes are strategically placed across the genome. This spatial organization may indicate that each gene contributes uniquely to the plant's physiological processes, particularly in stress-related responses [23-24]. The independent localization of GAD genes across different chromosomes could reflect the plant's adaptive strategy to ensure that multiple regions of the genome are involved in regulating responses to environmental stressors such as drought, salinity, and low temperatures [25-26]. The absence of gene duplication within individual chromosomes further suggests that each GAD gene has a specific role, without functional redundancy within the same chromosomal context. This distribution pattern highlights the importance of a diversified

regulatory mechanism across the genome, providing a genetic framework that allows specialized and flexible responses to environmental challenges [13-27].

3.2. Phylogenetic and protein – protein interaction (PPI) analysis of StGAD proteins

The phylogenetic tree of *StGAD* genes illustrates a likely gene duplication process (Fig. 2). *StGAD2* and *StGAD3* seem to have recently diverged and remain closely related, possibly retaining similar or overlapping functions. *StGAD1*, as the most divergent gene, might have evolved under different evolutionary pressures, taking on specialized or stress-related functions. These evolutionary patterns reflect the complex adaptive strategies employed by plants, where gene duplication allows for functional diversification and enhanced resilience to environmental changes [28-30].

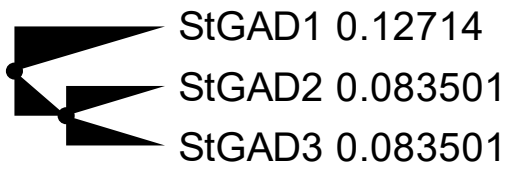

Figure 2: Phylogram of *StGAD* genes illustrating evolutionary divergence with branch lengths indicating genetic distances.

Protein–protein interactions (PPIs) are essential for coordinating numerous biological processes, including cellular regulation, metabolic pathways, and intercellular communication. Alterations or disruptions in these interactions can result in profound physiological and pathological consequences, highlighting their importance in maintaining homeostasis [31]. STRING Analysis showed that all three *StGAD* genes belong to the group II decarboxylase family and function as glutamate decarboxylase. StGAD1 and StGAD2 have 502 aa while StGAD3 has 497 aa. Despite the sequence homology between StGAD2 and StGAD3 (Fig), StGAD1 and StGAD2 genes share more protein homology and co-occurred based on STRING analysis (Fig. 3). This network highlights potential functional relationships and evolutionary conservation among the GAD proteins

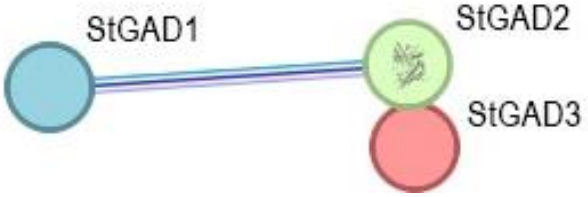

Figure 3: Protein-Protein Interaction (PPI) Network of GAD proteins in *S. tuberosum*: The connections between the nodes represent protein homology, gene co-occurrence, and interactions documented in curated databases.

3.3. 3D modelling and subcellular localization of StGAD proteins

The three StGAD proteins—StGAD1, StGAD2, and StGAD3—share a conserved globular core composed of α-helices, suggesting that all three proteins perform related enzymatic or structural roles. However, their terminal regions exhibit distinct structural differences, influencing their specific subcellular localization and functions (Fig. 4).

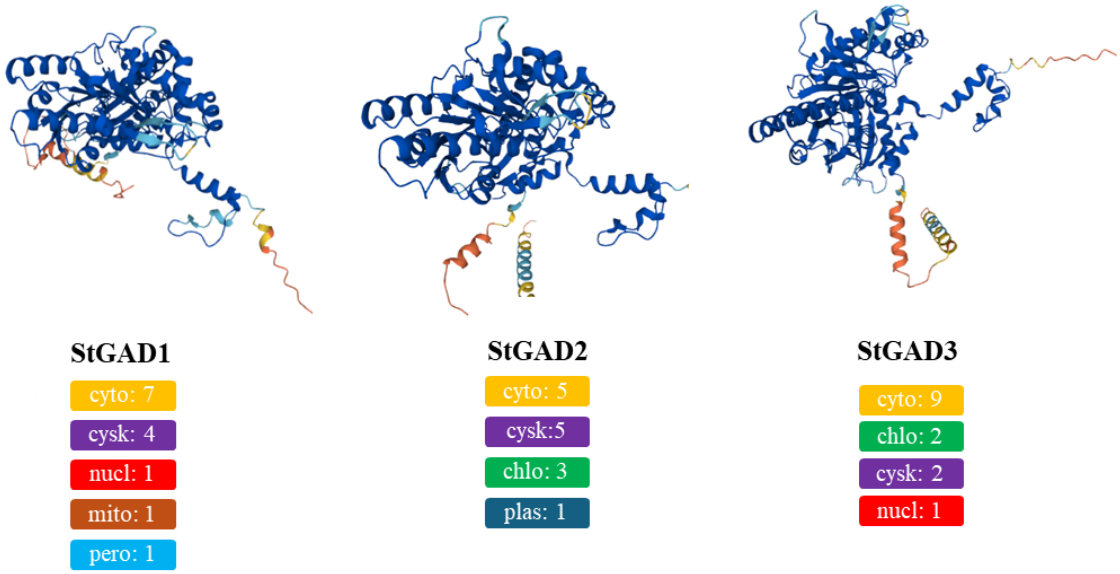

Figure 4: 3D Homology modeling and subcellular localization of StGAD proteins (cyto: Cytoplasm, cysk: Cytoskeleton, nucl: Nucleus, mito: Mitochondria, pero: Peroxisome, chlo: Chloroplast, plas: Plasma Membrane).

StGAD1 is localized to the cytoplasm and cytoskeleton, which aligns with its compact structure and short yellow helix. This design indicates that it functions in metabolic activities or acts as a structural scaffold associated with the cytoskeleton, maintaining stability and requiring minimal conformational flexibility. StGAD2 is predicted to localize to the mitochondria and peroxisome, as indicated by its extended red helix, which suggests a role in membrane interaction. This feature implies that StGAD2 may participate in energy metabolism within mitochondria or help with molecular transport in peroxisomes.

StGAD3 is found in the nucleus and plasma membrane, as highlighted by its flexible long coil and outward-extending red helix. This structural flexibility suggests that StGAD3 is involved in dynamic processes, such as nuclear signaling, gene regulation, or transport across membranes. Its design aligns with the need for proteins that interact with transport receptors or regulate membrane-bound activities.

Overall, StGAD1, StGAD2, and StGAD3 share a conserved core structure that supports related functions, but their variable terminal regions enable them to adapt to specific subcellular

compartments. StGAD1 operates in the cytoplasm and cytoskeleton, StGAD2 localizes to mitochondria and peroxisomes, and StGAD3 functions within the nucleus and plasma membrane. These structural differences empower them to perform specialized roles such as scaffolding, energy metabolism, molecular transport, and signaling, according to the demands of their respective environments.

3.4. Expression Profiles of *StGAD* Genes Under Drought and Salt Stresses

The expression analysis reveals distinct responses of the *StGAD1* and *StGAD2* genes to drought and salt stress in both root and leaf tissues. In root tissues, *StGAD1* was upregulated under drought stress, indicating that it plays an essential role in drought adaptation. This result is consistent with previous studies, which have shown that *GAD* genes contribute to stress tolerance by modulating γ-aminobutyric acid (GABA) accumulation, helping to maintain osmotic balance and mitigate oxidative stress [4]. Under the same drought conditions, *StGAD2* was significantly downregulated, with a negative value of -1.27. This repression suggests that *StGAD2* is not involved in drought response, while *StGAD1* functions as the primary gene regulating the root's metabolic adjustments under drought stress.

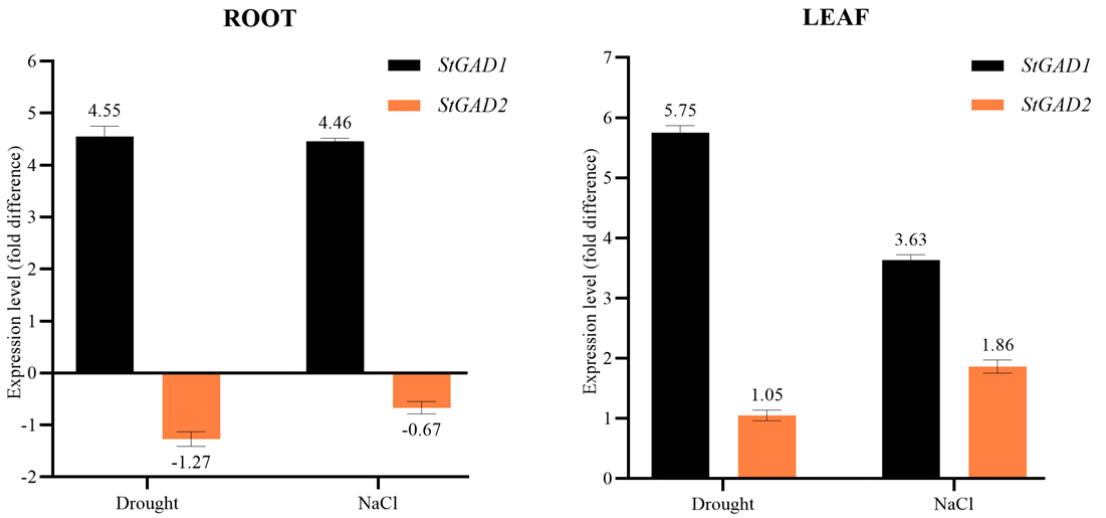

Figure 5: Expression profiles of *StGAD* genes in potato roots and leaves under drought and salt stress, assessed via RT-qPCR. Bars above the x-axis indicate upregulation, while values are presented on a $\log_2$ scale to clearly reflect the extent of both upregulation and downregulation. Error bars represent the standard deviation of the mean (SDOM; n = 3).

In leaf tissues, *StGAD1* expression was strongly upregulated, with a value of 5.75 under drought stress, confirming its prominent role in leaf stress adaptation. This upregulation aligns with reports that enhanced GAD activity promotes GABA accumulation, which serves as a signaling molecule to regulate stomatal closure and maintain water balance [32]. The data in the

present study confirm that *StGAD1* plays a critical role in maintaining cellular homeostasis under drought conditions, likely by preventing oxidative damage and supporting osmotic balance in leaf tissues. However, *StGAD2* did not exhibit a significant increase in expression under either drought or salt stress, indicating that this gene is not actively involved in the leaf's response to these abiotic stresses.

These findings reveal that the expression of *StGAD1* and *StGAD2* is regulated in a tissue-specific manner under drought and salt stress, with *StGAD1* serving as the primary stress-responsive gene in both roots and leaves. *StGAD1*'s upregulation under drought conditions underscores its role in stress adaptation, particularly through the GABA pathway, which supports water-use efficiency and oxidative stress management. In contrast, the downregulation of *StGAD2* in roots suggests that its function is not essential during drought, reflecting a shift in metabolic priorities. Furthermore, the lack of significant expression of *StGAD2* in leaves under stress conditions indicates that its involvement in abiotic stress response is limited compared to *StGAD1*.

The differential expression patterns observed in this study are consistent with previous findings. Li et al. (2016) [32] have reported that GAD genes are involved in regulating GABA signaling under osmotic stress, while Shelp et al. (2012) [4] demonstrated that GABA plays a crucial role in alleviating oxidative stress in plants. The upregulation of *StGAD1* in both roots and leaves during drought suggests a conserved function for this gene in stress adaptation, while the suppression of StGAD2 points to a functional divergence between the two isoforms, with *StGAD1* being the dominant regulator in the glutamate-GABA pathway during abiotic stress.

Notably, no expression of *StGAD3* was detected in either leaves or roots under both drought and salt stress. This absence suggests that *StGAD3* may not play a role in these specific environmental conditions. It is possible that *StGAD3* could be involved in other stress types, developmental stages, or tissues not covered in this study. Alternatively, it could be that the gene is expressed only under specific hormonal signals or environmental triggers, such as biotic stress or nutrient imbalances, which were not evaluated here [1]. This finding underscores the importance of further research to explore the broader functions of *StGAD3* and its role in plant development and stress responses under different conditions.

These findings highlight the distinct roles of *StGAD1* and *StGAD2* in stress response, with *StGAD1* emerging as a key player in drought tolerance, particularly in leaves, where it drives GABA synthesis and modulates cellular responses. *StGAD2* provides complementary support, maintaining basal GABA levels across both drought and salt conditions.

These results emphasize the stress- and tissue-specific regulation of GABA-related genes in plants and suggest that GABA metabolism is more relevant for drought adaptation than salinity tolerance in potatoes. Future studies should investigate the interaction between GABA metabolism and other physiological pathways to better understand the crosstalk between drought and salinity responses. Additionally, the role of *StGAD3* under different conditions or in other tissues should be explored to determine its potential involvement in stress adaptation beyond those examined in this study.

4. Conclusion

This study investigates the identification and functional roles of *GAD* genes in potato in response to drought and salt stress. *StGAD1* plays a key role in drought adaptation by promoting GABA accumulation, which regulates osmotic balance, reduces oxidative stress, and improves water-use efficiency. In contrast, *StGAD2* contributes minimally, maintaining baseline GABA levels but not actively participating under stress. No expression of *StGAD3* was observed, suggesting it may function under different conditions or developmental stages.

The chromosomal distribution shows each *GAD* gene located on distinct chromosomes, suggesting unique functions. Phylogenetic analysis reveals that *StGAD1* has evolved specialized stress-related roles, while *StGAD2* and *StGAD3* share closer evolutionary origins. Subcellular localization predictions indicate that *StGAD1* operates in the cytoplasm and cytoskeleton, *StGAD2* in mitochondria and peroxisomes, and *StGAD3* in the nucleus and plasma membrane. Protein-protein interaction (PPI) analysis shows functional relationships among the *GAD* proteins, particularly between *StGAD1* and *StGAD2*.

This research concludes that GABA metabolism, driven by *StGAD1*, is more relevant for drought adaptation than for salt tolerance, where other pathways like ion regulation take precedence. Future studies are needed to explore the role of *StGAD3* under different conditions and the interactions between GABA and other physiological pathways. These insights are valuable for improving crop resilience to environmental stressors through targeted genetic interventions.

Acknowledgments

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