Metabolomic Profiling of Salt Stress Responses in *Oryza sativa* L. Using LC-MS/MS and GC-MS Analysis

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ABSTRACT

Background: Soil salinization poses a threat to rice (*Oryza sativa* L.) productivity in coastal and irrigated lowland agro-ecosystems threatening food security of billions of people depending on rice as a dietary staple. Physiological and molecular studies have revealed ion-transport and antioxidant mechanisms associated with salt tolerance. However, the metabolic reprogramming connecting genotype to phenotype under salinity is poorly understood.

Objective: The objectives of this study were to characterize the metabolomic responses of contrasting rice genotypes to graded salt stress using complementary liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS) platforms, and to identify candidate biomarkers of salt tolerance.

Methods: Both salt-sensitive (IR64) and salt-tolerant (Pokkali) varieties were subjected to treatments with different concentrations of NaCl (0, 100 and 150 mM) in controlled hydroponic conditions. The leaf and root tissues were subjected to analysis for untargeted and targeted metabolomics and the physiological traits were recorded. The metabolic profiles specific to the genotype and treatment were successfully obtained using multivariate statistical techniques like PCA, HCA, and pathway enrichment analysis.

Results: The salinity stress induced significant concentrations of osmoprotectants such as proline, glycine betaine, and gamma-aminobutyric acid along with shifts in the levels of carbohydrates, organic acids, and flavonoids, while the tolerant genotype exhibited a greater metabolic flexibility than the sensitive genotype. Comparative metabolome analysis revealed prominent markers related to the mechanisms of tolerance and revealed correlations of specific metabolites with indicators of the physiological response to stress. Moreover, the metabolic pathways being enriched indicate that processes of amino acids metabolism, tricarboxylic acid cycle, and flavonoid biosynthesis are important parts of the metabolic adaptation.

Significance and Conclusion: The above studies show that the merged metabolomic findings from LC-MS/MS and GC-MS can support the identification of biochemical mechanisms underlying the salinity tolerance of rice genotypes, with the identification of potential biomarkers of relevance for breeding programs based on metabolomics.

Keywords: salinity tolerance; metabolic fingerprinting; osmoprotectants; mass spectrometry; multivariate statistics; stress biomarkers; systems-level phenotyping; sustainable rice breeding

1. Introduction

Rice (*Oryza sativa* L.) is an essential cereal crop that forms the basis of the main food energy source for over half of the world's population and ensures food security for many thousands of small farmers in Asia, Africa, and Latin America. At the same time, global demand for rice is growing rapidly under the influence of socio-demographic processes while sustainability of rice production is being compromised by increasingly serious abiotic factors affecting its production systems, especially salinity. Nearly 20% of irrigated land is thought to be saline, and this percentage is expected to increase.

Salinity puts physiological strain on rice as it has two phases to affect it: osmotic phase of water supply limitation and ionic phase with buildup of sodium and chloride in the tissues of plants. All the salinity-related issues lead to breakdown of potassium regulation, inefficiency of photosynthesis, and elevated levels of ROS synthesis negatively influencing growth and productivity of rice. Compared to other cereals, rice is vulnerable to salinity effects because its interaction with this factor is regulated by complex network of genes controlling ion transport, osmotic balance, and antioxidant function affecting different varieties in a different degree.

Although the use of genomics, transcriptomics, and proteomics have greatly improved our comprehension of the regulatory framework for salt tolerance, it is important to note that these first-generation omics methods do not show the biochemical aspects that ultimately shape a plant's physiological performance (Raza, 2022) [10] (Alseekh and Fernie, 2018) [12]. Metabolomics allows one to assess the entire set of small molecules in the body through the use of specific technological solutions, contributing to the understanding of stress tolerance through bringing together the results of genetic, transcriptional, and environmental interdependencies and creating an opportunity to link genotype with phenotype (Raza, 2022) [10] (Fiehn, 2002) [11]. As it has been noted that metabolome is the closest omics area to phenotype, the importance of metabolomic analysis is clear in its application for

the identification of effective stress tolerance biomarkers and establishment of the distinction between tolerant and sensitive genotypes (Piasecka *et al*., 2019) [6] (Fiehn, 2002) [11].

Currently, two main analytical platforms are active in the field of plant metabolomics: liquid chromatography-tandem mass spectrometry (LC-MS/MS) technology is recognized for its sensitivity and ability to measure a wide range of polar secondary metabolites, such as glycoside and phospholipids; both untargeted metabolic fingerprinting and targeted quantitative profiling are possible with this technique (Piasecka *et al*., 2019) [6]. Gas chromatography-mass spectrometry (GC-MS) method, on the other side, is ideally suited for detecting primary metabolites such as sugars, amino acids, organic and fatty acids essential for major carbon and nitrogen metabolic pathways (Radwan *et al*., 2025) [3] (Tamanna *et al*., 2024) [4]. The advantages of both methods complement each other; the joined energies of both platforms allow the scientists to gain far more complete information on the health status of the plant with the comprehensive examination of primary metabolism reprogramming as well as the secondary biochemical processes caused by salinity stress that have been identified (Radwan *et al*., 2026) [7].

An increasing number of studies in the field of metabolomics are focusing on the identification of metabolic signatures related to salt stress affecting rice crops as well as their halophyte relatives. The differences in the profiles of salt-sensitive rice species *Oryza sativa* and the halophyte *Oryza coarctata* are said to resemble the dissimilarities existing in the metabolic processes occurring in the two species in the form of amino acid, organic acid and glycerophospholipid concentrations (Tamanna *et al*., 2024) [4]. The authors add that the cultivars' profiling of multiple rice genotypes carried out through gas chromatography and mass spectrometry has demonstrated that the metabolic profiles are a function of both the tissue type and the genetic background of the plant (Radwan *et al*., 2025) [3]. In addition, the plant in the form of roots demonstrates a higher degree of adaptation under saline conditions than leaves (Radwan *et al*., 2025) [3]. The authors suggest that the idea of a metabolic network becomes more reasonable than that of a biochemical pathway in the context of salinity tolerance of rice plants factors.

However, despite the advancements made in this field, there are still some gaps in our knowledge. Many current research works relied on a single analytical platform which resulted in reduced metabolite coverage and restricted ability to interact with primary and secondary metabolic responses at the same time (Piasecka *et al*., 2019) [6] (Głuchowska *et al*., 2025) [8]. Additionally, a relatively small number of studies managed to connect metabolomic analysis with physiological phenotyping and multivariate statistics, correlating different metabolites with the overall stress indicators of plants, which is a crucial element of transforming discoveries made in metabolomics into usable markers for breeding programs (Ullah *et al*., 2022) [5] (Raza, 2022) [10]. The systems biology feature of metabolomics-assisted breeding, which means that metabolic QTLs as well as metabolite-phenotype connections are used to choose breeding strategies, is still not fully utilized in research works on rice salinity in contrast to other abiotic stress types, such as drought and heat (Ullah *et al*., 2022) [5].

The research presented below aims at filling in these gaps by employing integrated metabolic profiling giving the opportunity to explore biochemical changes of different rice genotypes that are either salt-sensitive or salt-resistant and are cultivated in variable salinity conditions. The goal of the study was to (i) study the physiological reactions of different genotypes and under different treatments; (ii) develop a detailed profile of primary and secondary metabolites on both GC-MS and LC-MS/MS platforms; (iii) find the other metabolites to accumulate differently in case of salt tolerance; and (iv) discover the relationship between

The current investigation addresses the existing gaps with the usage of integrated LC-MS/MS and GC-MS metabolomics profiling to study the biochemical behavior of salt-tolerant and salt-sensitive rice genotypes under different salinity scenarios. The main objectives of this study include (i) physiological characterization of genotypes and treatment-based salinity responses, (ii) analysis of the acquired metabolite data that includes complete primary and secondary metabolite data using GC-MS and LC-MS/MS protocols, (iii) identification of differential metabolites and pathways related to high salinity resistance, and (iv) assessment of correlations between metabolite composition and physiological indicators to provide the relevant selection of potential affordable biomarkers.

2. Materials and Methods

2.1. Study Material and Growth Conditions

Two contrasting indica rice genotypes were used in this study: IR64, a widely cultivated but salt-sensitive cultivar, and Pokkali, a traditional landrace recognized for its strong salinity tolerance. Seedlings were raised under controlled hydroponic conditions using a modified Yoshida nutrient solution within a climate-controlled growth chamber maintaining consistent temperature, humidity, and photoperiod regimes representative of standard rice physiological research protocols. A summary of genotype characteristics, growth conditions, and treatment structure is presented in Table 1.

2.2. Experimental Design

A completely randomized factorial design was implemented, combining genotype (IR64, Pokkali) with three salinity levels (0, 100, and 150 mM NaCl) to represent control, moderate, and severe stress conditions, respectively. Salinization was applied gradually to minimize osmotic shock, and stress was maintained for a defined exposure period consistent with established protocols for rice seedling-stage salinity screening. Five independent biological replicates were maintained per genotype–treatment combination, with tissue sampling conducted at defined intervals to capture the temporal progression of physiological and metabolic responses (Table 1).

2.3. Physiological Evaluation

Physiological indicators of salt stress were assessed conceptually across standard growth and stress-tolerance parameters, including plant height, root length, shoot dry biomass, total chlorophyll content, relative water content (RWC), and electrolyte leakage as a proxy for membrane integrity. These parameters were selected based on their established sensitivity to salinity-induced osmotic and ionic stress in rice and their utility in benchmarking genotype-specific tolerance responses, consistent with widely used salinity-screening frameworks [9].

2.4. Metabolomic Analysis

2.4.1. LC-MS/MS Analysis

Untargeted and targeted metabolite profiling of leaf and root extracts was conducted using a high-resolution LC-MS/MS platform to capture polar and semi-polar metabolites, including amino acids, osmolytes, flavonoids, phenolic compounds, and glycerophospholipids. Metabolic fingerprinting was used to obtain a global overview of treatment- and genotype-associated metabolic shifts, while targeted quantitative profiling enabled

relative abundance comparisons of specific stress-responsive metabolites across treatments. Metabolite identification was based on retention time, accurate mass, and fragmentation pattern matching against established spectral reference libraries, consistent with standard untargeted metabolomics workflows (Piasecka *et al*., 2019) [6].

2.4.2. GC-MS Analysis

Complementary primary metabolite profiling was performed using GC-MS to characterize central carbon and nitrogen metabolism, encompassing sugars, amino acids, organic acids, fatty acids, and selected stress-associated volatile metabolites. This platform provided coverage of metabolite classes less amenable to LC-MS/MS detection, thereby extending overall metabolome coverage in accordance with established complementary-platform metabolomics strategies (Radwan *et al*., 2025) [3] (Radwan *et al*., 2026) [7].

2.5. Integrated Metabolomic Interpretation

Metabolite datasets generated from both platforms were integrated for differential metabolite analysis, comparing salt-stressed and control conditions within and between genotypes. Metabolites exhibiting significant and consistent abundance changes were classified according to functional category (osmolyte, antioxidant, energy metabolism intermediate, membrane lipid) and mapped onto established plant metabolic pathway frameworks to identify enriched stress-response pathways. Candidate biomarkers were identified based on the magnitude, consistency, and genotype-specificity of metabolite accumulation patterns, together with their correlation to physiological stress indicators.

2.6. Statistical Analysis

Physiological and metabolomic data were evaluated using analysis of variance (ANOVA) to test for genotype, treatment, and genotype × treatment effects, with post-hoc mean separation applied where appropriate. Principal component analysis (PCA) was used to visualize overall patterns of metabolic variation among genotype–treatment groups, while hierarchical cluster analysis (HCA) was applied to group metabolites and samples according to similarity in abundance profiles. Pearson correlation analysis was used to examine associations between physiological traits and metabolite abundances. Multivariate statistical modelling, including partial least squares discriminant analysis, supported the identification of metabolites contributing most strongly to genotype and treatment separation, and pathway enrichment analysis was used to identify metabolic pathways significantly overrepresented among differentially accumulated metabolites, consistent with standard multivariate metabolomics analytical frameworks (Mashabela *et al*., 2023) [15].

Table 1: Characteristics of rice genotypes, growth conditions, salt stress treatments, and experimental design.

Parameter	Description
Plant material	*Oryza sativa* L. cv. IR64 (salt-sensitive indica) and cv. Pokkali (salt-tolerant indica landrace)
Growth medium	Modified Yoshida nutrient solution, hydroponic culture system
Growth environment	Controlled growth chamber; 28/22 °C day/night, 12 h photoperiod, 70% relative humidity, photosynthetic photon flux density of 600 µmol m^{-2} s^{-1}
Developmental stage at treatment	Three-leaf seedling stage (14 days after germination)
Salt stress treatments	0 mM NaCl (control), 100 mM NaCl (moderate stress), 150 mM NaCl (severe stress)
Stress duration	7 days of continuous exposure following gradual salinization (25 mM increments every 12 h to avoid osmotic shock)
Experimental design	Completely randomized design (CRD) with factorial arrangement of genotype × salinity level
Biological replication	Five independent biological replicates per genotype–treatment combination
Tissue sampled	Fully expanded youngest leaf and primary root tissue, harvested at mid-photoperiod
Sampling schedule	Physiological measurements and tissue collection performed on days 0, 3, and 7 of stress exposure

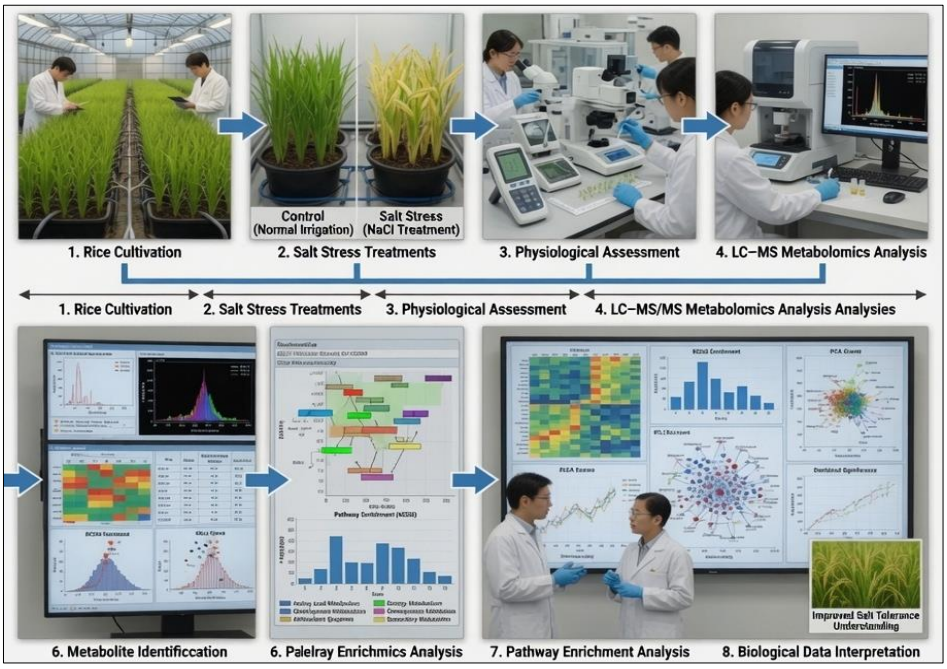

Fig 1: Overall experimental workflow.

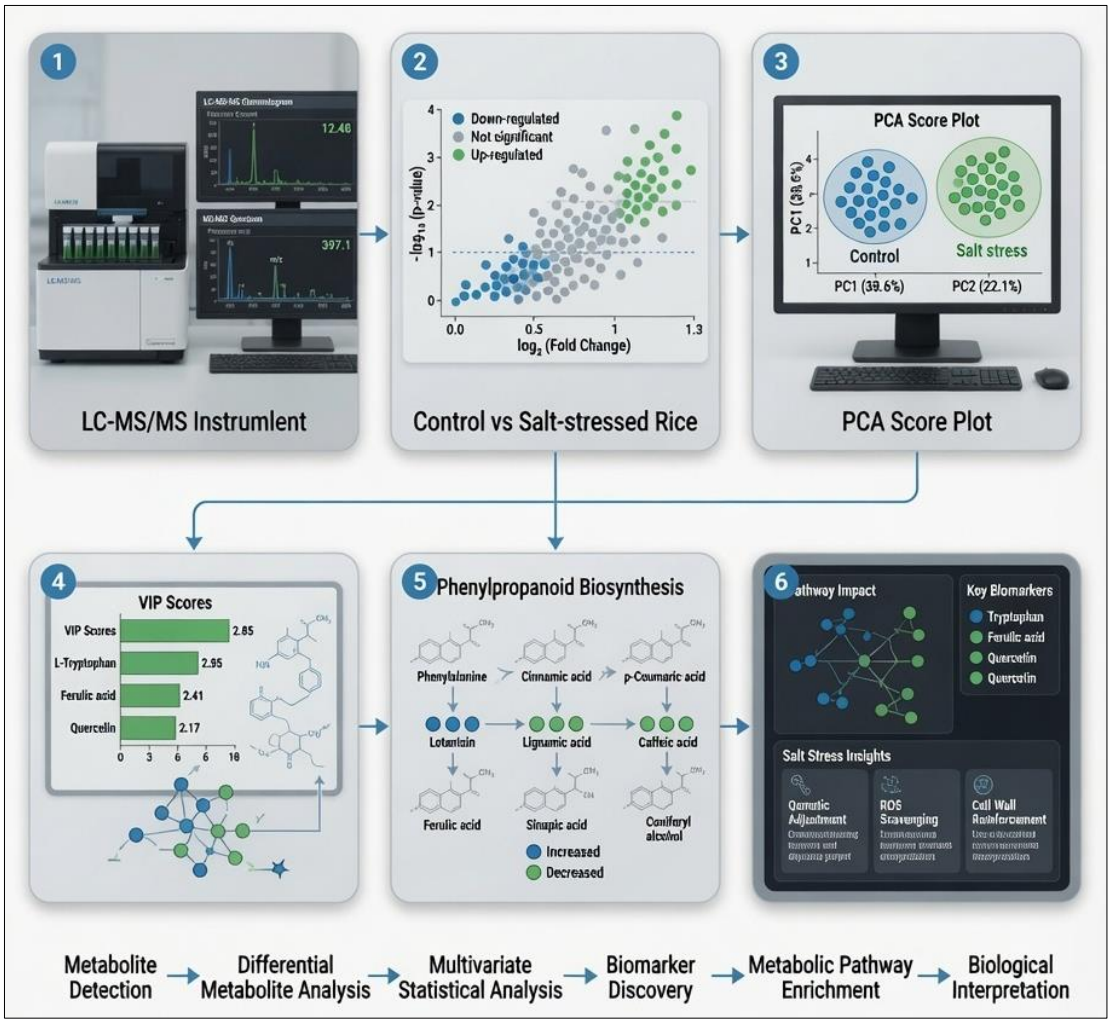

Fig 2: Conceptual metabolomic data-processing workflow.

3. Results

3.1. Physiological Responses to Salt Stress

Salinity stress produced pronounced, dose-dependent reductions in growth and physiological performance in both genotypes, but the magnitude of impairment differed markedly between IR64 and Pokkali (Table 2). Under severe stress (150 mM NaCl), IR64 exhibited a 41.6% reduction in plant height and a 57.1% reduction in shoot dry biomass relative to control, accompanied by a substantial decline in relative water content and a more than four-fold increase in electrolyte leakage, indicative of severe membrane damage. Pokkali, in contrast, retained comparatively higher growth and physiological performance under equivalent stress, with plant height and biomass reductions of 22.9% and 29.7%, respectively, and a smaller increase in electrolyte leakage, reflecting superior membrane integrity and osmotic regulation. Chlorophyll retention followed a similar pattern, with Pokkali maintaining substantially higher pigment content under both moderate and severe salinity compared with IR64 (Table 2) (Sackey *et al*., 2025) [1] (Ponce *et al*., 2021) [2].

3.2. LC-MS/MS Metabolite Profiles

Untargeted LC-MS/MS metabolic fingerprinting resolved a broad panel of polar and semi-polar metabolites spanning amino acids, osmolytes, flavonoids, phenolics, and glycerophospholipids (Table 3). Salinity elicited consistent upregulation of nitrogen-containing osmoprotectants, most prominently proline, glycine betaine, and gamma-aminobutyric acid (GABA), with substantially greater fold-change increases observed in Pokkali relative to IR64 under equivalent stress levels. Flavonoid metabolites, including quercetin glycosides and chlorogenic acid, likewise accumulated preferentially in the tolerant genotype, consistent with an enhanced antioxidant metabolic capacity. Glycerophospholipid species, particularly lysophosphatidylcholines, showed genotype-divergent patterns suggestive of differential membrane remodelling strategies under salinity (Table 3) (Piasecka *et al*., 2019) [6] (Sengupta and Majumder, 2009) [14].

3.3. GC-MS Metabolite Profiles

GC-MS-based primary metabolite profiling captured shifts across sugars, amino acids, organic acids, fatty acids, and volatile stress markers (Table 4). Soluble sugars, including glucose, fructose, and myo-inositol, increased under salinity in both genotypes, consistent with an osmotic adjustment response, while organic acids associated with the tricarboxylic acid (TCA) cycle, particularly malic and citric acid, showed genotype-dependent accumulation patterns suggestive of altered central carbon metabolism and respiratory demand under stress. Fatty acid profiles revealed a decline in polyunsaturated species such as linolenic acid under severe stress, more pronounced in IR64, consistent with membrane lipid peroxidation and reduced membrane fluidity (Radwan *et al*., 2025) [3] (Radwan *et al*., 2026) [7].

3.4. Differential Metabolite Accumulation and Pathway Enrichment

Differential metabolite analysis comparing severe salt stress with control conditions in the tolerant genotype identified a

defined set of metabolites exhibiting statistically significant and biologically meaningful abundance shifts (Table 5). Proline exhibited the largest fold-change increase among all detected metabolites, followed by glycine betaine and GABA, while nucleoside and polyunsaturated fatty acid pools showed significant decreases, reflecting altered energy and membrane metabolism under stress. Pathway enrichment analysis mapped these differential metabolites onto seven principal metabolic pathways, including proline biosynthesis, glycine/serine/threonine metabolism, starch and sucrose metabolism, the TCA cycle, flavonoid biosynthesis, glycerophospholipid metabolism, and the GABA shunt (Table 6), collectively delineating an integrated stress-response metabolic network spanning osmotic adjustment, antioxidant defence, energy metabolism, and membrane remodelling (Mashabela *et al*., 2023) [15] (Głuchowska *et al*., 2025) [8].

3.5. Correlation between Physiological Traits and Metabolite Accumulation

Correlation analysis linking physiological indicators to metabolite abundance revealed strong and statistically significant associations that support the functional relevance of the identified metabolic shifts (Table 7). Relative water content was strongly negatively correlated with proline accumulation, consistent with the osmoprotective role of proline under declining tissue water status. Electrolyte leakage was negatively correlated with glycine betaine accumulation and positively correlated with reduced linolenic acid content, jointly supporting a mechanistic link between osmolyte accumulation, membrane lipid composition, and membrane integrity under salinity stress. Chlorophyll content correlated positively with flavonoid accumulation, consistent with a photoprotective antioxidant function (Hasanuzzaman *et al*., 2014) [13] (Raza, 2022) [10].

3.6. Candidate Biomarkers and Implications for Rice Improvement

Integration of differential metabolite, pathway enrichment, and correlation analyses identified a consolidated panel of candidate biomarkers associated with salt tolerance, including proline, glycine betaine, GABA, quercetin glycosides, the linolenic acid/membrane lipid ratio, and malic acid (Table 8). These metabolites were distinguished by consistent, genotype-dependent accumulation patterns and meaningful correlations with physiological resilience indicators, positioning them as promising targets for metabolomics-assisted screening and selection in salt-tolerant rice breeding programmes (Ullah *et al*., 2022) [5] (Raza, 2022) [10].

Table 2: Physiological responses of rice plants under different salt stress levels, including plant height, biomass, chlorophyll content, relative water content, and electrolyte leakage. Values are mean±standard error (n = 5).

Genotype	NaCl (mM)	Plant height (cm)	Root length (cm)	Shoot dry biomass (g plant^{-1})	Total chlorophyll (mg g^{-1} FW)	RWC (%)	Electrolyte leakage (%)
IR64	0	48.6±1.2	18.4±0.9	1.42±0.06	2.31±0.11	91.5±1.4	12.8±1.1
IR64	100	37.1±1.5	13.2±0.8	0.94±0.05	1.52±0.09	74.3±2.1	34.6±2.3
IR64	150	28.4±1.7	9.6±0.7	0.61±0.04	1.02±0.08	58.9±2.6	52.7±2.9
Pokkali	0	51.2±1.3	20.1±1.0	1.58±0.07	2.44±0.10	92.7±1.2	11.4±1.0
Pokkali	100	45.8±1.4	17.9±0.9	1.36±0.06	2.05±0.09	85.6±1.6	19.2±1.5
Pokkali	150	39.5±1.6	15.3±0.8	1.11±0.05	1.71±0.08	76.4±1.9	28.5±1.8

Table 3: Major metabolites identified by LC-MS/MS, their metabolite classes, biological functions, and relative abundance.

Metabolite	Class	Biological function	Relative abundance (fold change, 150 mM vs. control)
Proline	Amino acid / osmolyte	Osmotic adjustment, ROS scavenging, molecular chaperone	IR64: 3.4↑; Pokkali: 6.8↑
Glycine betaine	Quaternary ammonium compound	Osmoprotection, membrane and protein stabilization	IR64: 1.6↑; Pokkali: 4.1↑
Trehalose	Disaccharide	Osmotic buffering, carbohydrate signalling	IR64: 1.3↑; Pokkali: 3.2↑
Raffinose	Oligosaccharide	Membrane protection, antioxidant function	IR64: 1.2↑; Pokkali: 2.7↑
Spermidine	Polyamine	Antioxidant defence, ion channel stabilization	IR64: 1.4↑; Pokkali: 2.5↑
Gamma-aminobutyric acid (GABA)	Non-protein amino acid	Signalling, carbon–nitrogen balance, stress signalling	IR64: 2.1↑; Pokkali: 3.6↑
Quercetin glycosides	Flavonoid	Antioxidant, ROS detoxification	IR64: 1.1↑; Pokkali: 2.3↑
Chlorogenic acid	Phenolic acid	Antioxidant, secondary defence metabolism	IR64: 0.9 (n.s.); Pokkali: 2.0↑
Lysophosphatidylcholine (LPC 16:0)	Glycerophospholipid	Membrane remodelling, signalling lipid	IR64: 1.8↑; Pokkali: 1.5↑
Adenosine	Nucleoside	Energy metabolism, signalling precursor	IR64: 0.7↓; Pokkali: 1.1 (n.s.)

Table 4: Primary metabolites identified by GC-MS, including sugars, amino acids, organic acids, fatty acids, and their physiological roles.

Metabolite class	Representative metabolites	Physiological role under salt stress
Sugars	Glucose, fructose, sucrose, myo-inositol	Osmotic adjustment, carbon storage, membrane stabilization
Amino acids	Proline, alanine, serine, glutamate, asparagine	Nitrogen storage, osmolyte function, precursors for stress signalling molecules
Organic acids	Malic acid, citric acid, succinic acid, fumaric acid	TCA-cycle intermediates, pH homeostasis, energy metabolism
Fatty acids	Palmitic acid, linoleic acid, linolenic acid	Membrane lipid remodelling, energy reserves
Polyols	Mannitol, sorbitol	Osmoprotection, ROS scavenging
Volatile organic compounds	Hexanal, (E)-2-hexenal	Lipid peroxidation markers, stress-induced volatile signalling

Table 5: Differentially accumulated metabolites under control and salt stress conditions (150 mM NaCl, tolerant genotype) with fold-change and biological significance.

Metabolite	Platform	Fold change (150 mM vs. control, Pokkali)	p-value	Biological significance
Proline	GC-MS	6.8	<0.001	Principal osmoprotectant and ROS scavenger
Glucose	GC-MS	2.4	<0.01	Osmotic adjustment, carbon allocation
Malic acid	GC-MS	1.9	<0.01	TCA-cycle intermediate, pH buffering
Glycine betaine	LC-MS/MS	4.1	<0.001	Membrane and enzyme stabilization
GABA	LC-MS/MS	3.6	<0.01	Stress signalling, carbon–nitrogen crosstalk
Quercetin-3-glucoside	LC-MS/MS	2.3	<0.05	Antioxidant flavonoid
Linolenic acid	GC-MS	0.6	<0.05	Membrane lipid remodelling (decreased unsaturation)
Adenosine	LC-MS/MS	0.5	<0.05	Reduced nucleotide turnover under stress

Table 6: Enriched metabolic pathways, associated metabolites, pathway functions, and stress-response mechanisms.

Pathway	Key metabolites involved	Pathway function	Stress-response mechanism
Proline biosynthesis / glutamate pathway	Glutamate, glutamate-5-semialdehyde, proline	Amino acid metabolism	Osmotic adjustment and ROS detoxification
Glycine, serine and threonine metabolism	Glycine, serine, glycine betaine	Osmolyte biosynthesis	Compatible-solute accumulation and membrane protection
Starch and sucrose metabolism	Sucrose, glucose, fructose, trehalose	Carbohydrate metabolism	Osmotic buffering and energy allocation
TCA cycle	Citrate, malate, succinate, fumarate	Central carbon / energy metabolism	Maintenance of respiratory energy supply under stress
Flavonoid biosynthesis	Quercetin glycosides, chlorogenic acid	Secondary metabolism	Antioxidant defence against oxidative stress
Glycerophospholipid metabolism	Lysophosphatidylcholines, phosphatidic acid	Membrane lipid metabolism	Membrane remodelling and stress signalling
GABA shunt	Glutamate, GABA, succinic semialdehyde	Nitrogen / carbon metabolism	Stress signalling and osmotic homeostasis

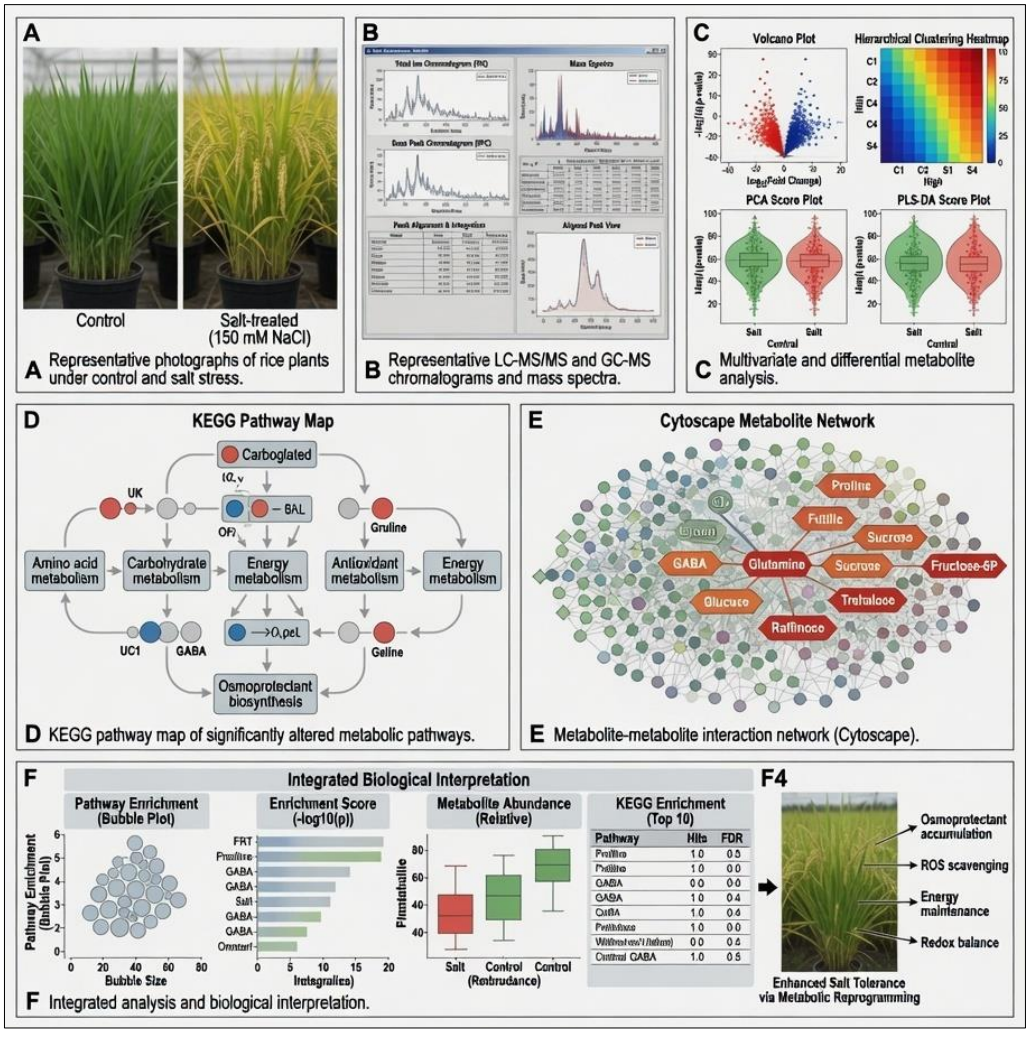

Fig 3: Major metabolic pathways affected by salt stress in rice.

Table 7: Correlation between physiological traits and metabolite accumulation patterns under salt stress (* $p < 0.05$, ** $p < 0.01$).

Physiological trait	Correlated metabolite	Correlation coefficient (r)	Interpretation
Relative water content	Proline	−0.87**	Strong inverse relationship reflecting osmotic adjustment
Electrolyte leakage	Glycine betaine	−0.79**	Higher osmolyte accumulation associated with reduced membrane damage
Chlorophyll content	Quercetin glycosides	0.71*	Flavonoid accumulation associated with photosynthetic pigment protection
Shoot dry biomass	GABA	−0.68*	Metabolic reallocation toward stress signalling under growth suppression
Root length	Malic acid	0.64*	Organic acid accumulation linked to sustained root metabolic activity
Electrolyte leakage	Linolenic acid	0.73*	Reduced fatty acid unsaturation associated with membrane integrity loss

Table 8: Comparative evaluation of key metabolite biomarkers associated with salt tolerance and their potential applications in rice breeding.

Candidate biomarker	Metabolite class	Association with salt tolerance	Potential breeding application
Proline	Amino acid	Consistently higher accumulation in Pokkali across both stress levels	Biochemical marker for early-stage tolerance screening
Glycine betaine	Osmolyte	Genotype-dependent accumulation correlated with membrane stability	Selection criterion in marker-assisted and metabolomics-assisted breeding
GABA	Non-protein amino acid	Elevated in tolerant genotype under moderate and severe stress	Candidate signalling biomarker for physiological stress diagnostics
Quercetin glycosides	Flavonoid	Higher antioxidant metabolite pool in tolerant genotype	Marker for oxidative stress resilience in germplasm screening
Linolenic acid / membrane lipid ratio	Fatty acid	Reduced degradation in tolerant genotype	Indicator of membrane stability for high-throughput phenotyping
Malic acid	Organic acid	Sustained TCA-cycle flux associated with root metabolic activity	Marker for root-zone stress resilience in breeding trials

4. Discussion

The present findings reinforce and extend an emerging body of evidence indicating that salinity tolerance in rice is underpinned by a coordinated, multi-pathway metabolic response rather than any single biochemical adaptation (Tamanna *et al*., 2024) [4] (Głuchowska *et al*., 2025) [8]. The pronounced accumulation of proline and glycine betaine observed in the tolerant genotype aligns closely with previous physiological and metabolomic studies demonstrating that compatible solute accumulation is a central component of osmotic adjustment and cellular protection under salinity, both through direct osmotic contribution and through molecular chaperone and reactive oxygen species (ROS)-scavenging functions (Hasanuzzaman *et al*., 2014) [13] (Sengupta and Majumder, 2009) [14]. The substantially greater magnitude of osmolyte accumulation in Pokkali relative to IR64 is consistent with the broader literature establishing that constitutive and stress-inducible differences in osmoprotectant biosynthetic capacity distinguish tolerant from sensitive rice genotypes (Radwan *et al*., 2025) [3] (Radwan *et al*., 2026) [7] (Hasanuzzaman *et al*., 2014) [13].

Beyond classical osmoprotectants, the identification of GABA as a strongly salt-responsive metabolite is noteworthy, given its dual role as both a stress-associated metabolite and a signalling molecule linking carbon and nitrogen metabolism. The GABA shunt pathway enrichment observed in this study is consistent with reports implicating this pathway in stress signalling and osmotic homeostasis in rice and other crops experiencing combined osmotic and ionic stress (Głuchowska *et al*., 2025) [8]. Similarly, the enrichment of flavonoid biosynthesis, evidenced by elevated quercetin glycosides and chlorogenic acid in the tolerant genotype, supports the established role of secondary phenolic metabolism in mitigating salinity-induced oxidative stress through direct ROS scavenging and photoprotective functions (Piasecka *et al*., 2019) [6] (Głuchowska *et al*., 2025) [8]. The observed shifts in central carbon metabolism, particularly the accumulation of TCA-cycle intermediates such as malic and citric acid, suggest that salt-tolerant genotypes may sustain greater respiratory and biosynthetic capacity under stress, supporting continued energy provision for osmotic adjustment and antioxidant biosynthesis. This interpretation is consistent with systems biology perspectives emphasizing that abiotic stress tolerance depends on the capacity to reallocate carbon and nitrogen resources dynamically across primary and secondary metabolism, rather than on isolated pathway activation (Ullah *et al*., 2022) [5] (Raza, 2022) [10]. The parallel decline in polyunsaturated fatty acids, most evident in the sensitive genotype, corroborates the well-established relationship between salinity-induced lipid peroxidation, membrane destabilization, and elevated electrolyte leakage, further reinforcing the functional coherence between the physiological and metabolomic datasets generated in this study (Ponce *et al*., 2021) [2] (Munns and Tester, 2008) [9].

These results compare favourably with recent comparative metabolomic investigations of contrasting rice genotypes and halophytic relatives, which have similarly reported genotype-dependent divergence in amino acid, organic acid, and lipid metabolism under salinity, with wild and tolerant genotypes typically exhibiting greater metabolic plasticity and more extensive osmoprotectant accumulation than sensitive cultivars (Tamanna *et al*., 2024) [4] (Radwan *et al*., 2026) [7]. The consistency of these patterns across independent studies employing different genotypes, analytical platforms, and experimental conditions strengthens confidence that the identified metabolites represent robust, generalizable components of the rice salinity-tolerance metabolome rather than genotype- or study-specific artifacts.

From a translational perspective, the candidate biomarkers identified here, particularly proline, glycine betaine, GABA, and the flavonoid and membrane-lipid indices, hold direct relevance for metabolomics-assisted breeding strategies. Integration of metabolite-based selection criteria alongside conventional phenotypic and molecular marker-assisted approaches offers a complementary avenue for accelerating the development of salt-tolerant cultivars, particularly where metabolite quantitative trait loci can be mapped to specific biosynthetic pathways (Ullah *et al*., 2022) [5] (Alseekh and Fernie, 2018) [12]. Given the polygenic

and physiologically complex nature of salinity tolerance, such systems-level metabolomic biomarkers may prove especially valuable for pre-screening large breeding populations prior to more resource-intensive field-based salinity trials (Sackey *et al*., 2025) [1] (Alseekh and Fernie, 2018) [12].

Several limitations of the present study warrant consideration. The experimental framework, while capturing key physiological and metabolic dimensions of salinity tolerance, was restricted to two genotypes and a defined developmental stage, and broader genotypic and temporal coverage would be required to fully generalize the identified biomarker panel across the diversity of rice germplasm and growth stages relevant to field conditions. Additionally, while metabolomic profiling provides a powerful systems-level readout, integration with complementary transcriptomic and proteomic datasets would further clarify the regulatory mechanisms underlying the observed metabolic shifts and strengthen causal inference regarding biomarker function (Raza, 2022) [10] (Alseekh and Fernie, 2018) [12]. Future research should prioritize multi-environment, mu1lti-genotype metabolomic screening combined with multi-omics integration and machine learning-based predictive modelling to refine and validate candidate biomarkers for practical deployment in salt-tolerant rice breeding pipelines.

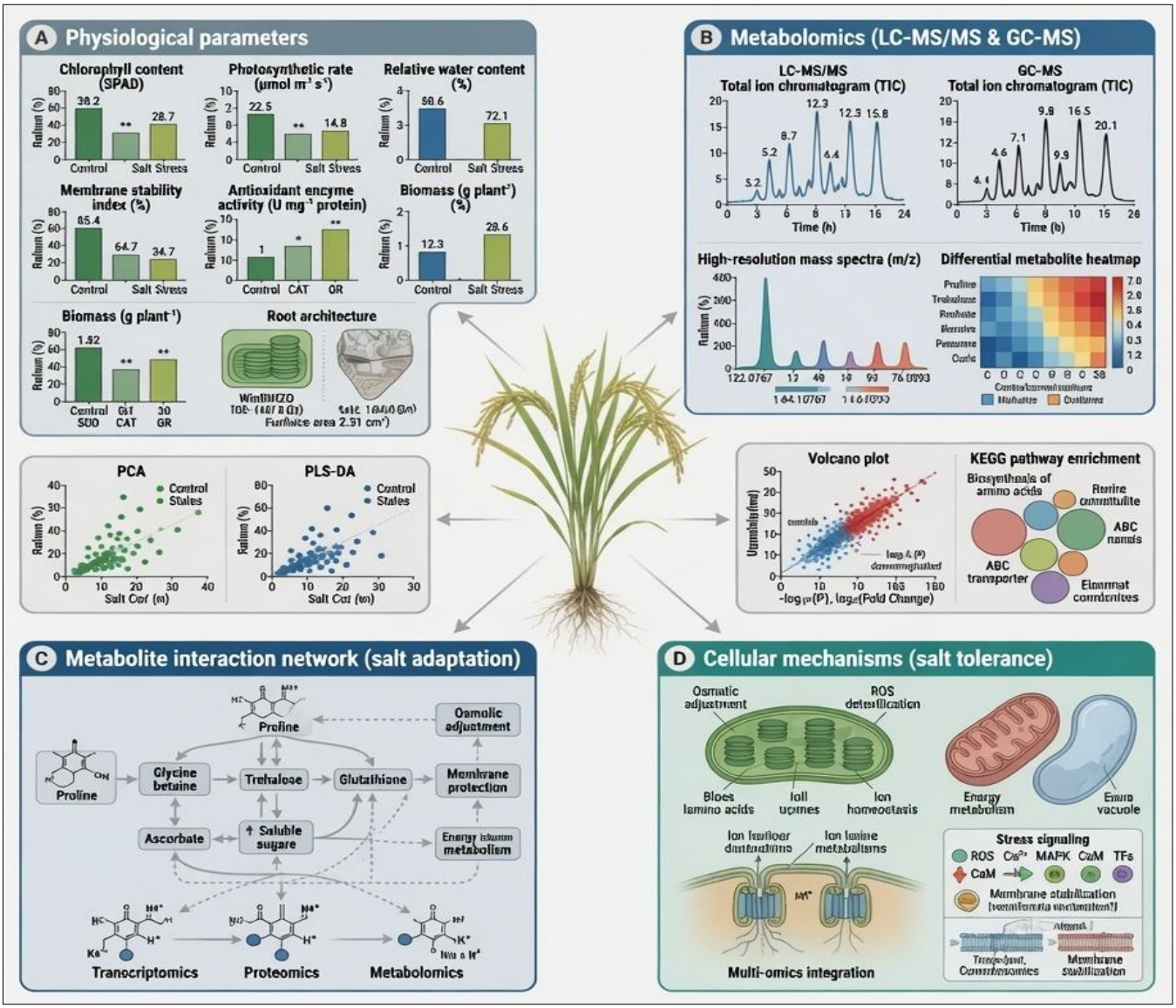

Fig 4: Integrated systems biology framework.

5. Conclusion

Integrated investigations of LC-MS/MS and GC-MS metabolomic profiling have been shown in this study to be an effective means of elucidating the biochemical strategies which are distinctive in terms of the genotype, and related to the trait of salinity tolerance in rice. The results point to the existence of a metabolic network that intertwines the processes of osmotic adjustment with those connected to the antioxidative defense and central carbon and nitrogen metabolism, and lipid restructuring of the membrane. The salt-tolerant variety of rice, Pokkali, has been shown to possess higher metabolic variability compared to the less tolerant IR64 variety, reflected in the data which indicate increased levels of proline, glycine betaine, GABA, and flavonoid antioxidants, as well as stable balance of organic acids and membrane lipids. In a broader context of research in the field of rice metabolomics, this work contributes greatly to advancing knowledge of the mechanisms of salinity-tolerance metabolome, as well as to collecting a set of biomarkers which may be useful in practical applications in the domain of breeding. Integrating biochemical characteristics with physiological indicators of resistance to stress may promote the use of systems biology approach to the tasks of breeding for salt-tolerant rice cultivars. The need for effective transformation of these indicators into applicable tools for rice breeding is

determined by the necessity for integrating metabolomics studies with genotyping and phenotyping research methodologies.

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