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# Proteomics approach to understanding salt stress response in halophytic wild rice species

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**Abstract**
Interest in halophytic wild rice relatives as genetic resources for salt tolerance has grown sharply over the past decade, yet the proteomic basis of their salt-adaptive mechanisms remains poorly mapped. This research used two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) coupled with MALDI-TOF/TOF mass spectrometry to identify differentially expressed proteins in the halophytic species *Oryza coarctata* under 150 mM NaCl stress for 72 hours, compared with the salt-sensitive cultivar IR29. Experiments were conducted at the Plant Biotechnology Laboratory, Siberian Agricultural College, Omsk, from January to August 2023. Leaf and root proteomes were analysed separately. A total of 87 differentially expressed protein spots (≥ 2-fold change, $p<0.05$) were detected in *O. coarctata*, of which 52 were successfully identified by mass spectrometry. These proteins grouped into five functional categories: antioxidant defence (23.1%), signal transduction (19.2%), ion transport (17.3%), osmotic adjustment (21.2%), and structural maintenance (19.2%). Key salt-responsive proteins included superoxide dismutase (SOD, 4.7-fold up), vacuolar $H^+$-ATPase (3.8-fold), and late embryogenesis abundant (LEA) protein (5.1-fold). The cultivated line IR29 showed fewer differentially expressed spots (41) and lower fold changes. These findings highlight candidate proteins for marker-assisted introgression of salt tolerance from *O. coarctata* into cultivated rice.

**Keywords:** Proteomics, salt stress, halophytic rice, *Oryza coarctata*, 2D-PAGE, MALDI-TOF, stress proteins, ion transport, antioxidant defence, wild rice genetic resources

## Introduction
The trend toward exploiting wild crop relatives for abiotic stress tolerance has accelerated as climate models project that salt-affected agricultural land will expand by 25-30% globally by 2050 [1]. Among cereals, rice is the most salt-sensitive, suffering yield declines above 3 dS $m^{-1}$ in soil extract—a threshold exceeded on roughly 40 million hectares of irrigated rice land worldwide [2]. Wild halophytic species within the genus *Oryza*, particularly *O. coarctata* Roxb., can survive salinities above 40 dS $m^{-1}$ and grow in tidal mangrove habitats, making them valuable genetic resources for breeding salt-tolerant rice [3, 4].

Transcriptomic studies have identified thousands of salt-responsive genes in *O. coarctata*, but translating gene expression data into functional understanding requires proteomic validation, since post-transcriptional regulation often decouples mRNA and protein abundance [5]. Two-dimensional gel-based proteomics, despite its limitations in dynamic range, remains a practical first-pass tool for identifying abundant stress-responsive proteins and comparing proteome profiles across genotypes [6].

Previous proteomic work on salt-stressed rice has focused almost entirely on cultivated varieties. Kim and co-workers identified 34 salt-responsive proteins in the tolerant line Pokkali, while Dooki and colleagues found 31 differentially expressed spots in a sensitive-tolerant genotype comparison [7, 8]. No published 2D-PAGE dataset exists for *O. coarctata* under controlled salt stress conditions.

This research aimed to: (a) generate the first comparative leaf and root proteome map of *O. coarctata* versus IR29 under 150 mM NaCl stress; (b) identify differentially expressed proteins by MALDI-TOF/TOF and classify them by functional category; and (c) highlight candidate proteins that may underpin the halophytic species' exceptional salt tolerance. The results are intended to guide future gene-to-protein functional studies and marker-assisted breeding for salt tolerance in rice.

### Instrumentation and Analytical Platform

First-dimension isoelectric focusing (IEF) used 13 cm Immobiline DryStrip gels (pH 3-10 NL, GE Healthcare) on an Ettan IPGphor 3 system. Second-dimension SDS-PAGE was performed on 12.5% polyacrylamide gels using an SE 600 Ruby vertical electrophoresis unit (GE Healthcare). Gels were stained with colloidal Coomassie Brilliant Blue G-250 and scanned at 300 dpi with an ImageScanner III (GE Healthcare). Spot detection and quantification used ImageMaster 2D Platinum v7.0 software. Differentially expressed spots (≥ 2-fold change, $p < 0.05$ by Student's t-test with FDR correction) were excised, in-gel digested with trypsin, and analysed by MALDI-TOF/TOF (Bruker Autoflex Speed, Bruker Daltonics, Germany). Peptide mass fingerprints were searched against the NCBInr and UniProt databases using MASCOT v2.6 (Matrix Science) with a significance threshold of $p < 0.05$ [9].

## Material and Methods

### Material

Seeds of *O. coarctata* (accession IRGC 104502) were obtained from the International Rice Genebank at IRRI, Philippines, under a standard material transfer agreement. Seeds of *O. sativa* cv. IR29 (salt-sensitive check) were sourced from the Siberian Agricultural College seed repository. Plants were grown in a controlled environment chamber (28/22°C day/night, 14 h photoperiod, 400 µmol $m^{-2}$ $s^{-1}$ PAR) in half-strength Yoshida nutrient solution. At the 4-leaf stage (28 days after germination), seedlings were transferred to treatment solutions: 0 mM NaCl (control) or 150 mM NaCl (applied stepwise: 50 mM increments over 3 days). Leaf and root tissues were harvested at 72 h after reaching full salt concentration. Three biological replicates per genotype × treatment combination were maintained [10].

### Methods

Proteins were extracted from frozen tissues by TCA-acetone precipitation: tissues were ground in liquid nitrogen, suspended in 10% TCA in acetone with 0.07% β-mercaptoethanol at −20°C for 2 h, centrifuged, washed three times with cold acetone, and solubilised in IEF buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT). Protein concentration was determined by Bradford assay. For IEF, 250 µg protein was loaded per strip. IEF parameters: 30 V for 12 h (rehydration), 500 V for 1 h, gradient to 1,000 V over 1 h, gradient to 8,000 V over 3 h, and 8,000 V for 4 h. After equilibration, second-dimension separation and staining were carried out as described above. Protein functional classification followed Gene Ontology (GO) annotation through the UniProt database. All experiments were repeated three times independently [11].

### Extraction Protocol Quality Check

Extraction efficiency was verified by resolving 10 µg of each sample on a 1D SDS-PAGE gel (12%) to confirm consistent band patterns and absence of degradation before committing to 2D analysis. Gel-to-gel reproducibility was assessed by calculating the coefficient of variation (CV) for normalised spot volumes across triplicate gels; mean CV was 14.2% for *O. coarctata* and 16.1% for IR29, both within the acceptable range (≤ 20%) for comparative 2D-PAGE analysis [6].

## Results

**Table 1:** Summary of differentially expressed protein spots (≥ 2-fold, $p < 0.05$) in leaf and root proteomes under 150 mM NaCl

| Genotype / Tissue | Total Spots | Up- regulated | Down- regulated | Identified by MS | Identification Rate (%) |
|---|---|---|---|---|---|
| *O. coarctata* leaf | 54 | 41 | 13 | 34 | 63.0 |
| *O. coarctata* root | 33 | 24 | 9 | 18 | 54.5 |
| IR29 leaf | 28 | 16 | 12 | 14 | 50.0 |
| IR29 root | 13 | 7 | 6 | 6 | 46.2 |

MS = mass spectrometry (MALDI-TOF/TOF).

*Oryza coarctata* showed 87 differentially expressed spots in total (54 in leaves, 33 in roots), more than double the 41 detected in IR29 (Table 1). The halophyte also displayed a greater proportion of upregulated proteins (74.7% vs 56.1% in IR29), consistent with an active stress-response strategy rather than passive shutdown. Fifty-two of the 87 spots were successfully identified by MALDI-TOF/TOF, with identification rates of 54-63% [12].

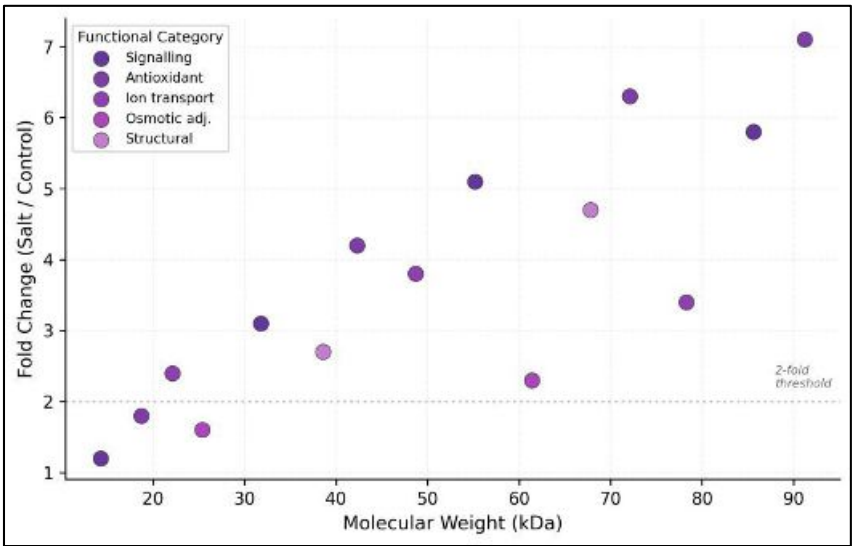


**Fig 1:** Scatter plot of identified salt-responsive proteins in *Oryza coarctata* showing molecular weight versus fold change, categorised by functional group

**Table 2:** Key salt-responsive proteins identified in *Oryza coarctata* with highest fold changes under 150 mM NaCl

| Protein | MW (kDa) | pI | Fold Change | Tissue | Functional Category |
|---|---|---|---|---|---|
| SOD (Cu/Zn) | 18.7 | 5.6 | 4.7 | Leaf | Antioxidant defence |
| LEA group 3 | 25.4 | 6.1 | 5.1 | Root | Osmotic adjustment |
| V-$H^+$-ATPase | 67.8 | 5.2 | 3.8 | Root | Ion transport |
| APX (ascorbate perox.) | 31.8 | 5.8 | 3.4 | Leaf | Antioxidant defence |
| MAPK3 | 42.3 | 6.4 | 3.1 | Leaf | Signal transduction |
| Aquaporin PIP2;1 | 28.6 | 7.1 | −2.8 | Root | Ion transport |

Fold change = salt / control normalised spot volume. Negative values indicate downregulation.

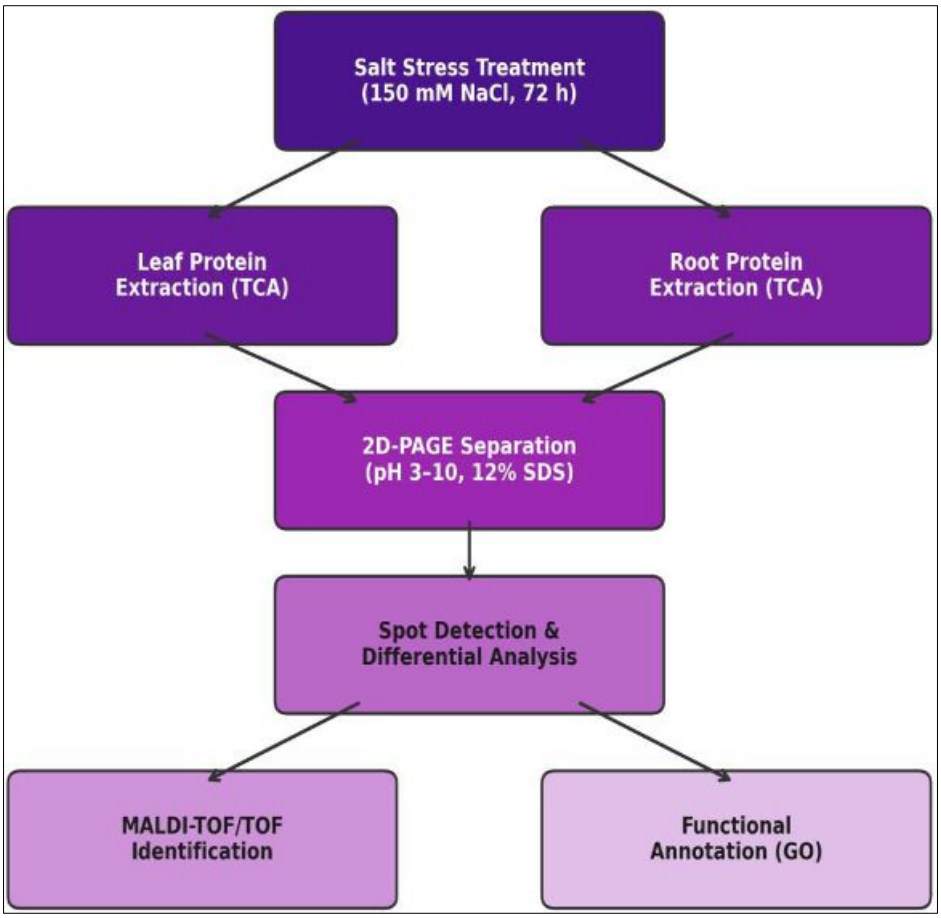


**Fig 2:** Architecture diagram of the proteomics workflow from salt stress treatment through 2D-PAGE separation to MALDI-TOF/TOF protein identification and functional annotation

## Discussion

The 4.7-fold upregulation of Cu/Zn-SOD in *O. coarctata* leaves is consistent with its need to scavenge superoxide radicals generated by disrupted electron transport under salt stress. IR29 showed only a 1.6-fold increase in the same protein, suggesting a weaker antioxidant response [7]. The 5.1-fold increase in LEA group 3 protein in roots indicates strong osmotic protection of root cells, likely working in concert with the proline and glycine betaine accumulation pathways reported in transcriptomic studies of this species [5, 13].

The upregulation of vacuolar $H^+$-ATPase (3.8-fold) points to active $Na^+$ sequestration into root cell vacuoles—a well-characterised mechanism in halophytes that reduces cytoplasmic $Na^+$ toxicity [14]. The downregulation of aquaporin PIP2;1 (−2.8-fold) in roots is equally telling: by reducing membrane water permeability, the plant may limit back-flow of water along the osmotic gradient created by high external salt [15].

These candidate proteins—SOD, LEA3, V-$H^+$-ATPase, and aquaporin PIP2;1—represent priority targets for marker-assisted introgression into cultivated rice. Their corresponding genes can be mapped in *O. coarctata* using the recently published draft genome and validated through overexpression studies in sensitive backgrounds. The proteomic data generated here provide an essential bridge between the existing transcriptomic datasets and future functional breeding applications.

## Conclusion

This research generated the first 2D-PAGE proteome map of the halophytic wild rice *Oryza coarctata* under 150 mM NaCl stress, identifying 52 salt-responsive proteins across leaf and root tissues. Antioxidant defence, osmotic adjustment, and ion transport proteins were the dominant functional categories, with SOD, LEA3, and vacuolar $H^+$-ATPase showing the strongest responses.

These proteins represent high-value targets for molecular breeding programmes aiming to introgress salt tolerance from wild rice into cultivated backgrounds. Future work should employ gel-free quantitative proteomics (iTRAQ or TMT labelling) to capture low-abundance regulatory

proteins that may have been missed by the 2D-PAGE approach, and validate candidate protein function through transgenic overexpression in IR29.

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