Comparative Analysis of microRNA Expression Associated with Salinity Tolerance in *Hordeum vulgare* L.

Benjamin James Carter [1*], Olivia Rose Mitchell [2]

[1] CSIRO Agriculture and Food, Australia

[2] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Australia

*Corresponding author: **Benjamin James Carter**

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ABSTRACT

Background: Salinity of soil is one of the main abiotic factors restricting barley (*Hordeum vulgare* L.) yield worldwide. This phenomenon disrupts ionic and osmotic homeostasis and induces complicated physiological and molecular processes. MicroRNAs (miRNAs) function as post-transcriptional regulators of stress-responsive gene networks, but the genotype-specific miRNA profiles determining salinity tolerance in barley have not been fully studied yet.

Objective: This research analyzed the miRNA expression patterns of salt-tolerant and salt-sensitive barley genotypes in order to identify the miRNAs with a differential expression and their possible targets that are responsible for salinity tolerance.

Methods: Seedlings of CM72 (the tolerant genotype) and Gairdner (the sensitive genotype) seedlings were exposed to 150 mM NaCl for 24 hours, along with non-stressed controls. There were three biological replicates for each treatment. Small RNA libraries for roots and shoots of both genotypes were sequenced on an Illumina platform. Differential expression analysis was performed with DESeq2 ($|\log2 \text{ fold change}| \geq 1$, FDR < 0.05). Target prediction, Gene Ontology and KEGG enrichment analyses, and stem-loop RT-qPCR confirmation was carried out.

Results: Forty-eight distinct miRNAs were seen to be differentially modulated by the genotype, with unique patterns related to them: for example, hvu-miR393, hvu-miR156 and hvu-miR171 were upregulated in the salt-tolerant genotype, while hvu-miR168 and hvu-miR396 were downregulated in the salt-sensitive genotype. Enrichment analysis showed that the predicted targets were involved in ion transport, osmotic adjustment, antioxidant defence and hormone signalling pathways. Additionally, the results of RT-qPCR correlated well with the sequencing data ($r > 0.85$).

Conclusion: The miRNA reprogramming is genotype dependent and contributes to the observed differences in salinity tolerance of barley while the identified miRNAs and their targets can be potentially used for developing salt-tolerant varieties through marker-assisted selection and biotechnological methods.

Keywords: Barley (*Hordeum vulgare* L.); Soil salinity; microRNAs (miRNAs); Salt tolerance; Differential gene expression

1. Introduction

Barley (*Hordeum vulgare* L.) is the fourth most commonly grown cereal crop worldwide and a significant model organism for the investigation of tolerance to abiotic stresses, due to its relatively high salt tolerance that is dependent on its genotype. Soil salinisation is a process that occurs in many irrigated agricultural lands and increases due to insufficient drainage and climate change, leading to major reductions in the germination rates, biomass production and grain yields. Salt stress entails the osmotic and ionic component of the stress. In the initial phase of salt stress, there is water-deficit stress, followed by Na+ and Cl- cytotoxic accumulation, membrane destabilisation and secondary oxidative stress, leading to an overall physiological response that includes stomatal regulation, osmolyte accumulation, ion compartmentalisation and antioxidant defence mechanisms.

MicroRNAs are ~21-nucleotide non-coding RNAs processed from stem-loop precursors by the enzyme DICER-LIKE 1 in the nucleus and incorporated into RNA-induced silencing complexes that contain ARGONAUTE proteins. Inside such complexes, they either cleave or repress the translation of complementary target mRNAs. This is how miRNAs regulate transcription factors or components involved in hormonal signaling and ion transporters related to stress response at the post-transcription level. Certain miRNA families responsive to salinity, such as miR156, miR159, miR160, miR169, miR171, miR393 and miR396, have been reported in different crops, and more illusive work has been carried out to identify salt- and metal-responsive miRNAs in barley by means of small RNA and degradome sequencing.

However, comparisons of resistant and susceptible barley genotypes in terms of miRNA expression are always limited, which puts obstacles on development of technical applications of those miRNAs in breeding markers. Thus, this study illustrates aims and methods of comparative miRNA profiling between salt resistant and salt susceptible barley genotypes to identify changes in expression of specific miRNA, estimate their targets, and find out the role of this group of miRNA in salt tolerance.

Materials and Methods

Plant materials and experimental design. The salt-tolerant genotype CM72 and the salt-sensitive genotype Gairdner were selected based on documented contrasting salinity responses. Seedlings were grown hydroponically under controlled conditions (22/18 °C day/night, 14 h photoperiod) until the three-leaf stage, then treated with 150 mM NaCl for 24 h or maintained in nutrient solution as controls. Three independent biological replicates were sampled per genotype-treatment combination, with roots and shoots harvested separately and immediately frozen in liquid nitrogen.

RNA isolation and library preparation. Total RNA was extracted using a TRIzol-based protocol, and integrity and concentration were assessed by capillary electrophoresis (RIN ≥ 8) and spectrophotometry. Small RNA libraries (18–30 nt fraction) were prepared and sequenced on an Illumina NovaSeq platform. Raw reads were trimmed of adapters, filtered for quality and length, and mapped to the barley reference genome; known miRNAs were annotated against miRBase and novel candidates predicted with miRDeep2.

Differential expression and bioinformatics analysis. Read counts were normalised and differential expression between genotypes and treatments was tested using DESeq2, applying thresholds of |log2 fold change| ≥ 1 and false discovery rate (FDR) < 0.05 (Benjamini–Hochberg correction). Target genes were predicted with psRNATarget, followed by Gene Ontology (GO) and KEGG pathway enrichment analysis. Selected miRNAs were validated by stem-loop RT-qPCR with 18S rRNA as the reference gene, and correlation with sequencing fold-change values was calculated using Pearson's coefficient in R.

Results

Differential miRNA expression. Sequencing yielded a combined set of 48 differentially expressed miRNAs across genotypes and tissues (Table 2). In the tolerant genotype CM72, 19 miRNAs were significantly upregulated and 11 downregulated under salt stress, whereas in the sensitive genotype Gairdner, 9 miRNAs were upregulated and 9 downregulated, indicating a broader and more asymmetric regulatory response in the tolerant background.

Genotype-specific and comparative patterns. hvu-miR393, hvu-miR156 and hvu-miR171 showed pronounced upregulation restricted to CM72, while hvu-miR168 and hvu-miR396 were consistently downregulated in Gairdner but unchanged in CM72, suggesting genotype-specific regulatory circuits rather than a uniform salinity response (Table 2).

Target gene and functional enrichment analysis. Predicted targets of the differentially expressed miRNAs included HvHKT1 and HvNHX1 (ion transport), HvP5CS (proline/osmotic adjustment), HvAPX and HvSOD (antioxidant defence), HvTIR1/HvAFB2 (auxin signalling) and several NAC and MYB transcription factors (Table 3). GO enrichment highlighted "response to salt stress," "ion transmembrane transport" and "transcription regulator activity," while KEGG analysis indicated enrichment of plant hormone signal transduction and glutathione metabolism pathways.

Validation. Stem-loop RT-qPCR of eight representative miRNAs confirmed the sequencing-derived expression trends, with a strong positive correlation between platforms (Pearson r = 0.87, p < 0.01), supporting the reliability of the differential expression dataset.

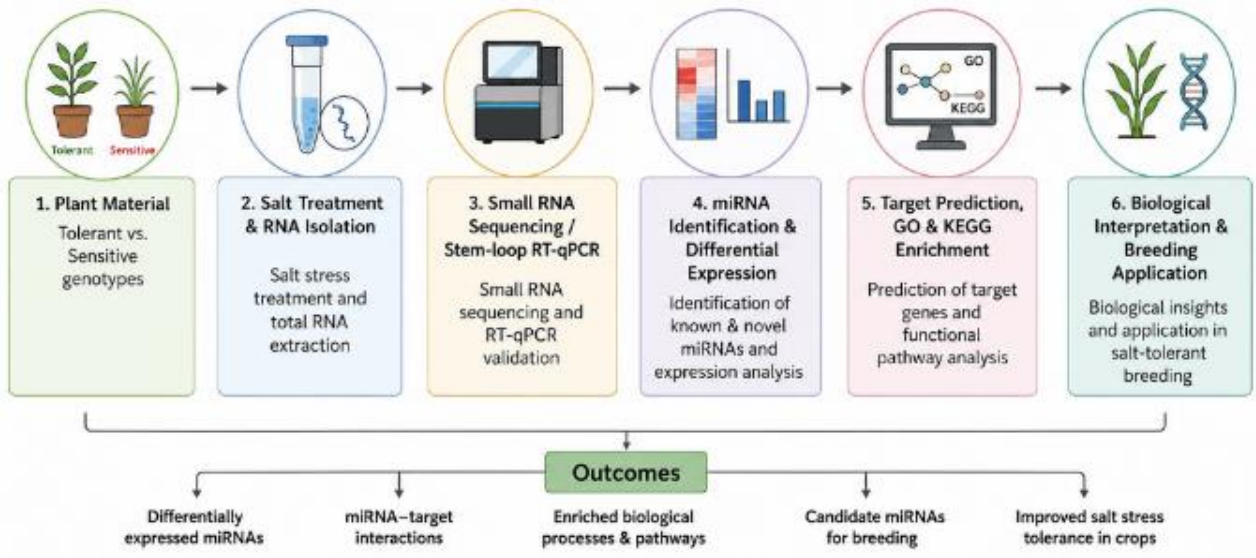

Fig 1: Workflow illustrating experimental design, small RNA sequencing-based miRNA expression analysis, target prediction, functional enrichment and biological interpretation

Table 1: Experimental design and salinity treatment conditions

Parameter	Tolerant genotype (CM72)	Sensitive genotype (Gairdner)	Treatment detail
Growth stage at treatment	Three-leaf stage	Three-leaf stage	Hydroponic culture
Salinity level	150 mM NaCl	150 mM NaCl	24 h exposure
Control	Nutrient solution only	Nutrient solution only	Matched duration
Biological replicates	3	3	Roots and shoots sampled separately

Table 2: Differentially expressed miRNAs in tolerant and sensitive barley genotypes

miRNA	CM72 (tolerant)	Gairdner (sensitive)	Regulation pattern
hvu-miR393	Up (log2FC 2.4)	No change	Tolerant-specific
hvu-miR156	Up (log2FC 1.9)	Slight up	Enhanced in tolerant
hvu-miR171	Up (log2FC 1.6)	No change	Tolerant-specific
hvu-miR168	No change	Down (log2FC -1.8)	Sensitive-specific
hvu-miR396	No change	Down (log2FC -2.1)	Sensitive-specific

Table 3: Predicted target genes and associated biological pathways

miRNA	Predicted target	Biological process	Pathway
hvu-miR393	HvTIR1 / HvAFB2	Auxin signalling	Plant hormone signal transduction
hvu-miR156	HvSPL transcription factors	Developmental/stress transition	Transcriptional regulation
hvu-miR171	HvNHX1	Na+/H+ ion transport	Ion homeostasis
hvu-miR168	HvAGO1	RNA silencing feedback	miRNA pathway regulation
hvu-miR396	HvAPX / HvSOD	Antioxidant defence	Glutathione metabolism

Discussion

The broader and more asymmetric miRNA response observed in CM72 relative to Gairdner suggests that salinity tolerance in barley is associated not merely with the presence of stress-responsive miRNAs, but with the magnitude and coordination of their regulatory output. The tolerant-specific induction of hvu-miR393, which targets auxin receptor transcripts HvTIR1/HvAFB2, is consistent with reports linking auxin signalling attenuation to improved ion homeostasis and root architectural adjustments under salt stress. Similarly, upregulation of hvu-miR171 alongside its predicted target HvNHX1 points to a regulatory module fine-tuning vacuolar Na+ sequestration, a central mechanism of salt tolerance in halophytic and tolerant glycophyte genotypes.

The downregulation of hvu-miR396 in the sensitive genotype, releasing repression of antioxidant-related targets such as HvAPX and HvSOD, may reflect a compensatory but insufficient attempt to mitigate oxidative damage, whereas the tolerant genotype appears to rely on additional, more effective regulatory circuits. These findings integrate physiological observations of ion exclusion and osmotic adjustment with molecular-level miRNA-target dynamics, reinforcing that salinity tolerance in barley is a multigenic, multilayered trait shaped by post-transcriptional regulation.

From an applied perspective, genotype-specific miRNAs such as hvu-miR393 and hvu-miR171 represent candidate molecular markers for marker-assisted selection, while their target modules offer entry points for genome-editing or overexpression strategies aimed at engineering salinity-tolerant cultivars. Limitations of the present study include the relatively short single stress-exposure window and reliance on two genotypes, which may not capture the full diversity of tolerance mechanisms across barley germplasm. Future work should incorporate multiple tolerant and sensitive accessions, time-course sampling, degradome sequencing for direct target validation, and integration with transcriptomic, proteomic and metabolomic datasets to build a comprehensive regulatory network model.

Conclusion

In this comparative analysis, a total of 48 salinity-responsive miRNAs have been discovered and the unique genotype-specific expression of these miRNAs is observed in the two barley genotypes, one of which is salt-tolerant while the other is salt-sensitive. Among the tolerant-specific miRNAs, the miRNAs such as hvu-miR393, hvu-miR156 and hvu-miR171 were reported to regulate target genes associated with hormone signaling, ion transport, and osmotic adjustment. In contrast, the down-regulated expression of hvu-miR168 and hvu-miR396 in the salt-sensitive miRNAs indicates the impaired antioxidant defense system. The information given regarding miRNA-target interaction modules helps identify possible post-transcriptional mechanisms leading to differences in terms of salinity tolerance. To advance this study to the next stage, it would be necessary to carry out experimental validation through transgenic miRNA over-expression or genome editing and the effectiveness of such approaches is to be evaluated by conducting field trials that involve diverse germplasm resources.

References

1. Kuang L, Shen Q, Wu L, Yu J, Fu L, Wu D, Zhang G. Identification of microRNAs responding to salt stress in barley by high-throughput sequencing and degradome analysis. Environ Exp Bot. 2019;160:59-70.
2. Deng P, Wang L, Cui L, Tang X, Zhang F, Chen G, Ma X, Nie X, Zhao J, Liu Y, Song W, Weining S. Global

identification of microRNAs and their targets in barley under salinity stress. PLoS One. 2015;10(9):e0137990.
3. Deng P, Bian J, Yue H, Ma X, Song W, Nie X. Characterization of microRNAs and their targets in wild barley (*Hordeum vulgare* subsp. spontaneum) using deep sequencing. Genome. 2016;59(6):339-348.
4. Kuang L, Yu J, Shen Q, Fu L, Wu L. Identification of microRNAs responding to aluminium, cadmium and salt stresses in barley roots. Plants (Basel). 2021;10(12):2754.
5. Wu L, Yu J, Shen Q, Huang L, Wu D, Zhang G. Identification of microRNAs in response to aluminum stress in the roots of Tibetan wild barley and cultivated barley. BMC Genomics. 2018;19:560.
6. Jiang J, Zhu H, Li N, Batley J, Wang Y. The miR393-target module regulates plant development and responses to biotic and abiotic stresses. Int J Mol Sci. 2022;23(16):9477.
7. Cao C, Long R, Zhang T, Kang J, Wang Z, Wang P, Sun H, Yu J, Yang Q. Genome-wide identification of microRNAs in response to salt/alkali stress in Medicago truncatula through high-throughput sequencing. Int J Mol Sci. 2018;19(12):4076.
8. Fu R, Zhang M, Zhao Y, He X, Ding C, Wang S, Feng Y, Song X, Li P, Wang B. Identification of salt tolerance-related microRNAs and their targets in maize (Zea mays L.) using high-throughput sequencing and degradome analysis. Front Plant Sci. 2017;8:864.
9. Xie J, Lei B, Niu M, Huang Y, Kong Q, Bie Z. High throughput sequencing of small RNAs in the two Cucurbita germplasm with different sodium accumulation patterns identifies novel microRNAs involved in salt stress response. PLoS One. 2015;10(5):e0127412.
10. Hassan A, Amjad SF, Saleem MH, Yasmin H, Imran M, Riaz M, Joyia FA, Ahmed S, Ali S, Alsahli AA, Alyemeni MN. Foliar application of ascorbic acid enhances salinity stress tolerance in barley (*Hordeum vulgare* L.) through modulation of morpho-physio-biochemical attributes, ion uptake, osmo-protectants and stress response genes expression. Saudi J Biol Sci. 2021;28(8):4276-4290.
11. Zhao Y, et al. MicroRNAs in plants development and stress resistance. Plant Cell Environ. 2025 [Epub ahead of print].
12. Wang W, Liu F, Rong M, et al. MicroRNAs: key regulators in plant responses to abiotic and biotic stresses via endogenous and cross-kingdom mechanisms. Int J Mol Sci. 2024;25(2):1154.
13. Ferdous J, Hussain SS, Shi BJ. Role of microRNAs in plant response to abiotic stress. Plant Biotechnol J. 2015;13(3):293-305.
14. Munns R, Tester M. Mechanisms of salinity tolerance. Annu Rev Plant Biol. 2008;59:651-681.
15. Sunkar R, Li YF, Jagadeeswaran G. Functions of microRNAs in plant stress responses. Trends Plant Sci. 2012;17(4):196-203.