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Genotype- and biostimulant-dependent organization of the cotton response to salinity: sequential screening and multivariate transcriptional analysis

Venera S. Kamburova
venera.k75@gmail.com
Center of Genomics and Bioinformatics of the Academy of Sciences of the Republic of Uzbekistan
Ilkhom B. Salakhutdinov
Center of Genomics and Bioinformatics of the Academy of Sciences of the Republic of Uzbekistan
Dmitriy A. Mirzabaev
Center of Genomics and Bioinformatics of the Academy of Sciences of the Republic of Uzbekistan
Farkhod S. Radjapov
Center of Genomics and Bioinformatics of the Academy of Sciences of the Republic of Uzbekistan
Shukhrat E. Shermatov
Center of Genomics and Bioinformatics of the Academy of Sciences of the Republic of Uzbekistan
Dinara I. Tojiboeva
Center of Genomics and Bioinformatics of the Academy of Sciences of the Republic of Uzbekistan
Elena F. Nazarova
Center of Genomics and Bioinformatics of the Academy of Sciences of the Republic of Uzbekistan

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Venera S. Kamburova[1]*, Ilkhom B. Salakhutdinov[1], Dmitriy A. Mirzabaev[1], Farkhod S. Radjapov[1], Shukhrat E. Shermatov[1], Dinara I. Tojiboeva[1], Elena F. Nazarova[1]

Affiliation

[1] Center of Genomics and Bioinformatics of the Academy of Sciences of the Republic of Uzbekistan, Kibray, Tashkent Region 111215, Uzbekistan

*** Corresponding author**

Venera S. Kamburova

Center of Genomics and Bioinformatics of the Academy of Sciences of the Republic of Uzbekistan

Kibray, Tashkent Region 111215, Uzbekistan

Email: venera.k75@gmail.com

Phone: +998 77 707 41 02

ORCID: 0000-0003-0817-6644

Author details

Ilkhom B. Salakhutdinov

Email: ilkhoms@yahoo.com; ORCID: 0000-0003-1159-6500

Dmitriy A. Mirzabaev

Email: dmitriy08m@gmail.com; ORCID: 0009-0000-7256-0953

Farkhod S. Radjapov

Email: farhod.radjapov@yandex.ru; ORCID: 0000-0002-6613-2140

Shukhrat E. Shermatov

Email: sshermatov@hotmail.com; ORCID: 0000-0002-1864-8126

Dinara I. Tojiboeva

Email: dinailxamovna96@mail.ru; ORCID: 0009-0006-8798-3979

Elena F. Nazarova

Email: elenafront1969@gmail.com; ORCID: 0009-0001-0347-1233

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[1] Center of Genomics and Bioinformatics of the Academy of Sciences of the Republic of Uzbekistan, Kibray, Tashkent Region 111215, Uzbekistan

*Corresponding author:

E-mail address: venera.k75@gmail.com (V.S. Kamburova)

Abstract

Salinity limits cotton growth, development, and productivity. One potential approach to mitigating the negative consequences of salt stress is the application of biostimulants. However, it remains poorly understood how biostimulant application modifies the cotton transcriptional response across genotypes and product identities. We conducted a single blocked experiment evaluated through a sequential analytical framework comprising cultivar screening, comparative evaluation of commercial biostimulants, and multivariate analysis of transcriptional changes. Based on morphological traits and the Multitrait Genotype–Ideotype Distance Index (MGIDI), Ishonch and Porloq-1 were identified as contrasting genotypes, whereas Ravnaq-1 represented an intermediate response to salinity. Rizokom-1, Mikrozim-1, and Serhosil were selected for subsequent analysis. Their effects were evaluated through the expression of genes assigned to the hormonal regulation, antioxidant defense, and metabolic/osmoprotective functional modules. Rizokom-1 provided the most favorable integrated morphological effect and generated the most pronounced transcriptional response, followed by Mikrozim-1 and Serhosil. The amplitude and functional structure of the response depended on both genotype and biostimulant. Salinity predominantly reduced response amplitude while the direction of the response was generally preserved, and the three functional modules remained strongly concordant across the analyzed genotype × salinity contexts. These findings support genotype-specific selection of biostimulants and identify promising cultivar × biostimulant combinations for subsequent physiological, biochemical, and agronomic validation.

Keywords *Gossypium hirsutum*; sodium chloride; seed germination; root development; marker genes; functional modules

Abbreviations ABA, abscisic acid; ANOVA, analysis of variance; cDNA, complementary DNA; Ct, cycle threshold; DNA, deoxyribonucleic acid; FC, fold change; GABA, γ-aminobutyric acid; GE, germination energy; GWAS, genome-wide association study; HSD,

honestly significant difference; MGIDI, Multitrait Genotype–Ideotype Distance Index; MGT, mean germination time; mRNA, messenger RNA; NaCl, sodium chloride; NCED, 9-cis-epoxycarotenoid dioxygenase; OPR, 12-oxophytodienoate reductase; PC, principal component; PCA, principal component analysis; PCR, polymerase chain reaction; POD, peroxidase; qPCR, quantitative polymerase chain reaction; RL, root length; RNA, ribonucleic acid; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; SD, standard deviation; SL, shoot length; SOD, superoxide dismutase.

Introduction

Soil salinity remains one of the most serious abiotic factors limiting the growth, development, and productivity of crops (Singh 2022). This problem is particularly relevant to cotton, which is widely cultivated in arid regions under irrigated agriculture (Khasanov et al. 2023), where secondary soil salinization is a major cause of reduced yield and fiber quality (Maryum et al. 2022). Salt stress disrupts water and ion homeostasis, intensifies oxidative processes, and induces metabolic reprogramming, thereby slowing seed germination, suppressing plant growth, and reducing productivity (Guo et al. 2022; Maryum et al. 2022; Muhammad et al. 2025). However, the magnitude of these effects depends not only on salinity intensity but also on a particular genotype's ability to mount an adaptive response (Munawar et al. 2021; Anwar et al. 2025; Gul et al. 2025). Differences among cultivars are particularly pronounced during the early stages of development, when the rate and completeness of germination, together with root and shoot growth, determine subsequent plant establishment under adverse conditions (Munawar et al. 2021; An et al. 2024; Anwar et al. 2025). Assessment of these traits therefore makes it possible to compare genotypes according to their responses to salinity and to identify variants that differ in their ability to maintain early growth processes. Characterizing such differences is also important for selecting genotypes in which the effects of treatments intended to mitigate the consequences of salt stress can be evaluated against different baseline levels of salt tolerance. In addition, comparisons across several NaCl concentrations enable identification of a stress level at which cultivar differences are most clearly expressed without completely suppressing germination and growth (An et al. 2024; Gul et al. 2025).

Transcriptional regulation is one of the levels at which differences in genotype responses to salinity are established (Muhammad et al. 2025). Salt stress alters the expression of genes involved in stress signaling, hormonal regulation, antioxidant defense, osmoprotection, and maintenance of metabolic homeostasis (Anwar et al. 2022; Muhammad et al. 2025). These changes do not occur in isolation but affect several functionally interconnected processes, whose coordinated involvement determines the internal organization of the transcriptional response (Anwar et al. 2022; Han et al. 2023). Therefore, characterizing genotype responses to salinity requires

consideration not only of changes in individual genes but also of their assignment to interconnected functional pathways (Han et al. 2023).

One promising approach to the targeted modulation of plant responses to stress is the use of biostimulants (Kaushal et al. 2023; Lau et al. 2025). Their application may be accompanied by changes in seed germination, root-system and shoot development, as well as in the expression of genes involved in hormonal, antioxidant, and metabolic regulation (Kaushal et al. 2023; Benito et al. 2024; Lau et al. 2025). However, the effectiveness of biostimulants is determined by the nature of the product, the crop genotype, and the application conditions (Detcheverry et al. 2026). Even products intended for similar purposes may differ in the magnitude and direction of their effects, and the same product may induce different responses across genotypes (Covaşă et al. 2025; Detcheverry et al. 2026). This necessitates an initial comparative assessment of biostimulants, followed by evaluation of how the selected products modify the transcriptional response to salinity.

Analysis of the literature showed that many studies of biostimulant action under salinity have focused on individual growth or physiological traits, or on selected genes and signaling pathways (Akbar et al. 2022; Kaushal et al. 2023; Benito et al. 2024; Khalid et al. 2024; Zhao et al. 2024; Hwang et al. 2025; Lau et al. 2025; Algasem et al. 2026). Recent studies in cotton have expanded this evidence by examining genotype-dependent responses to microbial, seaweed-based, and mycorrhizal biostimulants. Still, their analyses have remained focused primarily on morphological, physiological, biochemical, or selected molecular traits rather than on the coordinated behavior of several transcriptional modules (Batool et al. 2025; Calayır et al. 2026). Consequently, coordinated changes across multiple functional modules of the transcriptional response, considered together with plant genotype and biostimulant type, remain insufficiently studied. In cotton, it remains unclear how biostimulants modify the interconnected expression of genes involved in hormonal regulation, antioxidant defense, osmoprotection, and carbohydrate metabolism under salt stress, and to what extent the organization of this response depends on both genotype and biostimulant. This lack of information limits evidence-based selection of the most promising “genotype × biostimulant” combinations for subsequent physiological and agronomic validation.

We hypothesized that differences in biostimulant effectiveness under salt stress may be reflected both in the extent to which early morphological traits are maintained and in the associated, multicomponent transcriptional response. This study aimed to conduct a sequential screening of cotton cultivars based on their responses to salinity, assess the effects of five commercial biostimulants on cultivars contrasting in the preservation of morphological traits, and characterize the transcriptional response to the selected products in three cotton genotypes by analyzing 13 marker genes representing the hormonal regulation, antioxidant defense, and

metabolic/osmoprotective modules. The distinctive feature of the study is the sequential combination of morphological screening with an integrative characterization of the transcriptional response, enabling comparison of biostimulant effects and the preliminary identification of the most promising "cultivar × biostimulant" combinations for subsequent agronomic validation.

Materials and methods

Plant material

Experiments were conducted under controlled phytotron conditions using five commercial medium-fiber cotton (*Gossypium hirsutum* L.) cultivars representing different breeding approaches: the conventionally bred cultivars Ishonch, An-Bayovut-2, and Namangan-77; Ravnaq-1, developed using marker-assisted selection; and Porloq-1, obtained through biotechnological methods. The Genetic Resources Bank of the Center of Genomics and Bioinformatics of the Academy of Sciences of the Republic of Uzbekistan provided seeds.

Experimental design

The study was conducted as a single blocked experiment comprising the genotype × salinity × treatment combinations described below. The resulting data were evaluated through three sequential analytical stages.

In the first analytical stage, five cotton cultivars were comparatively screened under 0, 50, and 150 mM NaCl using the untreated variants established within the common experiment. This analytical subset included 15 cultivar × salinity combinations, each represented by five independent biological replicates. For the assessment of germination parameters, each biological replicate consisted of a separate Petri dish containing 10 seeds, whereas morphological traits were evaluated using a separate pot containing five plants. Cultivar responses to salt stress were assessed based on germination time, germination energy, root length, and shoot length. Based on the screening results, Ishonch and Porloq-1, which differed most markedly in their ability to maintain the investigated traits under salinity, were retained for focused evaluation in the second analytical stage.

In the second analytical stage, the effects of five biostimulants—Rizokom-1, Rizokom-2, Mikrozim-1, Mikrozim-2, and Serhosil—were evaluated in Ishonch and Porloq-1 under 0 and 150 mM NaCl. This analytical subset included six treatment conditions, including an untreated control, and comprised 24 cultivar × salinity × treatment combinations, each represented by five independent biological replicates. The untreated Ishonch and Porloq-1 groups at 0 and 150 mM NaCl were the same experimental units included in the cultivar-screening subset and served as shared reference groups in both analyses; no duplicate control groups were established. As in the first analytical stage, a separate Petri dish containing 10 seeds served as the biological replicate

for assessing germination parameters, whereas a separate pot containing five plants served as the biological replicate for assessing morphological traits. The 50 mM NaCl treatment was used only for cultivar screening, whereas the biostimulant comparison focused on 0 and 150 mM NaCl because 150 mM provided clearer differentiation among cultivar responses without completely suppressing germination and seedling growth.

For the third analytical stage, leaf samples corresponding to Rizokom-1, Mikrozim-1, Serhosil, and the untreated control were selected from the common experiment for transcriptional analysis. This subset included Ishonch and Porloq-1, representing contrasting responses to salinity, as well as Ravnaq-1, which occupied an intermediate position in the cultivar-screening analysis. The full-factorial transcriptional subset comprised three cultivars, four pre-sowing treatment conditions—three biostimulants and a distilled-water control—and two salinity levels, 0 and 150 mM NaCl. In total, 24 cultivar × salinity × treatment combinations were analyzed, each represented by five independent biological replicates.

At the transcriptional analysis stage, the same experimental units were used for morphological and transcriptional analyses. Each biological replicate consisted of a separate pot containing five plants; thus, the 24 experimental combinations comprised 120 independent experimental units. The pots were randomly positioned in the phytotron and periodically rearranged to minimize spatial heterogeneity in growth conditions. After the 21-day salt treatment, root and shoot lengths were first measured for each of the five plants and averaged within each pot; the resulting pot mean was used as the value of one biological replicate in the statistical analysis. Leaf tissue from the same five plants was then pooled into a single composite sample, representing a single biological replicate.

Pre-sowing biostimulant treatment and seed germination

The study used the commercial biostimulants Rizokom-1, Rizokom-2, Mikrozim-1, Mikrozim-2, and Serhosil, developed and manufactured by the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan. The composition of the products and the precise application parameters are proprietary information of the manufacturer. The shortened name Hosil was used only in the figures for the commercial biostimulant Serhosil.

Seeds were surface-sterilized with a 15% (w/v) sodium hypochlorite solution for 15 min, then rinsed at least 3 times with sterile distilled water. Specialists from the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan performed pre-sowing treatment with working solutions of the biostimulants in accordance with the manufacturer's instructions. Seeds in the control treatments were soaked in distilled water for the same duration. The treatment was applied once before germination.

To determine germination parameters, seeds were placed in Petri dishes on sterile filter paper moistened with distilled water or an NaCl solution at the corresponding concentration. Germination was monitored daily for 10 days.

For experiments assessing morphological traits and transcriptional responses, pretreated seeds were placed in Petri dishes on sterile filter paper moistened with distilled water and incubated in the dark at 28 °C for 3 days. The seedlings were then transferred to a 16-h light/8-h dark photoperiod at an illumination intensity of 5000 lx, a temperature of 25–26 °C, and a relative air humidity of 65 ± 5%. Under these conditions, the seedlings were maintained until transplantation into soil, but for no longer than three days.

Growth conditions and salt stress simulation

Seedlings were transplanted into 2-L plastic pots filled with a mixture of fine river sand and field soil at a 3:1 volume ratio. The substrate was disinfected in advance by heating in a muffle furnace at 100 ± 5 °C for 3 h. Plants were grown at 25–26 °C and 65 ± 5% relative air humidity under a 16-h light/8-h dark photoperiod with an illumination intensity of 5000 lx.

After an acclimation period of at least three days, salt treatment was initiated at the cotyledon stage using a modified protocol of Rodriguez-Uribe et al. (2011). Depending on the experimental stage, NaCl solutions with nominal concentrations of 0, 50, or 150 mM were applied. Their corresponding electrical conductivity equivalents were estimated as approximately 0, 5.0, and 15.0 dS m^{-1}, respectively, based on the conversion 10 mM NaCl ≈ 1.0 dS m^{-1}; electrical conductivity was not measured directly. Every two days, 100 mL of distilled water or the NaCl solution at the corresponding nominal concentration was added to each pot, maintaining the substrate near field capacity while allowing free drainage.

The duration of salt treatment in all pot experiments was 21 days. At the end of the treatment, when plants had reached the four- to five-true-leaf stage, shoot and root lengths were measured as described below. At the transcriptional analysis stage, leaf tissue from the same five plants in each pot was pooled into a single composite sample, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

Morphological trait assessment

After the 21-day salt treatment, each plant was photographed against a millimeter scale. Shoot and root lengths were determined from the resulting digital images using ImageJ v1.53e (https://imagej.net/ij/index.html), after calibrating the image scale against the millimeter scale. In the first and second experimental stages, measurements from the five plants in each pot were averaged, and the resulting pot mean was treated as a single biological replicate in the statistical analysis.

Germination parameter assessment

Germination parameters were assessed according to the method of Munawar et al. (2021). Depending on the experimental stage, seed germination was evaluated at nominal NaCl concentrations of 0, 50, or 150 mM. The number of germinated seeds was recorded daily for 10 days. The mean germination time (MGT) and germination energy (GE) were then calculated from the recorded data.

The mean germination time was calculated using the following formula:

$$MGT = \frac{\sum n_i \times t_i}{\sum n_i}$$

where n_i is the number of seeds that germinated during the i-th observation interval, and t_i is the time from the beginning of the experiment to the corresponding observation time, expressed in days.

Germination energy was calculated using the following formula:

$$\text{GE (\%)} = \frac{N_p}{\text{N}} \times 100$$

where N_p is the number of seeds germinated by the established peak germination time, and N is the total number of seeds tested.

RNA extraction, cDNA synthesis, and RT-qPCR

Total RNA was extracted from the pooled leaf-tissue sample of each biological replicate using the RNeasy Mini Kit (Qiagen, Germany) and treated with TURBO DNase (Ambion, USA) according to the manufacturers' instructions. The RNA was further purified by extraction with a phenol–chloroform mixture (5:1), followed by ethanol precipitation. RNA integrity was assessed by electrophoresis on a denaturing 1% agarose gel containing 2.2 M formaldehyde, and RNA concentration was determined using a GENESYS 10 UV spectrophotometer (Thermo Scientific, USA). Complementary DNA (cDNA) was synthesized using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Quantitative real-time PCR was performed using a LightCycler 480 Instrument II with LightCycler 480 SYBR Green I Master (Roche, Switzerland). The 20-µL reaction mixture contained 10 µL of SYBR Green I Master, 1 µL each of forward and reverse primers at 10 µM, 2 µL of fourfold-diluted cDNA, and 6 µL of double-distilled water. The amplification program consisted of an initial denaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s.

Gh_UBQ7 was used as the reference gene, based on its reported relative stability in cotton leaves under salt stress (Wang et al. 2013). Target genes were selected based on their roles in hormonal regulation, antioxidant defense, osmoprotection, and carbohydrate metabolism under abiotic stress. Primers were designed using Primer3web v4.0.0 and Vector NTI Advance v11 and

validated using Primer-BLAST. Primer sequences and the functional classification of the target genes are provided in Table 1.

Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). For each genotype, the corresponding treatment without biostimulant application and without NaCl addition was used as the calibrator, and its expression level was set to 1. Thus, the $\log_2$FC values presented in Figs. 3 and 4 represent expression relative to the untreated control of the corresponding genotype at 0 mM NaCl. To estimate the biostimulant-specific effect under salinity, $\Delta\log_2$FC was additionally calculated relative to the untreated control of the same genotype at 150 mM NaCl within the corresponding experimental block. The analysis included five independent biological replicates, with each biological sample analyzed in three technical RT-qPCR replicates. Measurements from the technical replicates were averaged, so that each biological replicate contributed a single relative expression value per target gene to the statistical analysis. The resulting relative expression values were transformed to the $\log_2$ scale for statistical and integrative analyses.

Table 1 Functional classification of the target genes and primer sequences used for RT-qPCR

Functional group	Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Hormonal regulation	*Gh_NCED5*	TGGAACGCTTGGGAGGAG	TCGGCAATGGCGAGATAA
	Gh_GH3.17	AAAGGCAGTGACAAAGGCAAA	TTCTTCCACCGTGGAGCAAC
	Gh_OPR3	GCTTACGGGCAAACCGAATC	AGGCGGCCGTAAGATACAAG
	Gh_ICS1	ATCGAGTGGCTCCATGCTCAAC	CGGCGGCACCAACAAGATTATG
Antioxidant defense	*Gh_SOD*	CATCTCTCACGCACTCTGTC	CCTTAGCCATTTCTGTCTGTG
	Gh_APX	TCGTTGCCGTTGAGATTAC	TGGTAGCATCAGGAAGACG
	Gh_CAT1	TGATAAGTTGCTCCAGACTCG	CTTCGTGGTGATTGTTGTGA
	Gh_POD1	CCAATCTGGGTCCGGCAAACC	CCAGGGGTCCCGTCACGTTT
	Gh_GPX	CAGTGCATTGTGGAAGGCAGGAA	GGGAGGCGTCTTCAGCCATGT
	Gh_GR	AGTTCAGTCGTGGATTCAGTT	AGTTATGGCCAACTCCTGCC
Metabolic/ osmoprotective	*Gh_P5CS*	AGTCTCGACCCGATGCTCTTG	TTTGCCACCTTTGAGCAATAGG
	Gh_SPS	CCTGCCTCTTTCATGTTCAA	CAGTCCTACTCGCTACTTCGTG
	Gh_SuSy	GGCTTTTTCTTGTCCGACCATA	AAAGGAAGAGGCGGGTTTTCC
Reference gene	*Gh_UBQ7*	GAAGGCATTCCACCTGACCAAC	CTTGACCTTCTTCTTCTTGTGCTTG

Statistical and integrative data analysis

The statistical unit was an independent biological replicate; five biological replicates were analyzed for each experimental combination. Across the entire experiment, replicates bearing the same number across genotype, salinity, and treatment combinations corresponded to the same experimental block. Results are presented as mean ± standard deviation.

For comparison of germination and morphological traits, relative performance was calculated after $\log_2$ transformation. In the cultivar-screening experiment, the mean value of the corresponding trait at 0 mM NaCl was used as the baseline separately for each cultivar. For germination energy, root length, and shoot length, relative performance was calculated as $\log_2(X/X_0)$, where X is the individual value at 50 or 150 mM NaCl and X_0 is the mean value at 0 mM NaCl. Because a decrease in mean germination time represents a more favorable response, this trait was expressed as the inverse ratio $\log_2(X_0/X)$. Cultivars were compared separately at 50 and 150 mM NaCl. In the biostimulant-screening experiment, the baseline was the mean value of the untreated treatment of the corresponding cultivar at the same NaCl concentration. Biostimulants were compared separately for each cultivar and NaCl concentration. Pairwise comparisons were performed using Tukey's HSD test, and residual variance was estimated using a linear model including the experimental treatment and block. For Fig. 1a and 2a, means and sample standard deviations were calculated from the five individual $\log_2$-transformed relative-performance values. Differences were considered statistically significant at $p < 0.05$.

To obtain an integrated ranking of the cultivars, the Multitrait Genotype–Ideotype Distance Index (MGIDI) was calculated separately for each NaCl concentration (0, 50, and 150 mM) using mean germination time, germination energy, root length, and shoot length. Mean germination time was specified as a trait for which lower values were desirable, whereas higher values were desirable for the remaining traits. Cultivars were ranked by their distance from the ideotype, with a lower MGIDI value indicating a more favorable combined trait profile. A selection intensity of 20% was used for the integrated ranking.

For the biostimulant-screening experiment, MGIDI was calculated separately for each cultivar × NaCl concentration combination using the five biostimulants as candidate treatments (Olivoto et al. 2022). The untreated control was excluded from the product ranking and was used only as the reference for $\log_2$ relative-performance normalization. The same trait directions and selection intensity of 20% were applied. For visualization in Fig. 1b and 2b, MGIDI values were displayed on a common inverted radial scale from 0 to 3 using a plotted radius of 3 − MGIDI. Consequently, lower MGIDI values are displayed farther from the center. The common radial limit encompassed the full observed range without truncation.

Individual relative-expression values were transformed to $\log_2$ scale. For each gene, a three-way analysis of variance was performed with genotype, NaCl concentration, and treatment as fixed factors, including all two-way interactions and the three-way interaction. Effect sizes were estimated using partial η^2 and ω^2. The normality of the residuals was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using the median-based Levene's test. Post hoc comparisons of genotype × treatment combinations were performed separately at each NaCl

concentration using Tukey's HSD test. Differences were considered statistically significant at $p < 0.05$.

Principal component analysis was performed on a matrix of 120 individual transcriptional profiles, each containing expression values for 13 genes. Before analysis, the data were $\log_2$-transformed and standardized separately for each gene using the overall mean and sample standard deviation. Principal components were calculated by singular value decomposition of the standardized matrix. For each genotype × NaCl concentration × treatment combination, the centroid of the five biological replicates was calculated. The distribution of the 60 biological observations at each NaCl concentration was visualized in the PC1–PC2 space using 95% covariance ellipses calculated from the corresponding covariance matrix and the 0.95 quantile of the chi-square distribution with two degrees of freedom. The transcriptional trajectory was defined as the vector from the centroid of the corresponding control to the centroid of the biostimulant-treated variant. Trajectory length was calculated as the Euclidean distance in the full 13-dimensional standardized space. In contrast, the change in trajectory direction under salt stress was quantified as the angle between the corresponding vectors at 0 and 150 mM NaCl. Confidence intervals were estimated using a nonparametric bootstrap with independent resampling of observations within each experimental group, 2000 iterations, a fixed random seed of 20260719, and percentile-based 95% intervals. The contribution of each functional module was calculated as the mean squared standardized centroid change divided by the number of genes assigned to that module; the resulting values were normalized to the sum of the three modules and expressed as percentages.

For correlation and integrative analyses, the effect of each biostimulant was calculated within the corresponding experimental block: $\Delta\log_2\mathrm{FC} = \log_2(X_{\mathrm{biostimulant}}) - \log_2(X_{\mathrm{control}})$.

For each biostimulant, Pearson correlation coefficients between genes were calculated using 30 individual effects corresponding to three genotypes, two NaCl concentrations, and five biological replicates. The resulting p-values were adjusted using the Benjamini–Hochberg method, separately for each biostimulant and for the 78 gene pairs evaluated. Similarity between pairs of biostimulant-specific correlation matrices was assessed by calculating the Pearson correlation between the 78 unique off-diagonal gene-pair coefficients extracted from their upper triangles. Root mean square error and mean absolute difference between corresponding coefficients were additionally calculated as descriptive measures of matrix dissimilarity. Hierarchical clustering of the 13 genes was performed using a combined matrix of 90 individual effects, Euclidean distances, and the average-linkage method, without additional standardization.

For each biological replicate, the module response was calculated as the mean $\Delta\log_2\mathrm{FC}$ of the genes assigned to the corresponding functional module. The integrated transcriptional response was defined as the equally weighted mean of the three module scores. Response retention under

salt stress was expressed as the ratio of the module effect at 150 mM NaCl to that at 0 mM NaCl, calculated for matched experimental blocks. The salt-dependent change in the biostimulant effect was defined as: $\Delta\Delta\log_2FC = \Delta\log_2FC_{150} - \Delta\log_2FC_0$.

The concordance of the functional modules was assessed using Pearson correlation coefficients calculated from their mean responses across six experimental contexts, defined by combinations of three genotypes and two NaCl concentrations.

The described statistical and integrative procedures were implemented computationally in Python 3.12.13. Data preparation and structuring were performed using pandas 2.2.3. Analysis of variance was conducted using statsmodels, whereas diagnostic tests, multiple comparisons, and correlation analyses were performed using SciPy 1.17.0. Principal component analysis was performed via singular value decomposition of the data matrix using NumPy 2.3.5, and hierarchical clustering was performed using scipy.cluster.hierarchy. The MGIDI was calculated in R 4.5.1 using the metan package in RStudio with a selection intensity of 20%. Results were visualized, and multipanel figures were assembled programmatically in Python using Matplotlib.

Results

Screening of Cotton Cultivars and Biostimulants to Select Experimental Variants for Subsequent Analysis

In the first analytical stage, differences among the five cotton cultivars were assessed using changes in mean germination time, germination energy, root length, and shoot length at 50 and 150 mM NaCl. At 50 mM NaCl, the deterioration in overall relative performance was relatively moderate. Blocked analysis of variance revealed a significant genotype effect for all four parameters; however, pairwise comparisons using Tukey's HSD test did not detect statistically significant differences among cultivars in mean germination time. Cultivar differences were more pronounced for germination energy, root length, and shoot length, with Porloq-1 showing the smallest reduction in relative performance for these parameters. Increasing the NaCl concentration to 150 mM intensified the adverse effect of salinity; the genotype effect was statistically significant for all four parameters, and Tukey's HSD comparisons provided clearer separation among the cultivars (Fig. 1a; Online Resource 1).

At 150 mM NaCl, Porloq-1 retained the highest relative performance values, with $\log_2$ changes of −0.35 for mean germination time, −0.26 for germination energy, −0.28 for root length, and −0.62 for shoot length. For all four parameters, Porloq-1 belonged to a distinct upper statistical group relative to the most sensitive cultivars. Ishonch showed the greatest reductions among the cultivars studied, with corresponding values of −0.53, −1.08, −1.08, and −1.25; across all parameters, its values were in the lowest Tukey HSD groups. Ravnaq-1 and Namangan-77 occupied intermediate

positions across the parameters considered, whereas An-Bayovut-2 also showed pronounced reductions in several parameters.

Integrated ranking using the MGIDI at a selection intensity of 20% confirmed the differentiation among the genotypes. Porloq-1 ranked first and had the smallest distance to the ideotype at 0, 50, and 150 mM NaCl, reflecting the most favorable combination of mean germination time, germination energy, root length, and shoot length across all conditions examined. In contrast, Ishonch had the greatest distance from the ideotype and was therefore selected as the relatively salt-sensitive genotype within the evaluated set of cultivars, based on its combined early-growth response to 150 mM NaCl (Fig. 1b; Online Resource 1). Based on the integrated ranking and contrasting response profiles, Ishonch and Porloq-1 were retained for the comparative analysis of biostimulant effects. In the subsequent analytical stages, 150 mM NaCl was retained as the stress condition because it provided the clearest differentiation among genotypes without completely suppressing germination and seedling growth.

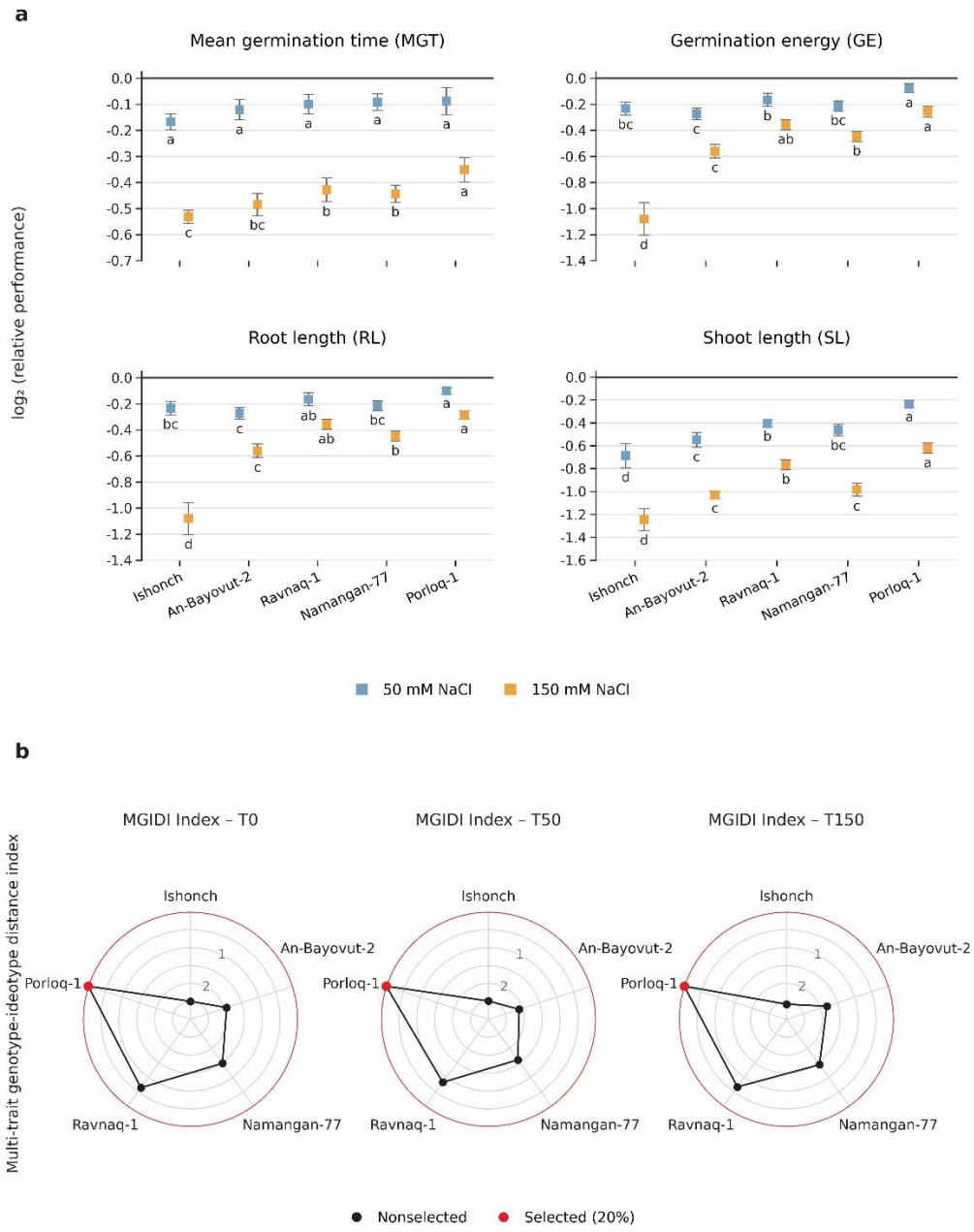


Fig. 1 Cultivar screening under 50 and 150 mM NaCl and MGIDI-based selection of contrasting genotypes. (a) Relative performance of mean germination time (MGT), germination energy (GE), root length (RL), and shoot length (SL), expressed as $\log_2$-transformed values relative to the corresponding 0 mM NaCl treatment. Squares represent means, and error bars represent sample standard deviations calculated from five individual $\log_2$-transformed relative-performance values. Blue and orange symbols denote 50 and 150 mM NaCl, respectively. Within each NaCl concentration and trait, different letters indicate statistically significant differences among cultivars according to Tukey's HSD test at $p < 0.05$. (b) MGIDI profiles of the five cultivars at 0, 50, and 150 mM NaCl based on MGT, GE, RL, and SL. MGIDI values are shown on an inverted radial scale from 0 to 3, with lower values plotted farther from the center. Red symbols indicate the genotype selected in each NaCl condition at a selection intensity of 20%; smaller MGIDI values indicate a shorter distance to the ideotype

The effects of the biostimulants depended on genotype, NaCl concentration, and the parameter analyzed. Blocked analysis of variance identified statistically significant differences among the treatment variants, while pairwise comparisons using Tukey's HSD test showed that the direction and magnitude of the effects differed among the biostimulants and cultivars (Fig. 2a; Online Resource 1).

In the absence of NaCl, Rizokom-1 and Mikrozim-1 exerted the most pronounced positive effects across the parameters examined in both cultivars. At 150 mM NaCl, Mikrozim-1 produced the greatest reduction in mean germination time in Ishonch, with a $\log_2$ change in relative performance of 0.55. Rizokom-1 produced the largest increases in root and shoot length, by 0.66 and 0.34, respectively. Rizokom-1, Mikrozim-1, and Serhosil increased germination energy in Ishonch to comparable extents, according to Tukey's HSD pairwise comparisons. In Porloq-1, the most consistent positive effect across parameters was observed with Rizokom-1; its effects did not differ statistically from those of Mikrozim-1 for mean germination time, germination energy, root length, or shoot length. In most cases, Mikrozim-2 and Rizokom-2 were less effective than the corresponding Mikrozim-1 and Rizokom-1 preparations.

Integrated MGIDI ranking confirmed the most favorable combined performance of Rizokom-1. It ranked first for both cultivars at both NaCl concentrations, whereas Mikrozim-1 ranked second in all four "genotype × salinity" contexts. Serhosil occupied the third position, while Mikrozim-2 and Rizokom-2 ranked lower (Fig. 2b; Online Resource 1).

Based on the comparative screening, Rizokom-1 and Mikrozim-1 were selected for transcriptional analysis as the biostimulants producing the most pronounced changes in morphological parameters. Serhosil was also selected because it produced a positive response with a distinct amplitude and response profile. The transcriptional subset of the common experiment included the contrasting genotypes Ishonch and Porloq-1, together with Ravnaq-1, which occupied an intermediate position in its response to 150 mM NaCl. This analytical design enabled us to assess the dependence of the transcriptional response on biostimulant identity across three distinct genotypic contexts.

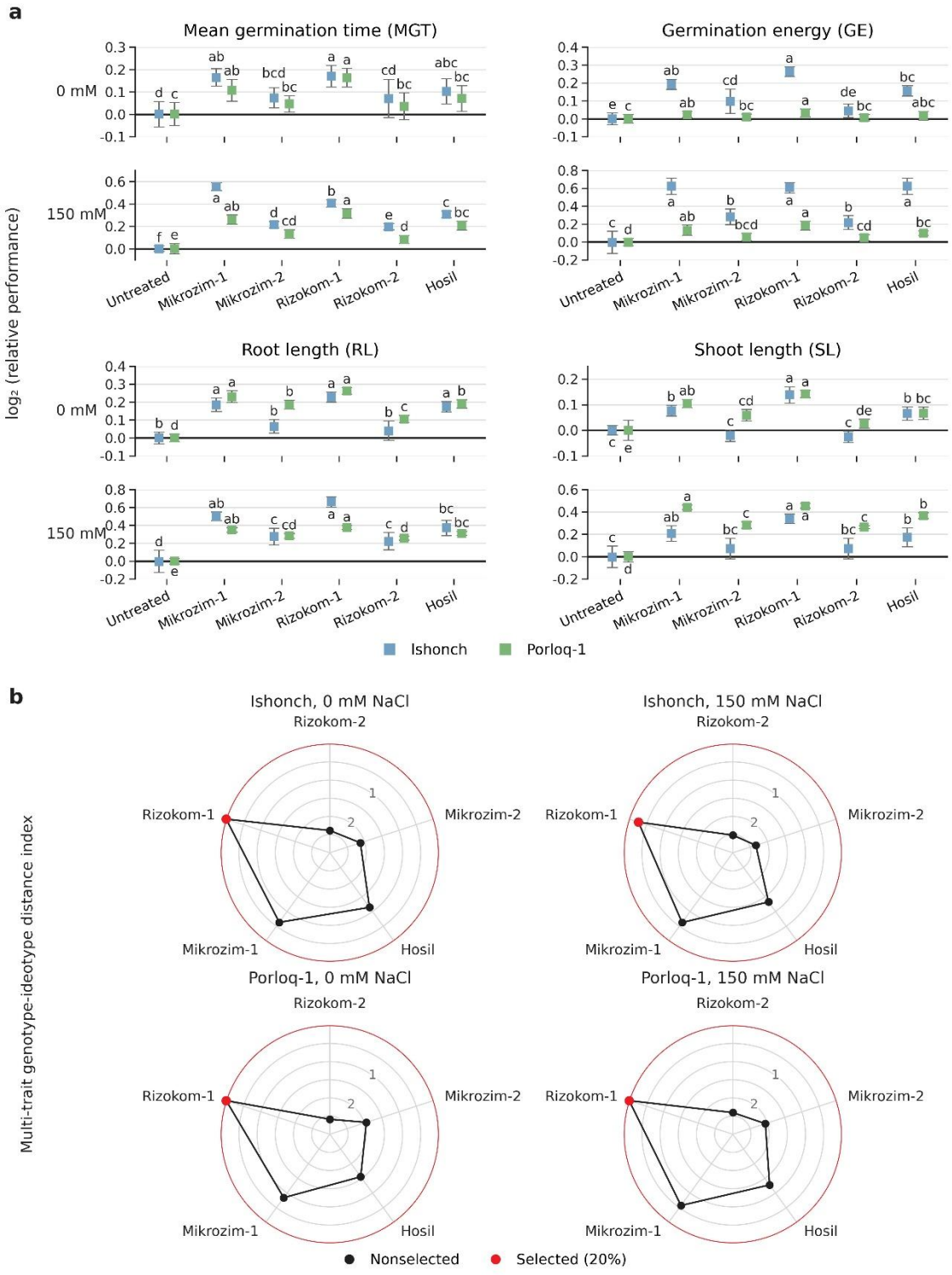


Fig. 2 Biostimulant screening and MGIDI-based ranking of treatment variants in Ishonch and Porloq-1. (a) Relative performance of mean germination time (MGT), germination energy (GE), root length (RL), and shoot length (SL) in untreated controls and following treatment with Rizokom-1, Rizokom-2, Mikrozim-1, Mikrozim-2, or Serhosil under 0 and 150 mM NaCl. Values are expressed as log_2-transformed relative performance calculated relative to the corresponding untreated treatment at the same NaCl concentration. Squares represent means, and error bars represent sample standard deviations calculated from five individual log_2-transformed relative-

performance values. Blue and green symbols denote Ishonch and Porloq-1, respectively. Within each genotype × NaCl concentration × trait combination, different letters indicate statistically significant differences among treatments according to Tukey's HSD test at $p < 0.05$. (b) MGIDI profiles for each genotype × NaCl concentration combination based on the four analyzed traits and the five candidate biostimulants. The untreated control was excluded from the MGIDI ranking. MGIDI values are shown on an inverted radial scale from 0 to 3, with lower values plotted farther from the center. Red symbols indicate the treatment selected within each genotype × NaCl concentration combination at a selection intensity of 20%; smaller MGIDI values indicate a shorter distance to the ideotype. "Hosil" is the shortened name used for the commercial biostimulant Serhosil

Changes in the Expression of Functional Module Genes in Response to Biostimulant Treatments

To characterize the transcriptional response of cotton, changes in the expression of genes representing the hormonal regulation, antioxidant defense, and metabolic/osmoprotective modules were analyzed.

In the absence of salt stress, all three biostimulants increased the expression of the analyzed genes relative to the untreated control. A statistically significant effect was detected for each of the 13 genes in all three genotypes. Regardless of functional gene classification, the response magnitude consistently decreased in the order Rizokom-1 > Mikrozim-1 > Serhosil, whereas the genotypes ranked by response magnitude as Ishonch > Ravnaq-1 > Porloq-1 (Figs. 3, 4; Online Resource 2). At 150 mM NaCl, the baseline response of untreated plants differed substantially among the genotypes. In Ishonch, the expression of all 13 genes was below the level of the corresponding control at 0 mM NaCl. In Ravnaq-1, positive $\log_2$FC values were recorded for 12 genes, except *Gh_GH3.17*, whereas Porloq-1 showed positive values for all genes analyzed. Therefore, the effects of the biostimulants under saline conditions were evaluated relative to the untreated control of the same genotype at 150 mM NaCl. Under this comparison, all three biostimulants increased the expression of every gene analyzed, with statistically significant differences observed in 115 of 117 gene × genotype × biostimulant combinations. The exceptions were the effects of Serhosil on *Gh_ICS1* and *Gh_SuSy* in Porloq-1 (Figs. 3, 4; Online Resource 2).

Despite the positive effects of the biostimulants relative to the corresponding saline controls, the extent to which expression recovered to baseline levels depended on the genotype and functional module. In Ishonch, the expression of all four genes involved in hormonal regulation and most antioxidant genes remained below baseline. Rizokom-1 produced positive $\log_2$FC values for *Gh_SOD* and *Gh_POD1*, as well as for all three genes of the metabolic/osmoprotective module.

Following Mikrozim-1 treatment, the expression of *Gh_P5CS*, *Gh_SPS*, and *Gh_SuSy* exceeded the baseline, whereas after Serhosil treatment, the expression of all analyzed genes remained suppressed. In Ravnaq-1 and Porloq-1, positive log_2FC values were maintained for all 13 genes after treatment with each biostimulant. Thus, the order Rizokom-1 > Mikrozim-1 > Serhosil was preserved under saline conditions, and the differences among genotypes were maintained (Figs. 3, 4; Online Resource 2).

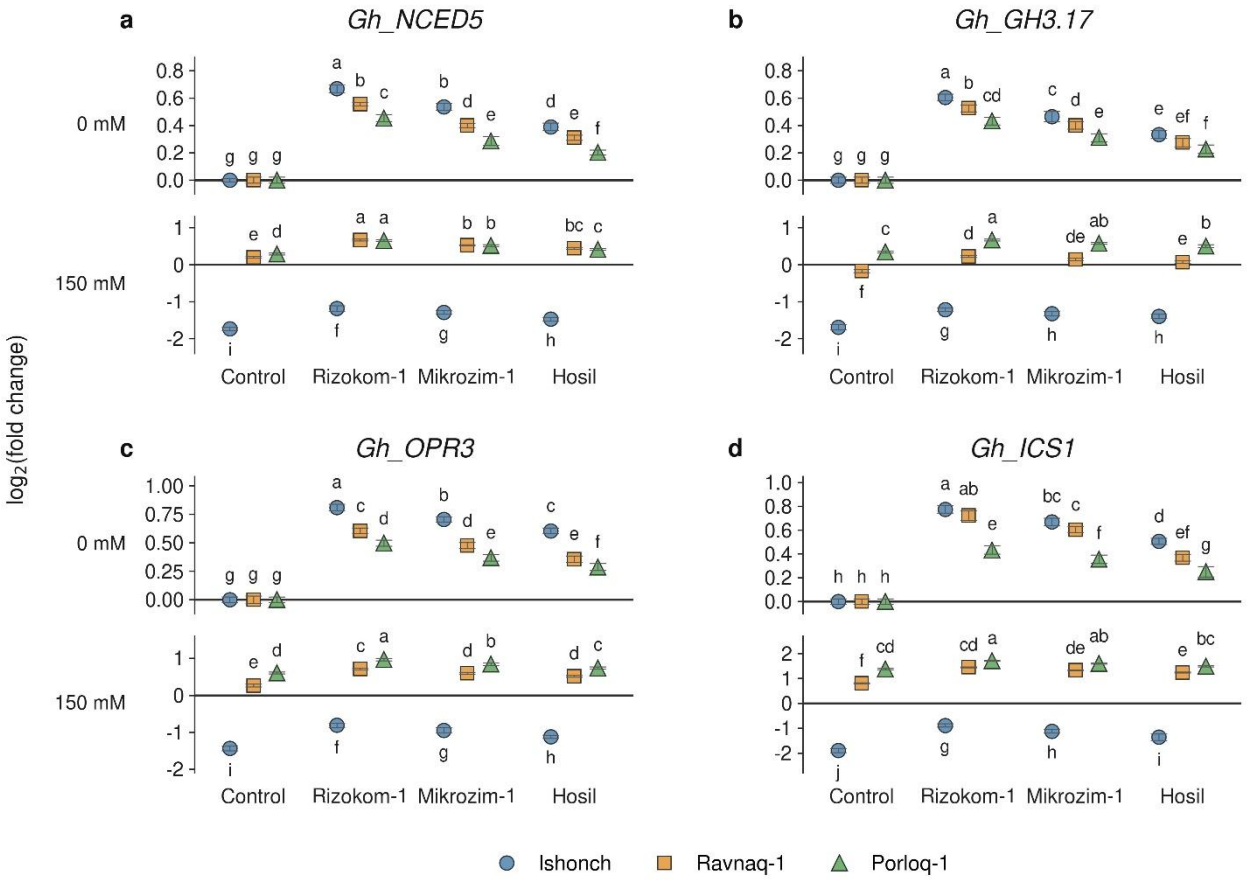


Fig. 3 Expression of genes representing the hormonal regulation module in response to biostimulant treatments at 0 and 150 mM NaCl. The panels show (a) *Gh_NCED5*, (b) *Gh_GH3.17*, (c) *Gh_OPR3*, and (d) *Gh_ICS1*. Values are presented as log_2 fold changes relative to the untreated control of the same genotype at 0 mM NaCl. Symbols represent means, and error bars represent standard deviations calculated from five independent biological replicates. Blue circles, orange squares, and green triangles indicate Ishonch, Ravnaq-1, and Porloq-1, respectively. The horizontal zero line indicates expression equal to the calibrator. Within each NaCl concentration, different lowercase letters indicate statistically significant differences among genotype × treatment combinations according to Tukey's HSD test ($p < 0.05$). "Hosil" is the shortened name used for the commercial biostimulant Serhosil

Three-way analysis of variance confirmed that the combined effects of genotype, NaCl concentration, and treatment determined the observed changes. The main effects of all three factors, as well as the "genotype × NaCl" and "genotype × treatment" interactions, were significant for every gene analyzed. The genotype × NaCl interaction was the main source of variability for 11 genes, whereas biostimulant treatment made the largest contribution for *Gh_SPS* and *Gh_SuSy* (Online Resource 2). Thus, the effects of the biostimulants extended to genes from all three functional modules. Still, the magnitude and configuration of the transcriptional response depended on the cotton genotype and salinity conditions.

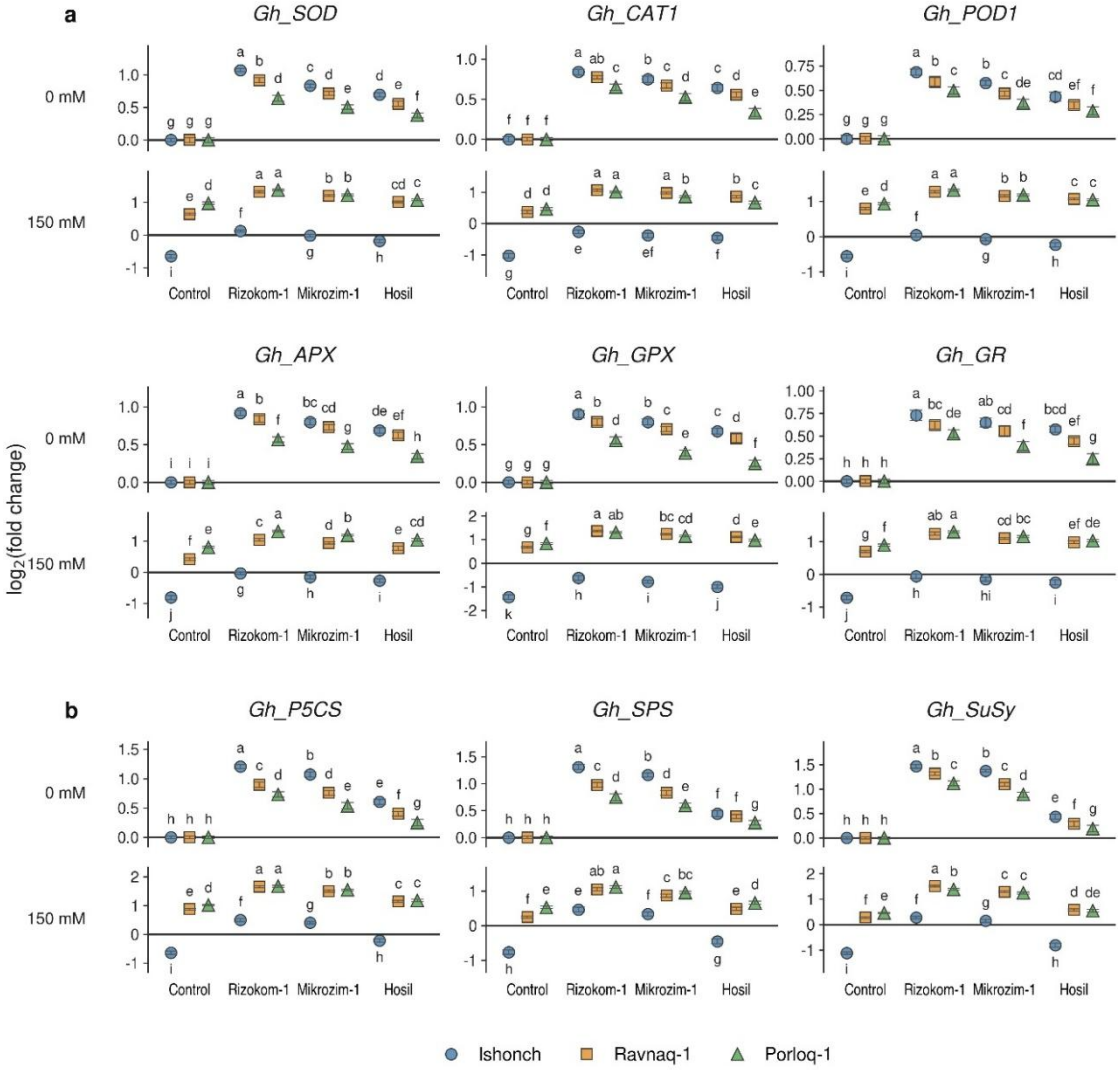


Fig. 4 Expression of genes representing the antioxidant defense and metabolic/osmoprotective modules in response to biostimulant treatments at 0 and 150 mM NaCl. (a) Genes of the antioxidant defense module: *Gh_SOD*, *Gh_CAT1*, *Gh_POD1*, *Gh_APX*, *Gh_GPX*, and *Gh_GR*. (b) Genes of the metabolic/osmoprotective module: *Gh_P5CS*, *Gh_SPS*, and *Gh_SuSy*. Values are presented as $\log_2$ fold changes relative to the untreated control of the same genotype at 0 mM NaCl. Symbols represent means, and error bars represent standard deviations calculated from five independent biological replicates. Blue circles, orange squares, and green triangles indicate Ishonch, Ravnaq-

1, and Porloq-1, respectively. The horizontal zero line indicates expression equal to the calibrator. Within each NaCl concentration, different lowercase letters indicate statistically significant differences among genotype × treatment combinations according to Tukey's HSD test ($p < 0.05$). "Hosil" is the shortened name used for the commercial biostimulant Serhosil

Multivariate Analysis of the Biostimulant-Induced Transcriptional Response

To assess the overall organization of the observed transcriptional changes, principal component analysis was performed using individual expression profiles of the 13 genes.

The first two principal components explained 94.83% of the total variance, with PC1 accounting for 89.28%. The high positive loadings of all genes analyzed on this component indicate that PC1 reflected the overall amplitude of the transcriptional response. PC2 was associated with the relative contribution of its functional components: positive loadings were observed predominantly for genes of the hormonal regulation module, whereas *Gh_P5CS* and several antioxidant genes were oriented toward the negative region. Thus, the positions of transcriptional profiles along PC1 were determined primarily by response magnitude, whereas their positions along PC2 reflected differences in functional response structure (Fig. 5a, b; Online Resource 3).

In the absence of salinity, the control groups of the three genotypes occupied similar positions, whereas the biostimulant treatment shifted them predominantly toward the positive end of PC1. The magnitude of this shift decreased in the order Rizokom-1 > Mikrozim-1 > Serhosil and was most pronounced in Ishonch. At 150 mM NaCl, the distribution of the groups varied among the genotypes: the strongest negative shift was observed for untreated Ishonch, whereas the Ravnaq-1 and Porloq-1 profiles remained predominantly in the positive PC1 region. Thus, salinity altered the overall configuration of the transcriptional profiles in a non-unidirectional manner, depending on the initial genotype response and the biostimulant applied (Fig. 5a; Online Resource 3).

Evaluation of the trajectories relative to the corresponding untreated controls showed that salt stress primarily reduced the amplitude of the induced response. At 150 mM NaCl, trajectory length decreased in all experimental combinations, while the order Rizokom-1 > Mikrozim-1 > Serhosil was retained for each genotype. The smallest reduction was observed for Rizokom-1, whereas for Serhosil it reached 47.5%. The response direction changed to a lesser extent: the angles between the corresponding trajectories at 0 and 150 mM NaCl did not exceed 6.14° for Rizokom-1, 9.82° for Mikrozim-1, and 15.65° for Serhosil. The greatest redirection of the response was observed in Porloq-1 treated with Serhosil (Fig. 5c; Online Resource 3).

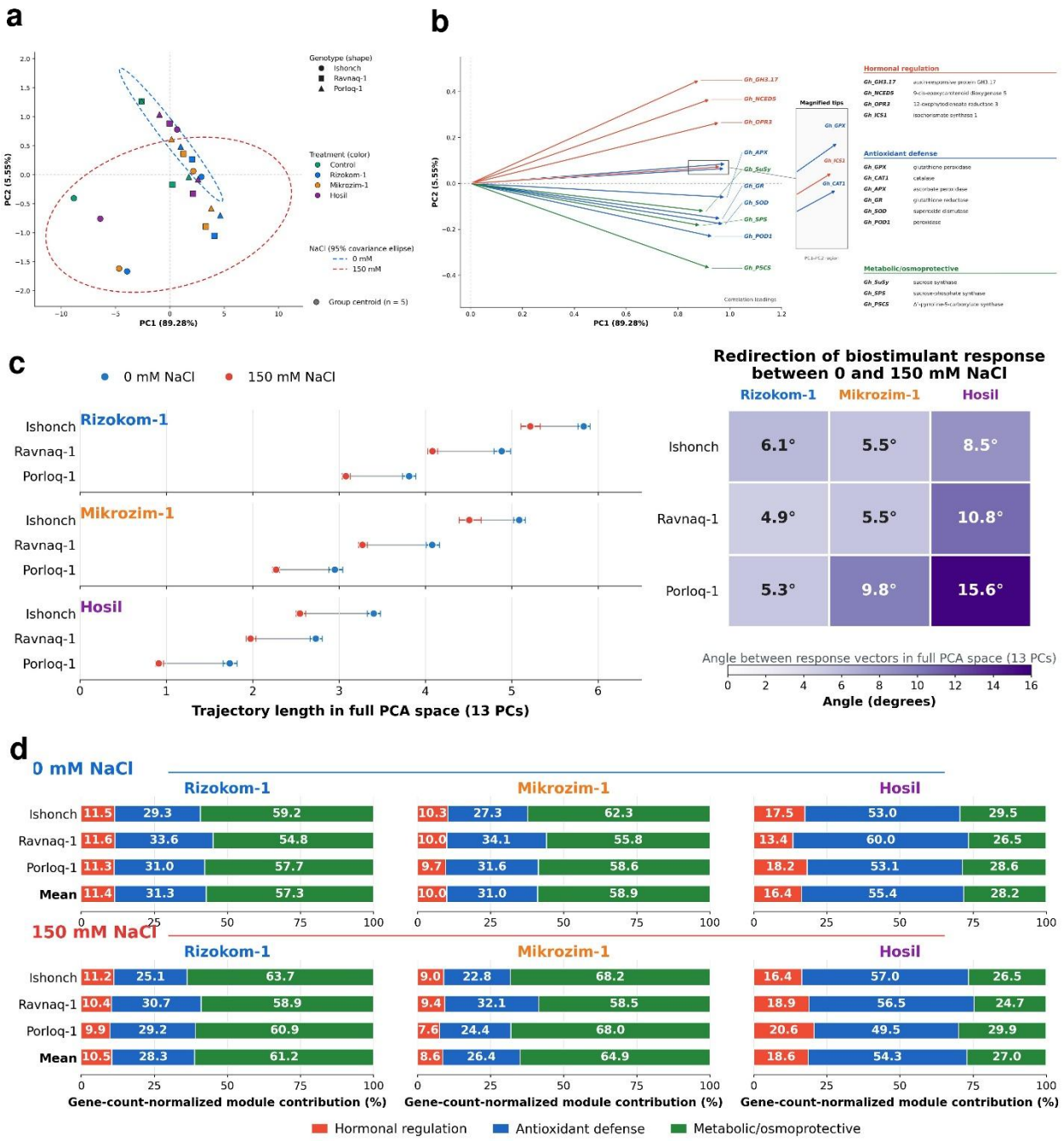


Fig. 5 Multivariate organization of the transcriptional response to biostimulant treatment under 0 and 150 mM NaCl. (a) Principal component analysis of 120 individual transcriptional profiles based on the expression of 13 genes. Genotypes are indicated by different point shapes and treatments by different colors; dashed ellipses represent 95% covariance ellipses describing the distribution of the 60 biological observations at each NaCl concentration, and larger gray points indicate group centroids. (b) Gene loadings in the PC1–PC2 space, with genes grouped into the hormonal regulation, antioxidant defense, and metabolic/osmoprotective modules; the boxed region is shown as an enlarged inset to resolve closely spaced loading tips. (c) Transcriptional trajectory lengths calculated in the full 13-dimensional standardized space and angles between the corresponding response vectors at 0 and 150 mM NaCl. Blue and red points indicate centroid-

based estimates at 0 and 150 mM NaCl, respectively, and error bars represent 95% bootstrap confidence intervals. (d) Gene-count-normalized contributions of the hormonal regulation, antioxidant defense, and metabolic/osmoprotective modules to the transcriptional response. "Hosil" is the shortened name used for the commercial biostimulant Serhosil

The functional structure of the trajectories also remained specific to the biostimulant. In the responses to Rizokom-1 and Mikrozim-1 at both NaCl levels, the metabolic/osmoprotective module predominated, contributing 54.8–68.2% of the response. For Serhosil, the antioxidant defense module remained predominant, contributing 49.5–60.0%. Thus, salinity reduced the overall response amplitude and partially altered its direction, but did not eliminate the characteristic balance of functional components associated with each biostimulant (Fig. 5d; Online Resource 3). The persistence of this overall response structure provided the basis for subsequent assessment of the relationships among changes in individual genes.

Correlation Structure of the Biostimulant-Induced Transcriptional Response

To assess the interrelatedness of the observed changes, correlations among gene-specific biostimulant effects calculated relative to the corresponding untreated controls were evaluated.

For each biostimulant, all 78 gene pairs showed positive correlation coefficients that remained statistically significant after Benjamini–Hochberg correction. Thus, variation in the biostimulant effect across genotypes, salinity regimes, and biological replicates for one gene was generally accompanied by changes in the same direction in other genes. Positive associations were detected both within and between functional modules. Among the strongest cross-module correlations were those between *Gh_NCED5* and *Gh_SOD* under Rizokom-1 and Serhosil treatment, and between *Gh_NCED5* and *Gh_SuSy* under Mikrozim-1 treatment (Fig. 6a; Online Resource 3).

The overall strength of the correlations differed among the biostimulants. The highest mean correlation coefficient was observed for Mikrozim-1 ($\bar{r}$ = 0.849), followed by Rizokom-1 ($\bar{r}$ = 0.822), whereas Serhosil showed the lowest value ($\bar{r}$ = 0.751). Thus, the greatest response amplitude observed for Rizokom-1 was not accompanied by the highest overall gene correlation: the most homogeneous coordinated variation in transcriptional effects was observed under Mikrozim-1 treatment. Comparison of the correlation matrices also showed that the response structure was most similar between Rizokom-1 and Mikrozim-1, whereas the similarity of each biostimulant to Serhosil was lower (Online Resource 3).

Hierarchical clustering separated *Gh_SuSy*, *Gh_P5CS*, and *Gh_SPS* into an independent group corresponding to the metabolic/osmoprotective module. The remaining genes formed a common cluster, alongside clusters combining genes from different functional modules. In particular, *Gh_NCED5* clustered with *Gh_POD1* and *Gh_OPR3* with *Gh_GR*, whereas *Gh_ICS1* clustered

with the antioxidant gene group. This distribution indicates that the organization of coordinated expression changes reflected not only predefined module membership but also similarities in gene-specific transcriptional-effect profiles across the analyzed conditions (Fig. 6b; Online Resource 3).

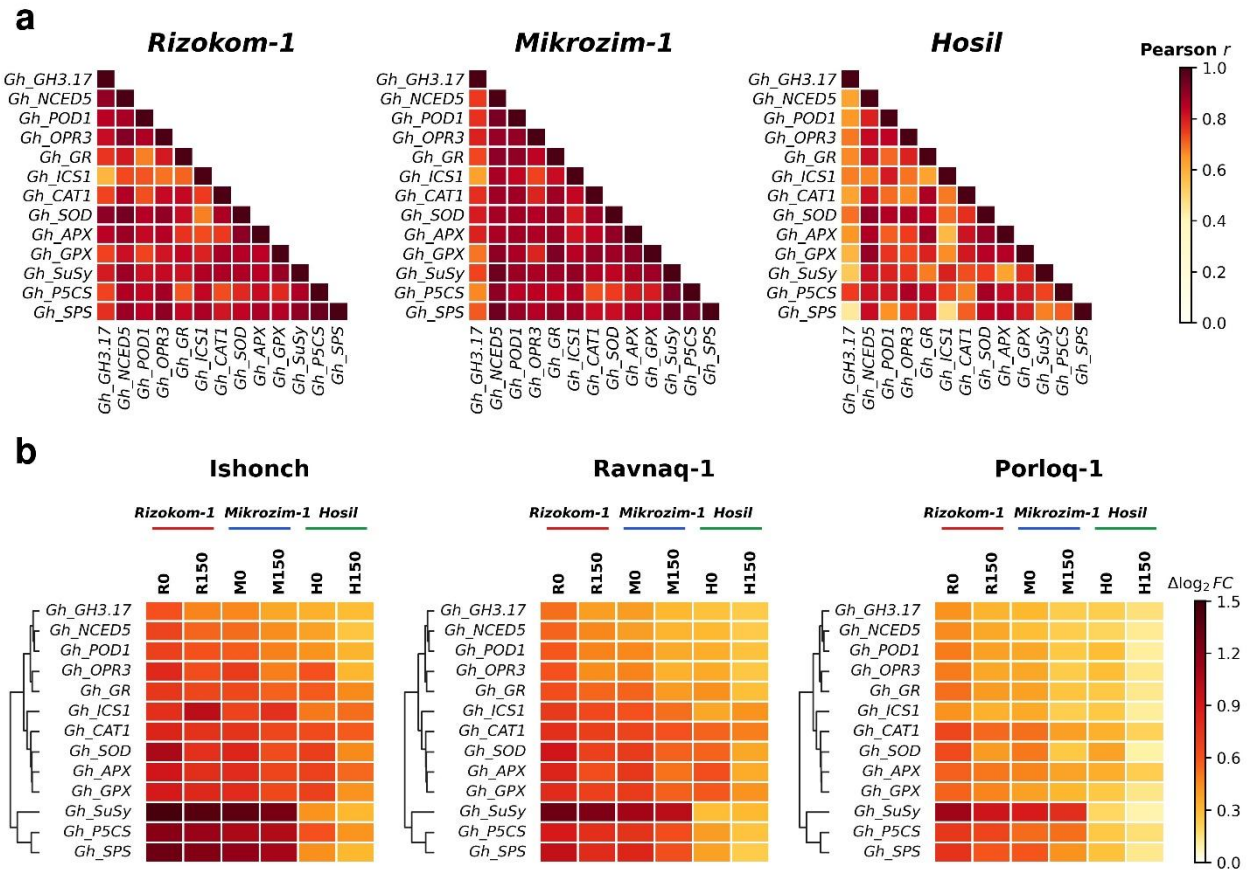


Fig. 6 Correlation and hierarchical clustering of transcriptional effects associated with biostimulant treatment. (a) Pearson correlation matrices for the 13 genes based on individual biostimulant effects calculated relative to the corresponding untreated controls. (b) Hierarchically clustered heatmaps of $\Delta\log_2$FC values for each genotype across the three biostimulants and two NaCl concentrations. R0 and R150 indicate Rizokom-1 treatment at 0 and 150 mM NaCl, respectively; M0 and M150 indicate Mikrozim-1 treatment; H0 and H150 indicate Serhosil treatment. Color intensity represents the magnitude of the transcriptional effect. “Hosil” is the shortened name used for the commercial biostimulant Serhosil

The ordered heatmaps retained the established gene structure while also reflecting the reduction in effect magnitude at 150 mM NaCl. This reduction was most pronounced in Porloq-1 and under Serhosil treatment, whereas in Ishonch, particularly following Rizokom-1 treatment, the structure of the positive effects was preserved most completely (Fig. 6b; Online Resource 3). Overall, these results show that the transcriptional effects of the biostimulants constituted an interconnected

system whose structure differed among the biostimulants and was not limited to correlations among genes within individual functional modules.

Integrated Assessment of the Modular Transcriptional Response to Biostimulant Treatment

To compare the overall magnitude and retention of the observed changes, individual gene effects were combined into scores for the three functional modules and an integrated transcriptional response index.

In the absence of NaCl, the magnitude of the integrated response consistently decreased in the order Rizokom-1 > Mikrozim-1 > Serhosil across all three cultivars. For each biostimulant, the highest index was observed in Ishonch, an intermediate value in Ravnaq-1, and the lowest value in Porloq-1. Exposure to 150 mM NaCl reduced the integrated response in all nine "genotype × biostimulant" combinations without altering this overall hierarchy. Under salt stress, 80.9–91.3% of the response observed in the absence of NaCl was retained following Rizokom-1 treatment, compared with 77.6–89.4% following Mikrozim-1 treatment and 52.7–76.7% following Serhosil treatment. The greatest reduction was observed in Porloq-1 treated with Serhosil (Fig. 7a; Online Resource 3).

Comparison of biostimulant effects at the two salinity levels showed that, for 112 of the 117 gene × cultivar × biostimulant combinations, the difference between the effects at 150 and 0 mM NaCl was negative. Thus, exposure to NaCl was predominantly associated with attenuation of the biostimulants' stimulatory transcriptional effects. The exceptions were four combinations involving *Gh_ICS1*—the three treatments in Ishonch and the Serhosil treatment in Ravnaq-1—and one *Gh_SuSy*–Ravnaq-1–Serhosil combination. Therefore, the reduction in response was systematic but not completely uniform and depended on the particular gene and genotype (Fig. 7b; Online Resource 3).

Analysis of response retention in the individual functional modules revealed differences in their sensitivity to salt stress. Under Rizokom-1 treatment, the metabolic/osmoprotective module was the most stable, retaining 84.4–94.5% of its initial response depending on the cultivar. A similar pattern was observed for Mikrozim-1, with response retention in this module ranging from 83.6% to 94.6%. For Serhosil, module-level indices showed a stronger dependence on genotype; in Porloq-1, response retention did not exceed 56.7% across any of the three modules. However, relative response retention did not always correspond to the absolute response magnitude; therefore, these measures reflected different properties of the transcriptional response—its initial intensity and its stability under NaCl exposure (Fig. 7c; Online Resource 3).

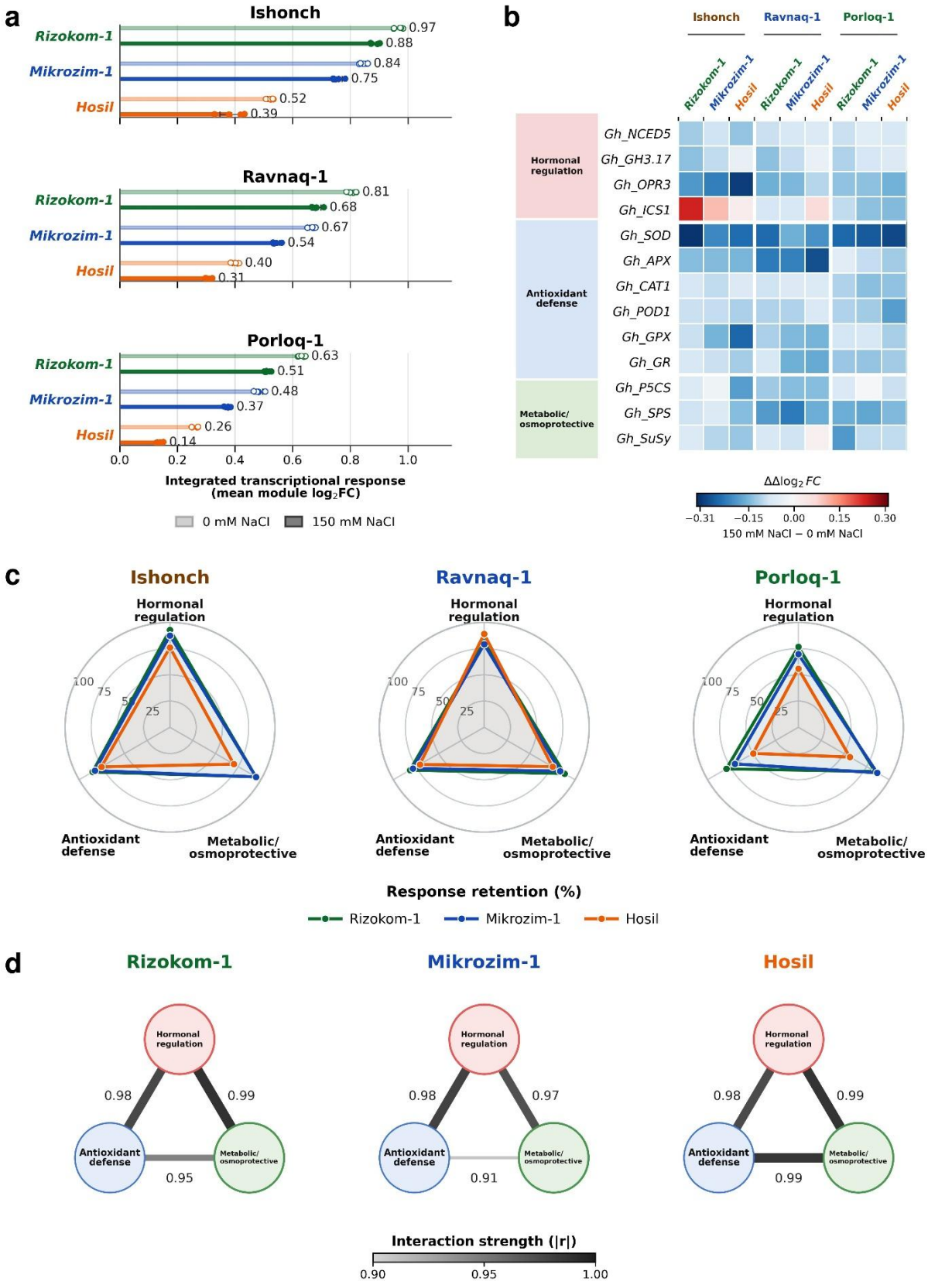


Fig. 7 Integrated assessment of the strength, salt-dependent change, retention, and module concordance of the transcriptional response. (a) Integrated transcriptional response, calculated as the equally weighted mean of the three functional-module scores, for each genotype and biostimulant at 0 and 150 mM NaCl. Open and filled circles represent individual biological

669 replicates at 0 and 150 mM NaCl, respectively; horizontal segments and adjacent numerical labels
670 indicate group means. (b) Salt-dependent changes in individual gene effects expressed as
671 $\Delta\Delta\log_2$FC, calculated as the difference between the effects at 150 and 0 mM NaCl. The colored
672 blocks indicate the hormonal regulation, antioxidant defense, and metabolic/osmoprotective
673 modules. (c) Retention of the response of each functional module at 150 mM NaCl relative to 0
674 mM NaCl. The radial scale indicates response retention (%), with higher values indicating greater
675 preservation of the response under salt stress. (d) Pearson correlations between the mean responses
676 of the three functional modules across six genotype × NaCl concentration contexts; edge labels
677 indicate the corresponding Pearson correlation coefficients (r), and edge thickness represents
678 interaction strength (|r|). "Hosil" is the shortened name used for the commercial biostimulant
679 Serhosil
680
681 Across the six "genotype × NaCl" combinations, the mean responses of the three functional
682 modules were strongly concordant despite differences in absolute response intensity and response
683 retention. Pairwise correlation coefficients between the module scores ranged from 0.945 to 0.989
684 for Rizokom-1, from 0.909 to 0.980 for Mikrozim-1, and from 0.975 to 0.989 for Serhosil ($p \leq$
685 0.0121). Thus, variation in the overall response amplitude across the analyzed contexts was
686 accompanied by concordant changes in the hormonal regulation, antioxidant defense, and
687 metabolic/osmoprotective modules (Fig. 7d; Online Resource 3).
688 Overall, the integrated analysis identified three characteristics of biostimulant action: the
689 amplitude of the transcriptional response, the extent to which it was retained under salt stress, and
690 the coordination of changes across functional modules. Rizokom-1 combined the highest response
691 amplitude with the greatest response retention; Mikrozim-1 showed an intermediate response
692 amplitude and a stable metabolic/osmoprotective component. In contrast, Serhosil produced a
693 weaker response that was more strongly dependent on genotype. At the same time, strong
694 concordance among changes in the analyzed functional modules was observed for all three
695 biostimulants.

696 **Discussion**

697 Plant tolerance to salinity is a multilevel process shaped by the plant's initial state, the intensity of
698 stress, and the coordination of the adaptive responses activated (Anwar et al. 2022; Muhammad et
699 al. 2025). The possibility of deliberately modulating this process is important for developing
700 approaches aimed at maintaining plant adaptive capacity under adverse conditions. At present, the
701 application of biostimulants is regarded as a promising approach for enhancing plant adaptive
702 capacity to various abiotic stresses, including salinity (Kaushal et al. 2023; Lau et al. 2025). A

more complete understanding of their effects, therefore, requires moving beyond descriptions of individual plant responses and assessing how these responses are integrated into a coherent adaptive response. At the same time, the effects of biostimulants are realized within a specific genotypic and stress context. They may vary depending on the plants' initial capacity to respond and the salinity intensity (Batool et al. 2025; Detcheverry et al. 2026). Accordingly, because seed germination and early root and shoot growth are informative traits for differentiating cotton genotypes under salinity (Munawar et al. 2021; An et al. 2024; Anwar et al. 2025; Gul et al. 2025), commercial cotton cultivars were screened at different levels of simulated salt stress at the first stage of the present study.

The screening showed that cultivar differentiation became more pronounced as the NaCl concentration increased. At 50 mM NaCl, reductions in most traits were relatively moderate, and variation in mean germination time did not clearly distinguish the cultivars. At 150 mM NaCl, Porloq-1 maintained germination energy and root and shoot growth more effectively, Ishonch showed the greatest combined reductions, and Ravnaq-1 occupied an intermediate position, whereas the relative performance of An-Bayovut-2 and Namangan-77 depended on the trait considered. This concentration- and trait-dependent pattern agrees with reports that genotypic differences in cotton under moderate salinity may be expressed in individual traits, while a higher salt load allows clearer differentiation based on a combination of early developmental responses (Shaheen et al. 2023; An et al. 2024; Anwar et al. 2025; Gul et al. 2025). It also confirms that maintaining germination under salinity is not necessarily accompanied by a comparable preservation of subsequent root and shoot growth, supporting the use of an integrated multitrait assessment. Accordingly, Ishonch and Porloq-1 were selected as contrasting cultivars for comparative screening of biostimulants, Ravnaq-1 was included in the transcriptional analysis as a genotype with an intermediate response, and 150 mM NaCl was selected as the discriminating stress level.

The contrast between the cultivars enabled us to assess the extent to which biostimulant effects depended on the initial genotype response to salinity. In the more sensitive cultivar Ishonch, the most pronounced mitigation of stress-induced changes was observed with Mikrozim-1 and Rizokom-1. In contrast, in the more tolerant Porloq-1, differences among treatments were less pronounced, and the most consistent positive effect was observed with Rizokom-1. A similar genotype-dependent pattern has been reported in cotton, where inoculation with a single plant-growth-promoting bacterium produced a stronger beneficial response in the more salt-sensitive genotype than in the more resilient genotype (Batool et al. 2025). More generally, the relative effect of biostimulation may be greater in genotypes experiencing greater stress inhibition, whereas more tolerant genotypes may show a smaller scope for additional improvement because

their untreated controls maintain higher performance (Kaushal et al. 2023; Lau et al. 2025). At the same time, the differences between Mikrozim-1 and Mikrozim-2, as well as between Rizokom-1 and Rizokom-2, indicate that the observed response was determined not only by genotype but also by the specific product. This interpretation is consistent with evidence that biostimulant effects depend on product properties, crop genotype, and stress conditions, and cannot be automatically extrapolated across products with similar intended applications (Covașă et al. 2025; Lau et al. 2025; Detcheverry et al. 2026). Rizokom-1, Mikrozim-1, and Serhosil, which produced positive changes in the examined parameters in both contrasting cultivars, were selected for subsequent transcriptional response analysis.

Because transcriptional regulation represents one of the levels underlying the formation of physiological and biochemical responses, the subsequent analysis focused on transcriptional changes associated with the action of the selected products (Anwar et al. 2022; Gechev 2025). Analysis of marker gene expression was used to characterize transcriptional changes associated with functionally related processes across three cultivars that differed in their responses to salinity. In the absence of NaCl, all three biostimulants increased the expression of genes belonging to the hormonal regulation, antioxidant defense, and metabolic/osmoprotective modules; however, the responses they induced differed in amplitude and in the relative contribution of the functional components. Similar activation of stress-associated genes following presowing treatment under favorable conditions has been reported in other studies and has been interpreted as a form of prior regulatory reprogramming that may prepare plants for subsequent stress exposure (Kaushal et al. 2023; Lau et al. 2025). At 150 mM NaCl, differences among the products were retained. Nevertheless, the transcriptional response was more strongly determined by genotype: in the sensitive cultivar Ishonch, biostimulant treatment was manifested primarily as attenuation of salinity-induced transcriptional repression, whereas in Ravnaq-1 and Porloq-1, positive regulation of the analyzed genes predominated. This pattern is consistent with evidence that contrasting cotton genotypes differ both in the initial state of their defense systems and in their responses to treatment with a plant-growth-promoting bacterium under salinity (Batool et al. 2025). In turn, the differences in response amplitude and functional structure observed for Rizokom-1, Mikrozim-1, and Serhosil are consistent with studies showing that similar positive effects of biostimulants on plant growth may be accompanied by distinct hormonal, antioxidant, and metabolic changes (Khalid et al. 2024; Gechev 2025; Mashabela et al. 2025). Thus, the observed changes did not conform to a simple model of nonspecific enhancement of gene expression; rather, the transcriptional response was determined jointly by the biostimulant identity, the cotton genotype, and the salt-exposure conditions.

This dependence was manifested not only in the overall response amplitude but also in the pattern of involvement of individual functional modules. Within the hormonal regulation module, it was reflected in the simultaneous but nonuniform changes in the expression of genes associated with ABA biosynthesis (*Gh_NCED5*), auxin metabolism (*Gh_GH3.17*), the jasmonate pathway (*Gh_OPR3*), and salicylic acid biosynthesis (*Gh_ICS1*). Recent integrative genome-wide association study (GWAS) and transcriptomic analyses of *Gossypium hirsutum* L. during germination under salinity likewise showed that genotype-dependent variation in early salt responses was associated with changes in genes involved in phytohormone-related and other stress-responsive pathways (Wang et al. 2026). Time-course transcriptomic analysis further demonstrated that hormone-related genes may exhibit different, and sometimes opposite, expression dynamics during prolonged salt exposure: *NCED* genes were continuously induced, whereas most genes involved in jasmonate biosynthesis, including *OPR* genes, were downregulated (Wang et al. 2020). This pattern indicates not a synchronous enhancement of all hormonal pathways but rather a shift in their relative contributions to stress regulation. Studies of microbial biostimulants under salinity also show that treatment can simultaneously modify genes belonging to several phytohormone-response pathways rather than affecting a single hormonal branch in isolation (Hwang et al. 2025). The differences among the responses of *Gh_NCED5*, *Gh_GH3.17*, *Gh_OPR3*, and *Gh_ICS1* observed in our study are consistent with this general pattern and suggest that the effects of the biostimulants were associated not with isolated activation of a single hormonal pathway but with a reorganization of the balance among several interconnected signaling processes. However, changes in the expression of genes involved in phytohormone biosynthesis and metabolism reflect only transcriptional regulation and do not permit direct conclusions about the concentrations of the corresponding hormones or the activity of the signaling pathways they control.

The possible significance of the reorganization of the hormonal regulation module lies in the involvement of phytohormones in regulating subsequent defense responses. In a cotton seed-priming study using mepiquat chloride, increased expression of ABA-biosynthesis genes and accumulation of this hormone preceded changes in GABA-dependent regulation and the ascorbate–glutathione cycle, whereas inhibition of ABA biosynthesis weakened the antioxidant response (Qi et al. 2023). In our study, changes in the expression of hormone-related genes were likewise accompanied by reorganization of the antioxidant defense module, which included *Gh_SOD*, *Gh_CAT1*, *Gh_POD1*, *Gh_APX*, *Gh_GPX*, and *Gh_GR*. These genes represent functionally related components involved in the removal of reactive oxygen species and the maintenance of cellular redox balance; therefore, their coordinated expression may reflect transcriptional coordination among several components of the antioxidant response (Wang et al.

2020). Similar genotype- and time-dependent organization of hormone- and antioxidant-related transcriptional responses has been reported in cotton under salinity (Bilal et al. 2026; Gao et al. 2026). This interpretation is consistent with studies showing that microbial and non-microbial biostimulants can modify hormone-related and antioxidant-associated responses under salinity. In cotton, inoculation with salt-tolerant plant-growth-promoting rhizobacteria altered hormone-signaling and ascorbate-related transcriptional pathways (Akbar et al. 2022), whereas treatment with *Bacillus mojavensis* under saline conditions was associated with changes in the expression of stress- and hormone-related genes (Zhao et al. 2024). Similar relationships among biostimulant application, hormonal regulation, and antioxidant or osmoprotective responses have also been reported across crop species, including lettuce and wheat (Deolu-Ajayi et al. 2022; Benito et al. 2024; Algasem et al. 2026). However, the transcriptional dynamics of individual members of the *SOD* and *POD* families do not always align with their respective enzyme activities, and integrated transcriptomic and proteomic analyses in cotton have revealed only partial correspondence between mRNA and protein abundance (Chen et al. 2021). Therefore, the present data indicate coordinated involvement of components of the antioxidant system at the transcriptional level but do not support conclusions about increased antioxidant enzyme activity or enhanced antioxidant protection in plants.

The transcriptional reorganization was not limited to the hormonal regulation and antioxidant defense modules but also involved the metabolic/osmoprotective module. The genes in this module perform distinct but functionally related roles: *Gh_P5CS* participates in proline biosynthesis, whereas *Gh_SPS* and *Gh_SuSy* are associated with sucrose metabolism and carbon redistribution. At 150 mM NaCl, positive regulation of this module was most consistently maintained after treatment with Rizokom-1 and Mikrozim-1, although its magnitude varied by cultivar. The functional significance of *P5CS* in the cotton response to salinity is supported by recent functional analysis of *GhP5CS1*, in which virus-induced gene silencing reduced proline accumulation, increased oxidative damage, and increased sensitivity to NaCl (Fang et al. 2025). Transcriptomic and metabolomic studies of cotton have likewise shown that salinity induces coordinated changes in amino-acid, carbohydrate, and energy metabolism, accompanied by the accumulation of sugars and other low-molecular-weight metabolites involved in osmotic adjustment (Guo et al. 2022; Han et al. 2023). Comparable involvement of osmoprotective and carbon-related responses has been reported following biostimulant application under salinity. In wheat, a natural biostimulant increased *TaP5CS* expression and proline accumulation (Algasem et al. 2026), whereas a combined microbial and non-microbial biostimulant treatment in lettuce altered primary metabolism, GABA accumulation, and hormone-associated pathways under salt stress (Benito et al. 2024). Seaweed-based biostimulants have also been associated with the

induction of *P5CS* and the accumulation of proline and soluble sugars under abiotic stress (Deolu-Ajayi et al. 2022). In this context, the concurrent changes in the expression of *Gh_P5CS*, *Gh_SPS*, and *Gh_SuSy* may reflect the formation of transcriptional prerequisites for osmotic regulation and carbon redistribution. However, without measuring proline, soluble sugars, and other metabolites, these data do not confirm actual accumulation of osmoprotectants or changes in the rates of the corresponding metabolic processes.

The nonuniform involvement of genes within the individual functional modules indicated that the biostimulant-associated transcriptional response should also be evaluated at an integrated level. Accordingly, multivariate analysis was used to assess its overall organization. At 150 mM NaCl, the effects of the biostimulants were associated with responses that generally retained the direction observed in the absence of salinity but showed lower amplitude and an altered balance among functional components. This was indicated by the relatively small angles between the response vectors at 0 and 150 mM NaCl in the full principal-component space, the reduced length of the transcriptional trajectories, and changes in the relative contributions of individual modules. Comparable integrative studies of biostimulant-treated plants under salinity have shown that microbial and non-microbial products can modify the relative contribution of hormonal, antioxidant, osmoprotective, and metabolic processes and often produce differential changes among stress-related responses (Akbar et al. 2022; Benito et al. 2024; Algasem et al. 2026). The dynamic nature of such responses is also consistent with cotton transcriptomic studies showing that the composition and functional distribution of salt-responsive genes vary with the intensity and duration of stress exposure (Wang et al. 2020; Gao et al. 2026). Therefore, the reduction in the integrated response amplitude at 150 mM NaCl should not be interpreted unambiguously as a weakening of biostimulant action. More likely, under salinity, their effects were associated with the retention of the overall direction of the transcriptional response, alongside a redistribution of activity among its functional components. This combination of stability in the overall direction and variability in internal structure is consistent with the view of the biostimulant-associated transcriptional response as a dynamic system that adapts its organization to prevailing exposure conditions.

The combination of a preserved overall response direction and changes in its internal structure was further reflected in the correlation and clustering analyses. At the level of individual genes, the magnitude and distribution of transcriptional effects varied among biostimulants and genotypes, whereas the gene–gene correlation structure differed among biostimulants. In contrast, the mean responses of the hormonal regulation, antioxidant defense, and metabolic/osmoprotective modules were highly correlated across the six genotype × NaCl contexts for each biostimulant. Integrative studies of biostimulant-treated plants under salinity have likewise shown that treatment can

simultaneously affect several hormone-related, antioxidant, osmoprotective, and metabolic processes, although the magnitude and composition of individual responses may vary with the product and experimental context (Akbar et al. 2022; Benito et al. 2024; Algasem et al. 2026). A similar combination of common and genotype-specific components has been identified in transcriptomic studies of cotton forms contrasting in salinity tolerance: under salinity, individual genes and regulatory networks differed among genotypes, but the main functional directions of the response – including hormonal regulation, antioxidant processes, membrane transport, and metabolic reorganization – were shared (Wang et al. 2020; Bilal et al. 2026; Gao et al. 2026). Our results suggest a similar principle of biostimulant action: the responses and relative contributions of individual genes within particular functions may vary across cultivars and products, whereas the mean responses of the main functional modules were strongly associated across the analyzed genotype × NaCl contexts (Khalid et al. 2024; Gechev 2025). Thus, coordination of the biostimulant-associated transcriptional response was more evident at the level of broader functional components than as an obligatory correspondence among the responses of all individual genes. However, the observed correlations characterize shared variation in expression and do not demonstrate direct regulatory interactions among the genes or modules (Cai and Des Marais 2023). The magnitude of the integrated transcriptional response also differed substantially among the cultivars. The greatest response amplitude and the highest retention under 150 mM NaCl were observed in Ishonch; intermediate values were recorded for Ravnaq-1, whereas the response was less pronounced in Porloq-1. Thus, a greater amplitude of expression changes did not correspond to greater salinity tolerance. This discrepancy is consistent with comparative studies of cotton genotypes, which have shown that salinity responses depend on the genotype, the trait evaluated, and the plants' initial physiological and metabolic state (Munawar et al. 2021; Guo et al. 2022; Anwar et al. 2025; Gul et al. 2025). In studies of biostimulant-treated plants, the magnitude and direction of the response likewise depended on the genotype, the product, and their interaction; therefore, a stronger molecular or physiological response did not necessarily indicate greater stress tolerance (Benito et al. 2024; Lau et al. 2025; Mashabela et al. 2025; Detcheverry et al. 2026). In this context, the strong response of Ishonch may reflect greater disturbance of its initial state and a greater need to recruit additional regulatory resources, whereas the lower response amplitude of Porloq-1 may be associated with a more stable organization of its initial response to salinity. These interpretations remain tentative, but they show that the intensity of biostimulant-associated transcriptional changes cannot be regarded as a direct measure of salinity tolerance. The observed differences primarily indicate that the realization of the transcriptional response to biostimulant treatment depends on the genotype's regulatory background.

Pronounced differences among the biostimulants accompanied the genotype dependence of the transcriptional response. Rizokom-1 produced the greatest response amplitude and the highest retention of the integrated response at 150 mM NaCl; Mikrozim-1 occupied an intermediate position, whereas the response to Serhosil was less pronounced and more strongly dependent on the cultivar. At the same time, the functional modules were highly correlated for Serhosil despite its lower overall response amplitude. Thus, the strength of concordance among transcriptional changes did not, by itself, determine the magnitude of the product effect: a relatively weak response could also show strong intermodule associations, and its characterization therefore requires simultaneous consideration of response amplitude, retention under salinity, and the balance among functional components. Similar product-specific differences have been reported in studies of microbial, non-microbial, and combined biostimulants, in which comparable effects on plant growth were accompanied by distinct hormonal, antioxidant, metabolic, and osmoprotective responses (Benito et al. 2024; Lau et al. 2025; Mashabela et al. 2025; Detcheverry et al. 2026). By integrating the results of the morphological screening and transcriptional analysis, Rizokom-1 may be preliminarily considered the most promising product for further investigation in Ishonch and Porloq-1, whereas Mikrozim-1 is of particular interest for the salt-sensitive cultivar Ishonch. Serhosil also mitigated the effects of NaCl on individual parameters; however, the pronounced genotype dependence of its effects indicates the need for more precise determination of the conditions under which it is most effective. These results provide a basis for genotype-specific selection of biostimulants and for subsequent evaluation of the selected "genotype × biostimulant" combinations using physiological, biochemical, and agronomic parameters.

Conclusion

Analysis across the three sequential analytical stages of a single experiment showed that biostimulants mitigated the negative effects of NaCl on early cotton development in a genotype- and product-specific manner and were accompanied by coordinated reorganization of the hormonal regulation, antioxidant defense, and metabolic/osmoprotective modules. The strong relationships observed among these modules across the analyzed genotype × salinity contexts, despite variation in response amplitude and internal structure, suggest a coordinated transcriptional response architecture associated with biostimulant treatment under salinity conditions. Based on the evaluation performed, Rizokom-1 was identified as the priority product for further investigation in Ishonch and Porloq-1, whereas Mikrozim-1 was identified as a candidate for further study in Ishonch. These results provide a basis for subsequent physiological, biochemical, and agronomic evaluation of the selected "cultivar × biostimulant" combinations and for determining their potential to mitigate salt stress in cotton.

Supplementary Information The online version of this article contains supplementary material, which is available to authorized users.

Online Resource 1 Cultivar and biostimulant screening data, statistical analyses, and MGIDI rankings. The file contains raw observations, descriptive statistics, $\log_2$-transformed relative-performance values, blocked ANOVA, Tukey's HSD comparisons, grouping letters, quality-control checks, and MGIDI input and output data.

Online Resource 2 RT-qPCR expression data and statistical analyses of 13 marker genes. The file contains relative-expression data, $\log_2$-transformed values, summary statistics, ANOVA results, diagnostic tests, Tukey's HSD comparisons, and functional-module annotations.

Online Resource 3 Multivariate and integrative analyses of the transcriptional response. The file contains PCA, scaling parameters, group centroids, covariance ellipses, transcriptional trajectories, module contributions, correlation and clustering analyses, heatmap data, integrated module responses, response retention, and module-concordance analyses.

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Author contributions Conceptualization: VK and IS. Methodology: FR. Investigation: DT and EN. Formal analysis, Data curation, and Visualization: DM. Writing—original draft preparation: SS. Writing—review and editing: VK and IS. Supervision: IS. Project administration: VK. All authors read and approved the final manuscript.

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. The calculation files supporting the reported analyses are provided as Online Resources 1–3.

Statements and Declarations

Competing interests The authors declare that they have no competing interests.

Ethics approval and consent to participate Not applicable. This study involved experiments with cotton plants and did not involve human participants or animals.

Use of AI-assisted tools An AI language model was used solely for language editing. All AI-assisted text was critically reviewed and edited by the authors, who take full responsibility for the

final content. AI was not used for data generation, statistical analysis, or the creation or modification of figures.

Consent for publication Not applicable.

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