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Integrative Multi-omics Analyses of Barley Rootzones under Salinity Stress Reveal Two Distinctive Salt Tolerance Mechanisms

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ABSTRACT

The mechanisms underlying rootzone-localized responses to salinity during early stages of barley development remain elusive. In this study, we performed the analyses of multi-root-omes (transcriptomes, metabolomes, and lipidomes) of a domesticated barley cultivar (Clipper) and a landrace (Sahara) that maintain and restrict seedling root growth under salt stress, respectively. Novel generalized linear models were designed to determine differentially expressed genes (DEGs) and abundant metabolites (DAMs) specific to salt treatments, genotypes, or rootzones (meristematic Z1, elongation Z2, and maturation Z3). Based on pathway over-representation of the DEGs and DAMs, phenylpropanoid biosynthesis is the most statistically enriched biological pathway among all salinity responses observed. Together with histological evidence, an intense salt-induced lignin impregnation was found only at stelic cell wall of Clipper Z2, compared with a unique elevation of suberin deposition across Sahara Z2. This suggests two differential salt-induced modulations of apoplastic flow between the genotypes. Based on the global correlation network of the DEGs and DAMs, callose deposition that potentially adjusted symplastic flow in roots was almost independent of salinity in rootzones of Clipper, and was markedly decreased in Sahara. Taken together, we propose two distinctive salt tolerance mechanisms in Clipper (growth-sustaining) and Sahara (salt-shielding), providing important clues for improving crop plasticity to cope with deteriorating global soil salinization.

Key words: barley root, transcriptomics, metabolomics, lipidomics, omics integration, salinity stress

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INTRODUCTION

Salinity is one of the major abiotic stresses severely affecting cereal crop yields worldwide. Improving salinity tolerance of one of the most widely cultivated cereals, barley (*Hordeum vulgare* L.), is essential to increase grain yields on saline agricultural lands. Barley is an essential feed, food, and brewing crop, and a model system for temperate cereals. As a glycophyte, barley suffers substantial yield loss when grown under saline conditions, with roots acting as the first sensors and responders (Glenn et al., 1999). Increased soil salinity exposes the roots to sodium (Na^+) and chloride (Cl^-) ions, which triggers a cascade of responses leading to differential gene expression, metabolism, and protein activity, as well as altered ion transport pathways, cell wall composition, and root morphology. Differential responses at the level of either cell types or developmental zones are part of a strategy for the root to respond and

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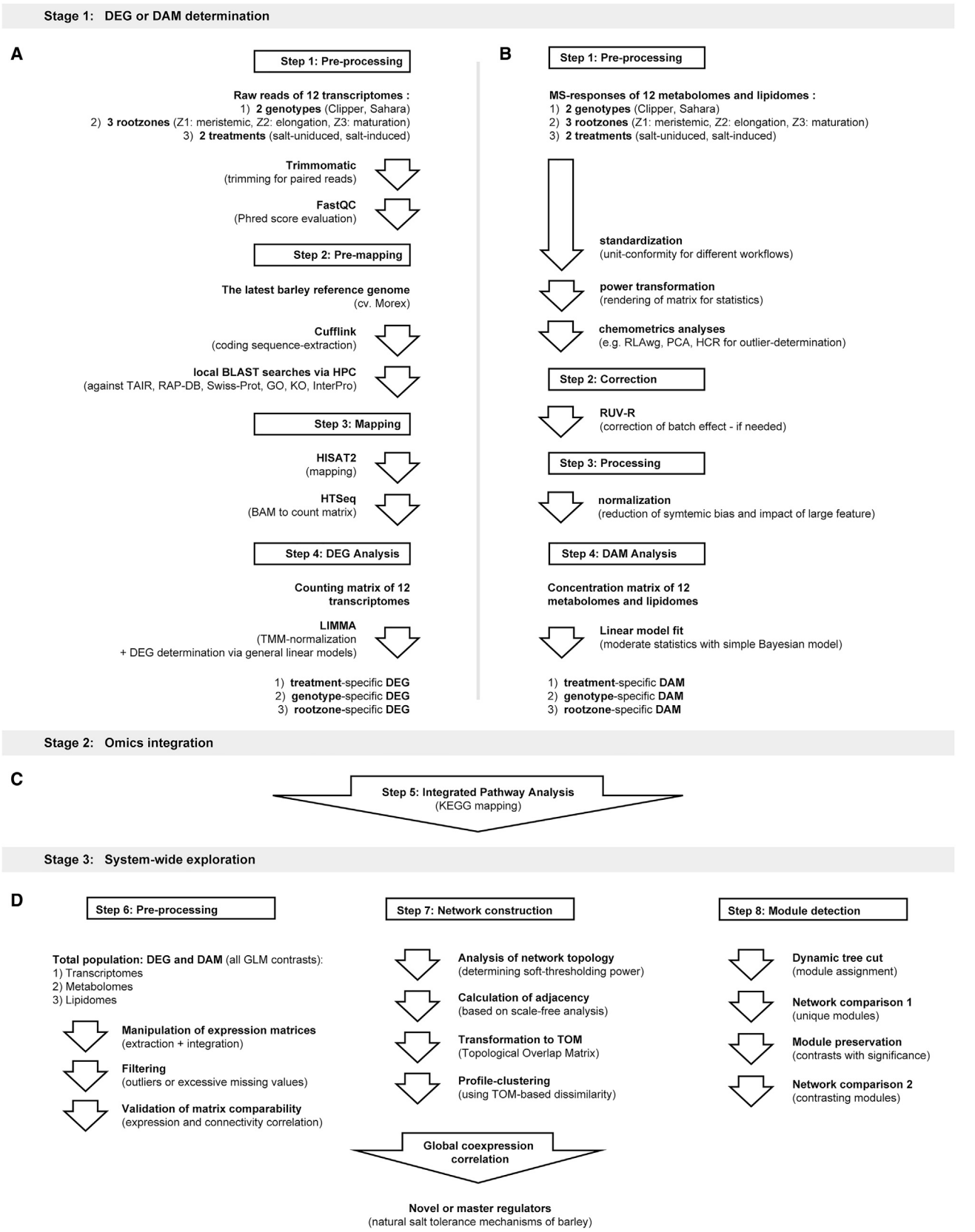
acclimate to salinity (Dinneny et al., 2008; Sarabia et al., 2018, 2020). Although a large number of studies have investigated salinity responses of plants at the physiological and molecular level (Hill et al., 2013; Shelden et al., 2013), relatively little is known about the early rootzone-specific response to salt stress in barley roots. Integrative 'omics approaches within large-scale experiments, including genomics, transcriptomics, ionomics, proteomics, and metabolomics, can help decipher the interplay of cellular functions at different levels.

In barley, several initial analyses indicate that different developmental zones within the root respond distinctly to salt stress in tolerant and sensitive genotypes. Two barley genotypes, Clipper (a domesticated cultivar) and Sahara (an African landrace) are of particular interest based on previously reported diversity in salt tolerance: with long-term salt exposure, Widodo et al. (2009) found that both Clipper and Sahara showed similar initial reductions in biomass after 3 weeks of 100 mM NaCl exposure when grown hydroponically under controlled conditions. Whereas, after 5 weeks of salinity treatment, Sahara was showing a recovery phenomenon and resumed growth and Clipper continued to show reduced growth relative to control despite containing a sodium exclusion locus (Shi et al., 2010). With shorter-term salt exposure (72 h post-germination of seedlings), Sahara showed the most significant inhibition of root elongation but not of root mass grown on 100 mM NaCl agar medium, whereas Clipper maintained its relative root growth rate (Shelden et al., 2013). Furthermore, barley salinity stress responses were shown to depend on the growth system (hydroponic or soil) (Tavakkoli et al., 2010). These observations suggest that developmental stage and growth environment as well as the timing and length of exposure can change the overall responses of barley to salinity stress. Consistent experimental setups are crucial to achieve reproducible and comparable outcomes in studies of salt stress in barley. In this work, we focus on the contrasting early growth responses to salt stress during the barley seedling development using agar medium, in which Clipper showed sustained seminal root growth, whereas Sahara showed decreased seminal root growth consistent to the previous work (Shelden et al., 2013).

In a subsequent study, two *de novo* transcriptome assemblies of Clipper and Sahara were constructed and generalized linear models (GLM) were applied to access spatial, treatment-related, and genotype-specific gene responses along the developmental gradient of barley roots (Hill et al., 2016). A gradual transition from transcripts related to sugar-mediated signaling at Z1 to those involved in cell wall metabolism in Z2 was observed. These findings are consistent with transcriptional analyses of salt treatments in other cereal crops and model plants, such as rice (Walia et al., 2005), maize (Zhao et al., 2014), and *Arabidopsis thaliana* (Hunter et al., 2019), which also show that the transcript levels of many cell wall- or callose deposition-related genes consistently change in response to salt stress. Changes in the chemical composition of cell walls as a result of salt stress are less well documented than the changes in gene expression. Previous studies have shown that salt stress induces changes in the root cell wall composition, including the increased deposition of lignin and suberin in endodermal and exodermal cells, which influence water and ion permeability and transport pathways (Byrt et al., 2018). For example, in two recent studies, (Kreszies et al. 2018b, 2020) found that cultivated barley varieties increased suberin levels in roots in response to osmotic stress as an adaptation to prevent water loss, whereas some wild barley varieties used suberization of specific root tissues or specific rootzones, while others showed no changes in suberization and more consistent water uptake rates compared with the barley cultivars.

De novo assembly approach is a powerful tool for studying transcriptomes, particularly when dealing with species with no reference genomes or with limited sequencing coverage. The assembled contigs resulting from the *de novo* approach, however, are prone to error and inevitably differ from the original transcriptomes (Engström et al., 2013). This had been the case for barley (International Barley Genome Sequencing Consortium, 2012), for which only a draft genome with limited sequencing depth and coverage was available before 2016. In the previous study (Hill et al., 2016), only around 23% of the *de novo* assemblies of Clipper and Sahara could retrieve positive functional annotations. Also, most of the sequencing reads could not be mapped to the assembled contigs and hence these reads were not taken into account during the subsequent differential gene expression analyses.

To take advantage of the latest version of the barley reference genome (Morex) (Mascher et al., 2017), in this study we built on our previous work (Hill et al., 2016) and re-visited the 12 transcriptomes using an improved bioinformatics pipeline with four major modifications: (i) rather than constructing the *de novo* assemblies of transcriptomes based only on the raw reads, the newly available Morex genome with increased sequencing depth and genome coverage was served as the mapping base of this study; (ii) instead of using Bowtie v2.1.0, we adopted the HISAT2, a mapping algorithm proven to have improved performance for gapped-read mapping of raw reads (Kim et al., 2015); (iii) for differential gene expression determination, we replaced EdgeR with limma (Ritchie et al., 2015) for better capacity to prevent type I and II errors; and (iv) we performed BLAST search against both databases of protein sequence (such as TAIR, Swissprot, TREMBL) and domain (InterPro) homology to maximize the functional annotations of the gene-of-interest. For this study, we also applied a novel combined targeted metabolomics and lipidomics approach to quantitatively determine the alteration of the corresponding primary metabolites and lipids in different rootzones of Clipper and Sahara with and without salinity treatment, to obtain further molecular insights into the impact of salinity at the metabolite level. We then designed a new GLM-based analysis approach to identify the treatment-, genotype-, and rootzone-specific differentially expressed genes (DEGs) concurrently with the differentially abundant metabolites and lipids (DAMs) in barley rootzones upon salt stress. Integrated pathway over-representation of the DEGs and DAMs showed that the salt treatment led to two differential modulations of phenylpropanoid biosynthesis, which likely contributed to the salinity-induced localization changes of cell wall components, such as lignin and suberin, in Clipper and Sahara. As a proof of concept, we further explored the interconnections between affected metabolites and gene expression pathways by construction of global coexpression-correlation networks specific to each barley



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genotype. Histochemical and immunochemical microscopy of both Clipper and Sahara roots to detect different depositions of lignin, suberin, and callose after salinity treatment proved the detected cell wall-related gene expression and metabolic changes in the phenylpropanoid pathway. Based on our system-wide exploration, we demonstrate that seedlings of Clipper and Sahara respond to salinity stress differentially, suggesting the distinctive dynamics underpinning the plasticity of different barley genotypes in response to salt stress.

RESULTS

Barley Transcriptome-Processing and -Annotation Pipeline

The improved workflow for transcriptome sequence pre-processing, pre-mapping, mapping, and transcript analyses is presented in Figure 1A. We achieved an average mapping efficiency of 95.7% ± 1.6% for the 192 sequenced libraries used in this study (Supplemental Figure 1A), demonstrating a high degree of sequence conservation among Morex, Clipper, and Sahara at the transcript level. In total, 247 281 out of the 333 926 predicted transcripts (74.1%) of the Morex genome were functionally annotated compared with around 37.4% and 40.1% annotation obtained for the *de novo* assemblies of Clipper and Sahara, respectively (Supplemental Figure 1B). From this, we constructed a new counting matrix composed of the trimmed mean of M values (TMM)-normalized counts per million (CPM) reads for the 12 transcriptomes (Supplemental Data 1).

Effects of Salinity on Barley Transcriptomes

We determined the DEG specific to treatment (0 or 100 mM NaCl), genotype (Clipper or Sahara), and rootzone (meristematic [Z1], elongation [Z2], or maturation [Z3]). Here, specific GLMs taking the interactions among three factors, namely treatments, genotypes, and rootzones into account, were applied to determine genotype- and rootzone-specific DEGs. Notably, for explaining a phenotype specific to either a particular genotype or rootzone, two possibilities exist: differences could either be due to the effect of DEGs unique to a genotype or rootzone (Figure 2A), or of DEGs common to both genotypes and rootzones, but with significant differences in expression (Figure 2B). To this end, both uniqueness and significance of expression differences were addressed through specific GLM designs as described in Supplemental Note 1 with the logic illustrated in Figure 2C–2F. Outcomes of the GLM-based differential analyses was integrated and summarized in Supplemental Figure 2A (see Supplemental Data 2 for annotated DEG lists).

To determine which biological processes are most prominent in the two genotypes upon salt stress, treatment-specific DEGs in each genotype were classified into seven groups according to their spatial distribution in barley roots. Each group was then subject to enrichment analysis of gene ontology (GO) with a focus on the category of biological processes (Figure 3; Supplemental Data 3).

Effects of Salinity on the Barley Metabolomes and Lipidomes

Next, we performed quantitative metabolomics and lipidomics analyses using the same root tissue samples to provide a complementary perspective to the early salt responses of barley seedling roots. A total of 154 compounds (22 sugars or sugar alcohols, 15 small organic acids, 32 amines or amino acids, 18 fatty acids, and 67 lipids) were quantified using four mass spectrometry-based metabolomics and lipidomics methods (Supplemental Data 4). The bioinformatics pipeline for elucidating the treatment, genotype, and rootzone-specific DAMs is illustrated in Figure 1B (see Supplemental Data 5 for annotated DAM lists). Notably, the same structure of GLM was used for the DAM and DEG determinations to facilitate the subsequent omics comparisons and integration.

To provide insight as to which metabolic groups are most markedly different between the two genotypes upon salt stress, treatment-specific DAMs in each genotype were classified into seven groups according to their spatial distribution in barley roots (Figure 4). Each group was then subject to metabolite set enrichment analysis with results detailed in Supplemental Data 6.

Effect of the Over-represented Salinity on the Barley Root-omes

Next, we utilized the Kyoto Encyclopedia of Genes and Genomes (KEGG) mapper to perform an integrated pathway analysis for the three omics datasets (Figure 1C) (Kanehisa et al., 2012). According to the number of matched DEG and DAM hits, biological pathways statistically over-represented at transcript and/or primary metabolite level in response to salinity were ranked in descending order, where the biosynthesis of phenylpropanoids (such as monolignols, flavonoids, lignins, and suberins) were enriched at both levels and identified at the top of the list (Supplemental Data 7). To visualize the specific post-salinity effect on the biosynthesis of phenylpropanoids, we calculated the *Z* scores of the TMM-normalized CPM for the transcripts involved and of the normalized concentration for the primary metabolites detected. In addition, we adopted an established method to perform a detailed quantification of the phenylpropanoid contents across our root samples (Supplemental Data 2) (Vanholme et al., 2012) and computed their *Z* scores. The relative abundance of transcripts, primary metabolites, and phenylpropanoids at different rootzones of the two barley genotypes were

Figure 1. Overview of the Bioinformatics Pipelines Implemented in This Study.

(A) Pre-processing, pre-mapping, mapping, and DEG analysis of 12 transcriptomes.

(B) Pre-processing, data-correction, normalization, and DAM analysis of 12 metabolomes and lipidomes.

(C) KEGG-based integrated pathway analysis of transcriptomes, metabolomes, and lipidomes.

(D) Pre-processing, network construction, and module detection for global co-expression correlation analysis of multi-omes in barley.

DAM, differentially abundant metabolite; DEG, differentially expressed genes; GLM, general linear model; GO, Gene Ontology; HCR, hierarchical clustering; HPC, high performance computation; KO, Kyoto Encyclopedia of Genes and Genomes Ontology; MS, Mass Spectrometry; PCA, principal component analysis; RAP-DB, Rice Annotation Project - Database; RLAwg, within-group relative log adjustment; TAIR, The Arabidopsis Information Resource; TMM, trimmed mean normalization; Z1, zone 1 (meristematic zone); Z2, zone 2 (elongation zone); Z3, zone 3 (maturation zone).

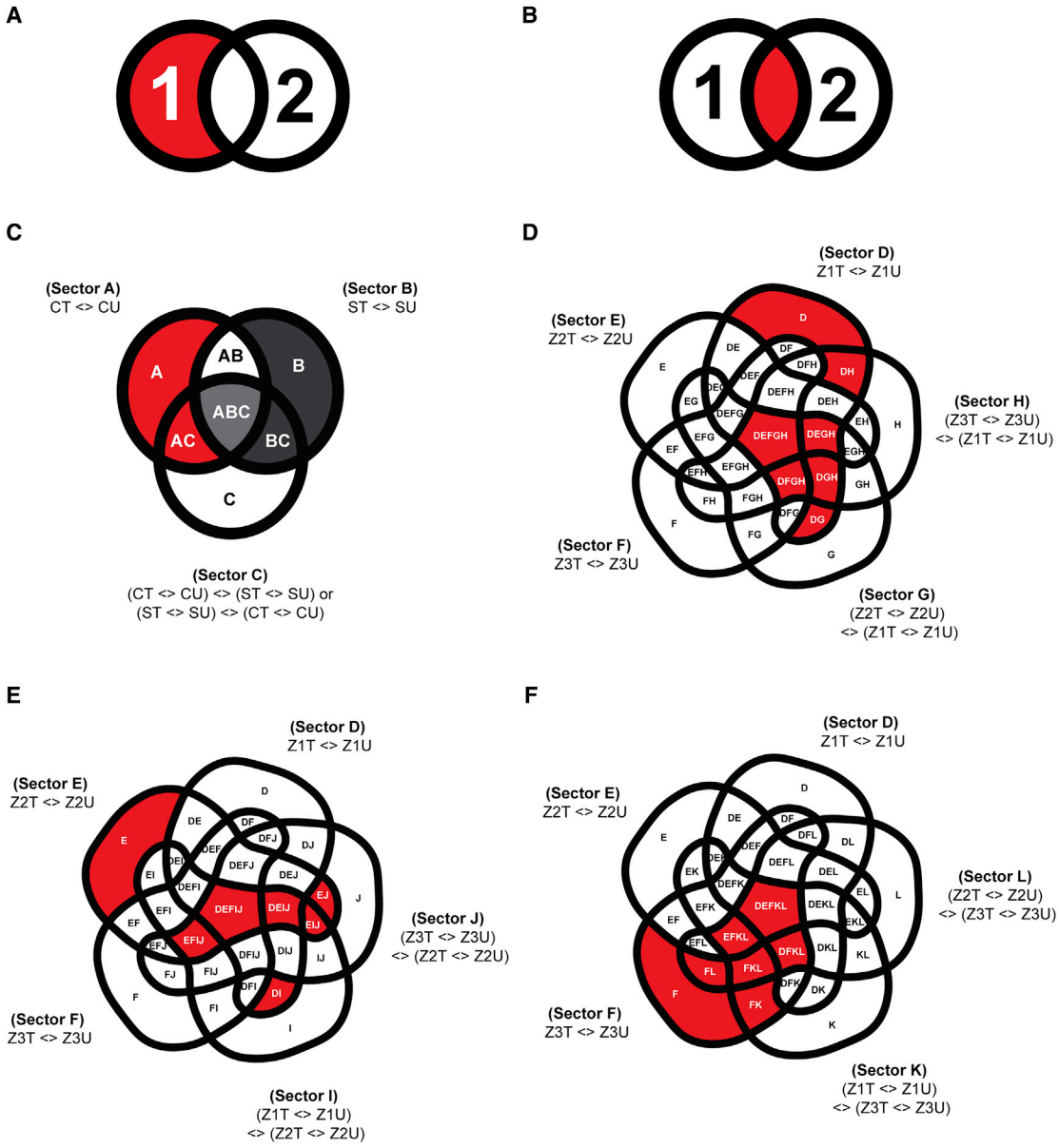


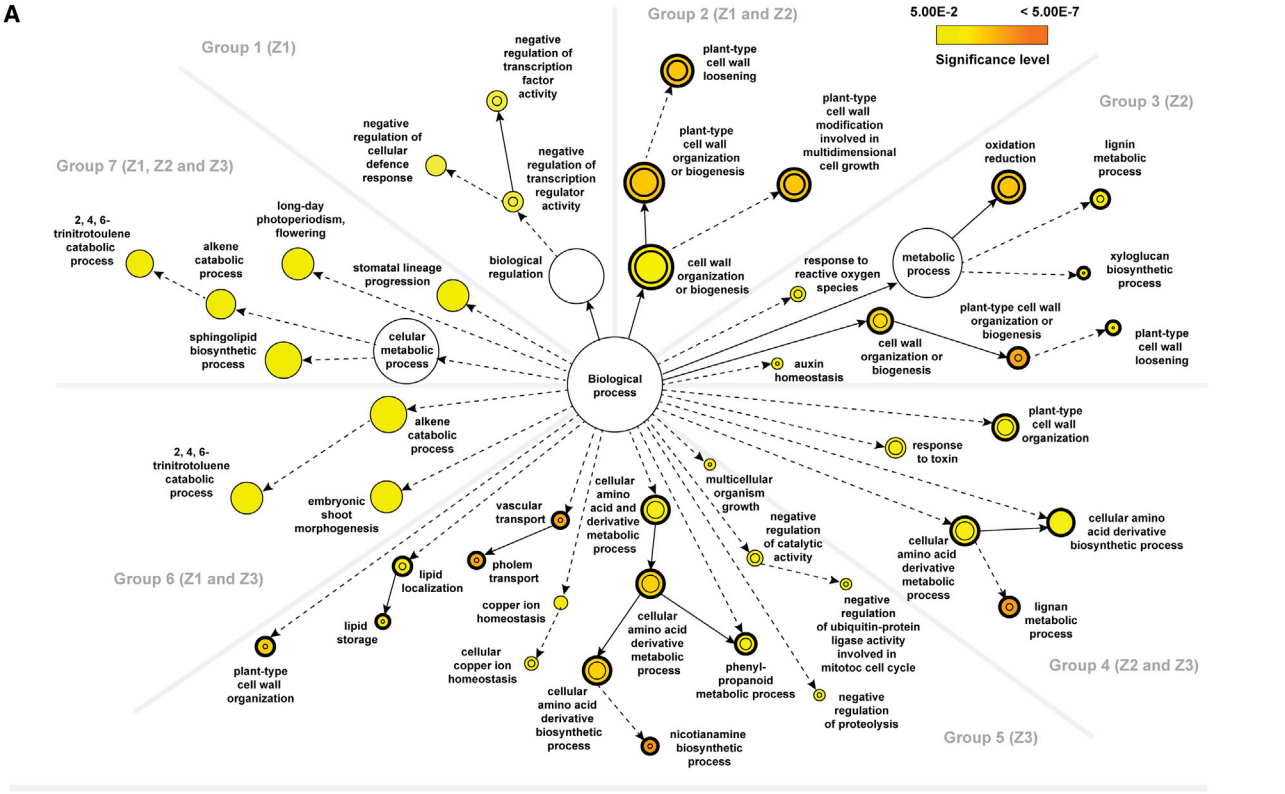
Figure 2. Design of GLM and Subsetting for the DEG or DAM Determination.

(A and B) Two possible sets of DEGs or DAMs that could account for the genotype- or rootzone-specific phenotypes (colored in red): **(A)** DEGs or DAMs unique to one genotype or rootzone; **(B)** DEGs or DAMs common to both genotypes or rootzones, but showed significant contrast in expression or abundance between the two. Numbers 1 and 2 in the figures denote two sets of DEGs or DAMs from two different genotypes/rootzones in comparison.

(C) Genotype-specific DEGs or DAMs for each rootzone. Subsectors correspond to the Clipper-specific DEGs/DAMs (including subsectors A, AC) and Sahara-specific DEGs or DAMs (including subsectors B, BC) in each rootzone are highlighted in red and dark gray, respectively. Subsector ABC are common to both Clipper and Sahara (colored in light gray), but defined by GLM contrast in opposite directions: (CT <> CU) <> (ST <> SU), and (ST <> SU) <> (CT <> CU), respectively.

(D–F) Rootzone-specific DEGs or DAMs of Clipper/Sahara at **(D)** meristematic zone (Z1), **(E)** elongation zone (Z2), and **(F)** maturation zone (Z3), respectively, and with the corresponding subsectors highlighted in red. Symbol "< >" denotes a "contrast/comparison" being tested during differential analysis through fitting of GLM.

CT, salt-treated Clipper; CU, untreated Clipper; DEGs, differentially expressed genes; DAMs, differentially abundant metabolites; ST, salt-treated Sahara; SU, untreated Sahara; Z1T, salt-treated Z1; Z1U, untreated Z1; Z2T, salt-treated Z2; Z2U, untreated Z2; Z3T, salt-treated Z3; Z3U, untreated Z3; <>, contrast of GLM.



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integrated and illustrated in the pathway frameworks modified based on the corresponding KEGG repository (Figure 5; Supplemental Figures 3 and 4).

Salinity-Induced Abundance and Localization Shifts of Phenylpropanoids in the Barley Rootzones

The biosynthetic pathway of phenylpropanoids can be generally divided into three main stages: the general phenylpropanoid pathway from phenylalanine to CoA-esters, the monolignol-specific pathway from CoA-esters to monolignols, and the lignin-specific pathway from monolignols to oligolignols or lignin polymers. For Z1 of both barley genotypes (Supplemental Figure 3), genes involved in all three stages of the biosynthesis remained weakly expressed as in the untreated controls. In line with the detection at the RNA level, negative standardized $\log_2$ concentration (*Z* scores) were recorded for almost all of the metabolic intermediates (Supplemental Figures 5 and 6). Histochemical staining also showed no observable difference in abundance and localization of phenylpropanoids, such as lignin and suberin after salt treatment (Supplemental Figures 7 and 8), implying that phenylpropanoid production in Z1 was not induced by salt.

In Clipper Z2 (Figure 5A), transcripts encoding for enzymes involved in the phenylpropanoid, monolignol, and lignin biosynthetic pathways were either increased in expression or maintained positive *Z* scores after salt treatment. The amount of the detected monolignols, including coniferyl alcohols (guaiacyl [G]-units of lignin) and sinapoyl alcohols (syringyl [S]-units of lignin), were significantly induced by salt (Supplemental Figure 6N and 6O). The active production of lignins at this rootzone was further supported by Basic Fuchsin staining, which showed a significant increase in lignin impregnation to cellulosic cell walls localized at the outer stelic regions (including endodermis, pericycle, and xylem) of Clipper roots after salt treatment (Supplemental Figure 7E, 7F, 7M, and 7N). Furthermore, gene products of *CHALCONE SYNTHASE* (*CHS*) are known to divert intermediates of the general phenylpropanoid pathway for flavonoid production (Heller and Hahlbrock, 1980). Weak expression of *CHS* and low levels of flavonoids, such as dihydroquercetin, were consistently detected in Clipper Z2. Notably, transcription of *GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 5* (*GPAT5*) and *FATTY ACID REDUCTASE 4* (*FAR4*), involved in biosynthesis and deposition of root suberin (Beisson et al., 2007; Domergue et al., 2010), were suppressed by the stress and maintained a negative *Z* score, respectively (Supplemental Figure 5I and 5J). These data are consistent with the observations of the salinity-induced decline in suberin levels visualized by Fluorol Yellow stain throughout Z2 (Supplemental Figure 8B and 8H).

For Sahara Z2 (Figure 5B), a significant increase of *CHS* expression diverted most phenylpropanoids toward the accumulation of dihydroquercetin (Supplemental Figure 6P). Together with the low expressions of *CINNAMOYL-COA REDUCTASE* (*CCR*) and *CINNAMYL-ALCOHOL DEHYDROGENASE* (*CAD*), the accumulation of monolignols and their precursors was restricted and no observable increase of lignin levels in the endodermal region could be detected histochemically after salt stress (Supplemental Figure 7G, 7H, 7O, and 7P). Furthermore, in contrast to Clipper Z2, there was higher abundance of *HYDROXYACID O-HYDROXYCINNAMOYLTRANSFERASE 1* (*HHT1*), *GPAT5*, and *FAR4* transcripts in Sahara Z2 (Supplemental Figure 5H–5J). The active biosynthesis of suberin inferred from the levels of biosynthetic enzyme transcripts was histologically confirmed, as increased levels of suberin were observed in the epidermis and across the subepidermal region of the rootzone (Supplemental Figure 8N and 8T).

In Z3 of Clipper (Supplemental Figure 4A), as in Z2, flavonoid production was inhibited. Salinity-induced accumulation of lignins was limited to G-units and localized at the endodermal and vascular regions (Supplemental Figure 7A and 7B). But, in contrast to Clipper Z2, an increased biosynthesis and deposition

Figure 3. Comparisons of the Statistical Over-Representation of GO Categories between Different Root Zones of the Two Barley Genotypes upon Salt Stress.

(A) Statistically over-represented GO categories unique to or shared between different rootzones of Clipper.

(B) Statistically over-represented GO categories unique to or shared between different rootzones of Sahara.

Group 1 composed of DEGs found only in Z1. GO analysis via BiNGO (Maere et al., 2005) and REVIGO (Supek et al., 2011) revealed that the most significant over-representation of this group were regulation of transcription and cellular defense response genes in Clipper, and biosynthesis of hemicelluloses, including xylan and its derivatives in Sahara. Group 2 included DEGs found in both Z1 and Z2. GO analysis indicated the genes to be mostly enriched in cell wall modification (in particular cell wall loosening) for Clipper, and phenylpropanoid metabolism for Sahara. Group 3 consisted of DEGs found only in Z2. While plant-type cell wall organization as well as lignin metabolism genes were strongly over-represented in Clipper, no significant enrichment of any GO category could be detected in Sahara. Group 4 represented DEGs found in Z2 and Z3. Lignan metabolism genes and related processes were drastically enriched in Clipper, but similar to Group 3, no significant over-representation was detected in Sahara. Group 5 consists of DEGs found only in Z3. In Clipper, nicotianamine metabolic process as well as vascular transport genes were ranked top in the overrepresentation list, compared with the enrichment of genes encoding proteins targeted to the mitochondrion, response to salt stress, and cell wall organization (xyloglucan metabolism) in Sahara. Group 6 represented DEGs found in both Z1 and Z3. This cluster was enriched in trinitrotoluene catabolism and related processes for Clipper, cell wall organization, or biosynthesis for Sahara. Group 7 contains DEGs found in all three rootzones. Sphingolipid biosynthesis genes were enriched in Clipper, whereas toxin metabolism was the most significantly overrepresented GO category in Sahara. Nodes represent GO categories and node-size is proportional to the number of detected genes for each node. Categories under the same GO hierarchy are linked by interconnected arrows (known as edges) and intensity of node-color indicates the significance level of statistical overrepresentation determined by Fisher's exact test with adjusted $p < 0.05$ as cutoff as per legend. For reference only, a threshold of 0.2 is set for those sectors showing no significant over-representation and white-colored nodes are used to visualize those ontologies closed to the threshold. Dotted edges indicate one or more hierarchies of GO, which have no statistical significance in the over-representation test and are determined as redundant via REVIGO, were not shown for clarity. DEGs statistically over-represented in both treatment-specific and genotype-specific analyses are denoted by nodes with thickened outlines. DEGs statistically over-represented in both treatment-specific and rootzone-specific batches are denoted by inner circle of nodes. Z1, zone 1 (meristemic zone); Z2, zone 2 (elongation zone); Z3, zone 3 (maturation zone).

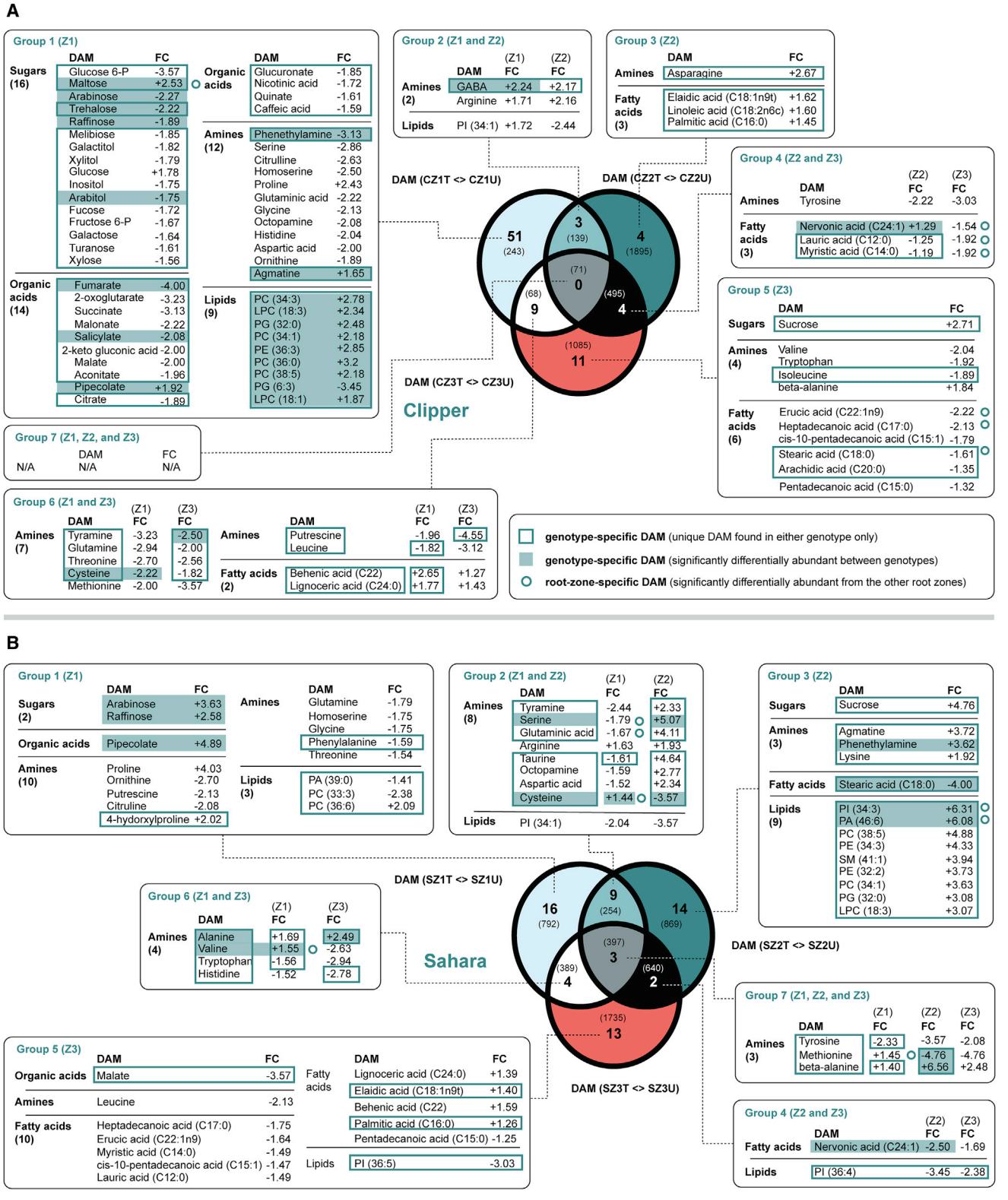

Figure 4. An Integrated View of the DAMs of the Two Barley Genotypes in Response to Salt Stress.

(A) DAMs unique to or shared among different rootzones of Clipper.

(B) DAMs unique to or shared among different rootzones of Sahara.

Parentheses in each text box refer to the number of metabolites within each metabolic subgroup. Parentheses in Venn diagrams: corresponding number of DEGs in each sector.

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of suberin at the endodermal and stele regions was supported by the consistently higher abundance of *GPAT5*, and *FAR4* transcripts (Supplemental Figure 5I and 5J) and by histochemical staining (Supplemental Figure 8A and 8G), respectively.

For Z3 of Sahara (Supplemental Figure 4B), relatively higher transcript abundance of *CCR* and *CAD* compared with Z2 upon salt treatment was detected. Intriguingly, the resulting metabolic changes led to higher accumulation of G-units of lignin and intense deposition of lignin mostly in the xylem vessels of this rootzone (Supplemental Figure 7C and 7D). Also, in line with the increased *Z* score for *CHS* and the suberin-related transcripts (such as *HHT1*, *GPAT5*, and *FAR4*) compared with Clipper Z3 (Supplemental Figure 5B and 5H–5J), a significant increase of dihydroquercetin and of endodermal and stele suberin deposition was recorded at Sahara Z3, respectively (Supplemental Figures 6P, 8M, and 8S). Taken together, the omics datasets at the transcriptional and metabolic levels combined with the histological observations suggest a strong differentiation in biosynthesis and localization of the phenylpropanoids between the two barley genotypes upon salt stress.

Global Intercorrelations of Salt Stress on the Barley Root-omes

Next, we extracted the abundance matrices of transcripts and metabolites that were significantly different in at least one of the GLM-based DEG or DAM determinations in Clipper (3802 transcripts, 83 metabolites) and Sahara (6477 transcripts, 61 metabolites). Global co-expression-correlation networks specific to the two barley genotypes were constructed via WGCNA (Langfelder and Horvath, 2008) to illustrate the system-wide consequences induced by salinity stress (Figure 1D). In these networks, each "leaf" or short vertical line represents an abundance profile of one transcript, metabolite, or lipid. Any interconnected lines within the same "branch" indicate profiles with highly correlated pattern of abundance. Based on the ''guilt-by-association'' principle, as defined in Saito et al. (2008), co-regregulated genes and metabolites among each co-expression cluster or branch are likely to have common functional roles. To systematically define the branch, we applied dynamic tree cut (Langfelder et al., 2008) to each network and the module assignment was performed to color code each highly correlated cluster (aka module) (Supplemental Figure 10).

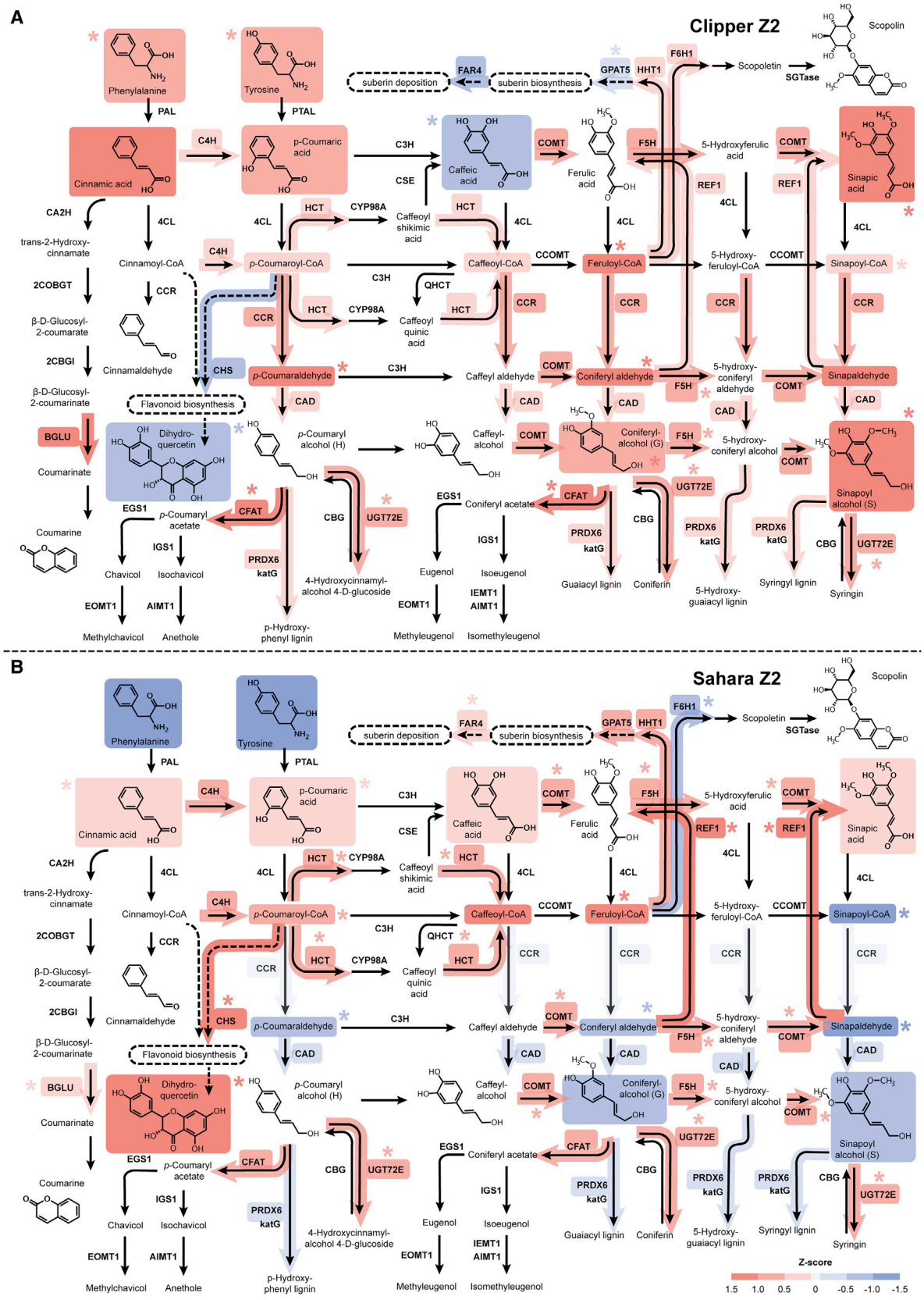
For modules that were unique to either Clipper or Sahara, or common to both genotypes but with significantly contrast abundance patterns in response to salt stress (Supplemental Table 1), we generated parallel profiles to visualize their variations of abundance in response to salt stress (Figure 6 and Supplemental Figure 11). Co-expression clusters were annotated by statistically significant enrichment (adjusted $p \leq 0.05$) of GO categories, and their specific biological roles were assigned through manual curation of the enrichment outcomes (Supplemental Data 8). Notably, the module eigengene (ME) corresponds to the first principal component of each module, and module membership (kME), a measure of the ME-based intramodular connectivity, is calculated by correlating the abundance profiles of modular members to their ME (Langfelder and Horvath, 2008). Providing that the importance of each regulator for a particular functional role is determined by its degree of contribution to the module variance and by its connection strength with the other intramodular members, ranking of members according to their kME in each module (Supplemental Data 9) can shed light on the key or master regulator(s) for a given biological role. Each cluster is categorized, explored, and discussed in detail in Supplemental Note 2. Biological processes in each rootzone and genotype, with module members being either induced or maintained at a high abundance level after the salt treatment, are summarized in Supplemental Table 2.

To validate the credibility of the global networks constructed for plants, we put one salt-induced biological process identified in Sahara, suppression of callose deposition, to the test and verified the callose abundance at four different tissue layers (focusing on epidermis, cortex, endodermis, and stele) using an immunochemical approach (Supplemental Figure 9). In contrast to the comparable amount of callose deposited in all layers of the three rootzones of Clipper after salt treatment (Supplemental Figure 9A–9F), as deduced from the global analysis, we detected declines of callose deposition throughout the layers underneath the epidermis of Sahara in all rootzones. Such declines (as indicated by the fading of orange fluorescence) were especially apparent at the plasmodesmata of cortical cell in Z3, plasmodesmata in stele and endodermis of Z2, and throughout the walls of stele and cortical cells in Z1) (Supplemental Figure 9G–9L). Furthermore, ABERRANT GROWTH AND DEATH 2 (AGD2) is a known suppressor of callose deposition (Rate and Greenberg, 2001). In line with the outcome of the immunochemical detection, we consistently showed that the expression of *AGD2* was categorized in Module C of the correlation network, which is characterized by modular members with stronger salinity-induced abundance for all rootzones in Sahara than in Clipper (Figure 6C). Altogether, these findings support the precision and feasibility to apply this intercorrelation approach to understand the salinity responses in barley roots.

DISCUSSION

The barley malting cultivar Clipper and landrace Sahara are two barley genotypes with known contrasting phenotypic traits in response to salt stress at an early stage of development: Clipper maintains root elongation, while in Sahara root elongation is significantly reduced in response to short-term salt stress (Shelden et al., 2013). In this study, we investigated system-wide responses of seedling roots of both genotypes to moderate

CZ1T, salt-treated Z1 in Clipper; CZ1U, untreated Z1 in Clipper; CZ2T, salt-treated Z2 in Clipper; CZ2U, untreated Z2in Clipper; CZ3T, salt-treated Z3 in Clipper; CZ3U, untreated Z3 in Clipper; DAMs, differentially abundant metabolites; DEGs, differentially expressed genes; FC, fold change; LPC, lysophosphatidylcholines; PA, phosphatidic acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidylglycerols; PI, phosphatidylinositols; PS, phosphatidylserines; SM, sphingomyelins; SZ1T, salt-treated Z1 in Sahara; SZ1U, untreated Z1 in Sahara; SZ2T, salt-treated Z2 in Sahara; SZ2U, untreated Z2 in Sahara; SZ3T, salt-treated Z3 in Sahara; SZ3U, untreated Z3 in Sahara; Z1, zone 1 (meristematic zone); Z2, zone 2 (elongation zone); Z3, zone 3 (maturation zone); -P, phosphate.



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salinity, and quantified spatial salt-induced perturbations in transcriptomes, metabolomes, and lipidomes of individual rootzones in each of the barley genotypes. By means of statistical overrepresentation of DEGs and DAMs (Figures 3 and 4), we investigated the datasets from the perspective of their "extremes" and illustrated the most differential salinity responses in three different rootzones of the two genotypes through integrated pathway analysis (Figure 5; Supplemental Figures 3 and 4). Using global co-expression correlation network analysis (Figures 6 and 7), we approached the datasets from the perspective of "intercorrelations" among the induced pathways to demonstrate the system-wide impacts on the genotypes triggered by salinity stress (Supplemental Table 2).

Through integration of the spatial omics information obtained from these approaches, we provide a novel and system-wide insight to the salt-induced modulations of apoplastic (lignin, suberin) and symplastic flows (callose) in barley roots (Figure 7). Besides providing a comprehensive multi-omics data resource allowing deep mining of salinity-induced changes in seedlings of barley at the rootzone level, we demonstrated seedling roots of different genotypes of barley could be in distinctive salinity response phases to cope with the stress, illustrating differential salt tolerance strategies could exist among the same plant species.

Salinity-Induced Lignin Precursor Production to Sustain Clipper Root Growth

Through modification and amplification of a very limited set of core structures derived from shikimate, phenylpropanoid metabolism generates an enormous array of plant secondary metabolites ranging from monomers (such as flavonoids, coumarins) to polymers (such as lignins, suberins) (Vogt, 2010). Upon short-term salt stress, our study shows that the building blocks of phenylpropanoids were diverted from the synthesis of flavonoids and suberins to the production of G- and S-units of lignins in Clipper Z2 (Figure 5A). Flavonoids, such as quercetin, are known as inhibitors of auxin efflux carriers in a variety of plant tissues (Jacobs and Rubery, 1988). Low levels of dihydroquercetin (Supplemental Figure 6P), an immediate upstream precursor of quercetin, may therefore, supress the inhibition of auxin efflux carriers in Clipper Z2 and facilitate the propagation of auxin signals, and auxin-mediated cell division and expansion. This finding is consistent with the previous phenotypic study of the two barley genotypes, in which Clipper maintains a greater root elongation rate than Sahara, even under moderate salt stress (Shelden et al., 2013). This also further validates our integrated pathway analysis approach to identify their molecular differences.

The Casparian strip is a specialized wall modification in the endodermis, which serves as a diffusion barrier to limit apoplastic flow and re-direct solute movement back to the symplastic stream through the plasma membrane (Steudle and Peterson, 1998). The strip is mainly composed of rings of lignin deposited around endodermal cells and interference in lignin biosynthesis has been shown to abrogate the early strip formation in *Arabidopsis* (Naseer et al., 2012). In monocotyledonous species, the lignin-like polymers of the Casparian strip are composed of a mixture of G- and S-units (Zeier et al., 1999). In Clipper Z2, both transcript and phenylpropanoid profiling results consistently show that the production and abundance of these units are significantly increased by the stress (Figure 5A; Supplemental Figure 6N and 6O). Also, an intense localization of lignins was detected at the outer stele region of the rootzone after the salt treatment (Supplemental Figure 7E and 7F). Similar to previous results in maize (*Zea mays* L.) (Shen et al., 2015), the salt-induced lignin production and its intense localization at the endodermis of Clipper Z2 likely contributed to the development of the Casparian strip closer to the root tip in response to the salt stress. There, passage of water and solutes have to undergo selective uptake via ion channels of the membranes (Apse and Blumwald, 2007). Filtering of excessive sodium ions might therefore be achieved in Clipper Z2 by this mechanism.

For most cereal crops and *Arabidopsis thaliana*, deposition of suberin can be induced at cell layers, such as the epidermis, outer cortex, and stele in response to salt and osmotic stresses (Schreiber et al., 2005; Kreszies et al., 2018a, 2018b, 2020).

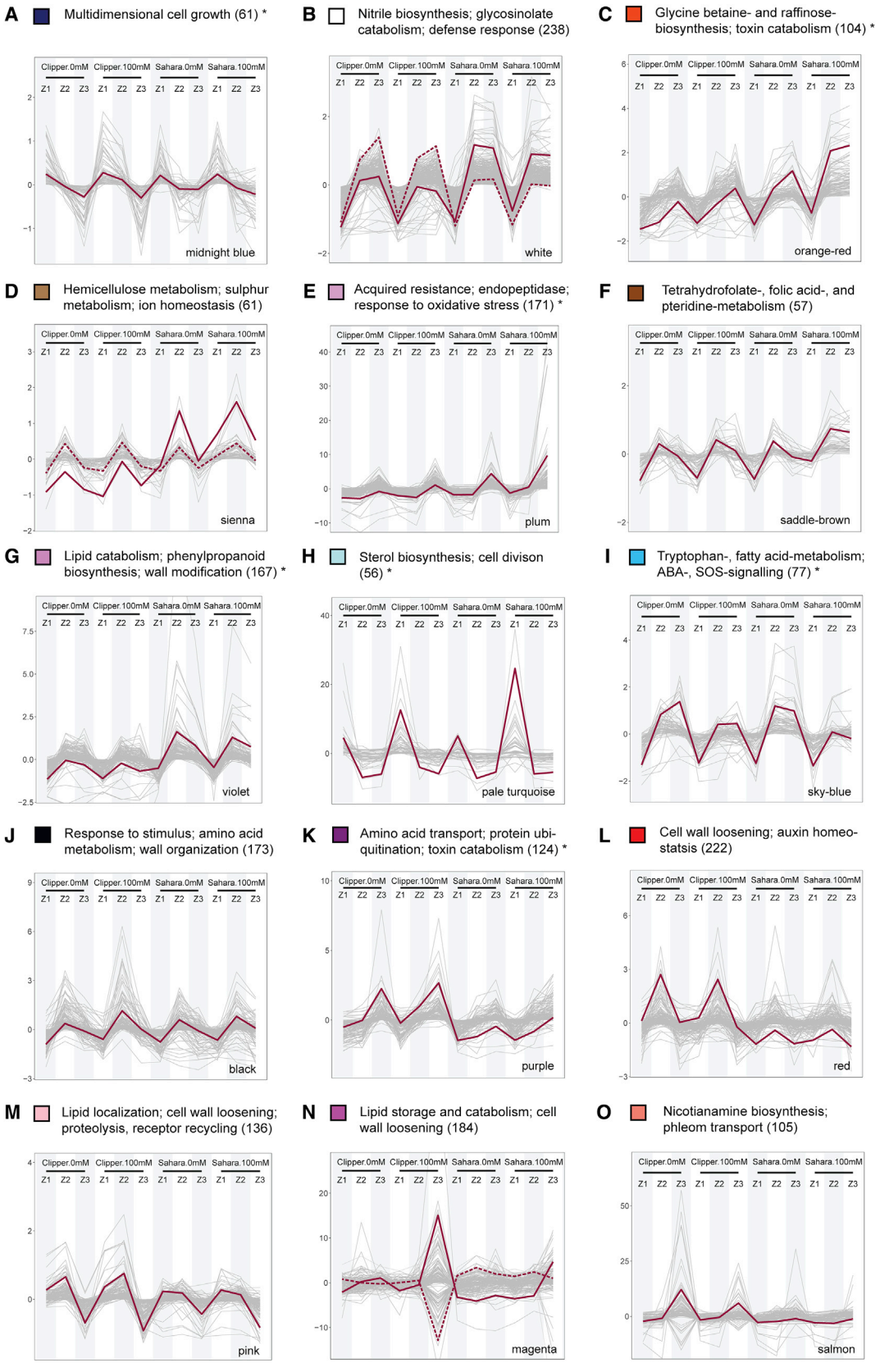
Figure 5. Standardized Abundance of Transcripts and Metabolites Involved in Phenylpropanoid Biosynthesis at the Elongation Zone (Z2) of the Two Barley Genotypes under Salt Stress.

(A) The abundance of transcripts and metabolites involved in the biosynthesis at Clipper Z2.

(B) The abundance of transcripts and metabolites involved in the biosynthesis at Sahara Z2.

Standardized abundances of transcripts and metabolites shown are the *Z* scores for TMM-normalized CPM and median-normalized concentration respectively. Level of the standardized abundance (i.e., positive, negative, and zero *Z* score) is indicated by intensity of shading in red, blue, and pale gray, respectively. Asterisks denote statistically significant differentiation of transcript- and metabolite-abundance (with Benjamini–Hochberg adjusted $p < 0.05$) after the salt stress compared with the untreated control. Standardized abundance of only the transcripts with significant degree of sequence similarities to the characterized homologs (E < 1.00E-3) and the metabolites within the limit of detection of methodologies and instrumentations adopted in this study are shown. Abundance details of these pathway components at different rootzones of the two barley genotypes before and after the salt treatment can be found in Supplemental Figures 5 and 6.

AIMT1, *trans*-anol O-methyltransferase; BGLU, beta-glucosidase; CA2H, cinnamic acid 2-hydroxylase; CBG, coniferin beta-glucosidase; 2CBGI, 2-coumarate β-D-glucoside isomerase; CAD, cinnamyl-alcohol dehydrogenase; CCOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; CFAT, coniferyl alcohol acyltransferase; C3H, p-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CHS, chalcone synthase; 4CL, 4-coumarate-CoA ligase; COBGT, 2-coumarate O-beta-glucosyltransferase; COMT, caffeic acid 3-O-methyltransferase; CSE, caffeoylshikimate esterase; CYP98A, coumaroylquinate(coumaroylshikimate) 3′-monooxygenase; EGS1, eugenol synthase; EOMT1, eugenol/chavicol O-methyltransferase; F5H, ferulate-5-hydroxylase; F6H1, feruloyl-CoA *ortho*-hydroxylase; FAR4, fatty acid reductase 4; GPAT5, glycerol-3-phosphate acyltransferase 5; HCT, shikimate O-hydroxycinnamoyltransferase; HHT1, hydroxyacid O-hydroxycinnamoyltransferase 1; IEMT1, (iso)eugenol O-methyltransferase; IGS1, isoeugenol synthase; katG, catalase-peroxidase; PAL, phenylalanine ammonia-lyase; PTAL, phenylalanine/tyrosine ammonia-lyase; PRDX6, peroxiredoxin 6; REF1, coniferyl-aldehyde dehydrogenase; SGTase, scopoletin glucosyltransferase; QHCT, quinate O-hydroxycinnamoyltransferase; UGT72E, coniferyl alcohol glucosyltransferase.



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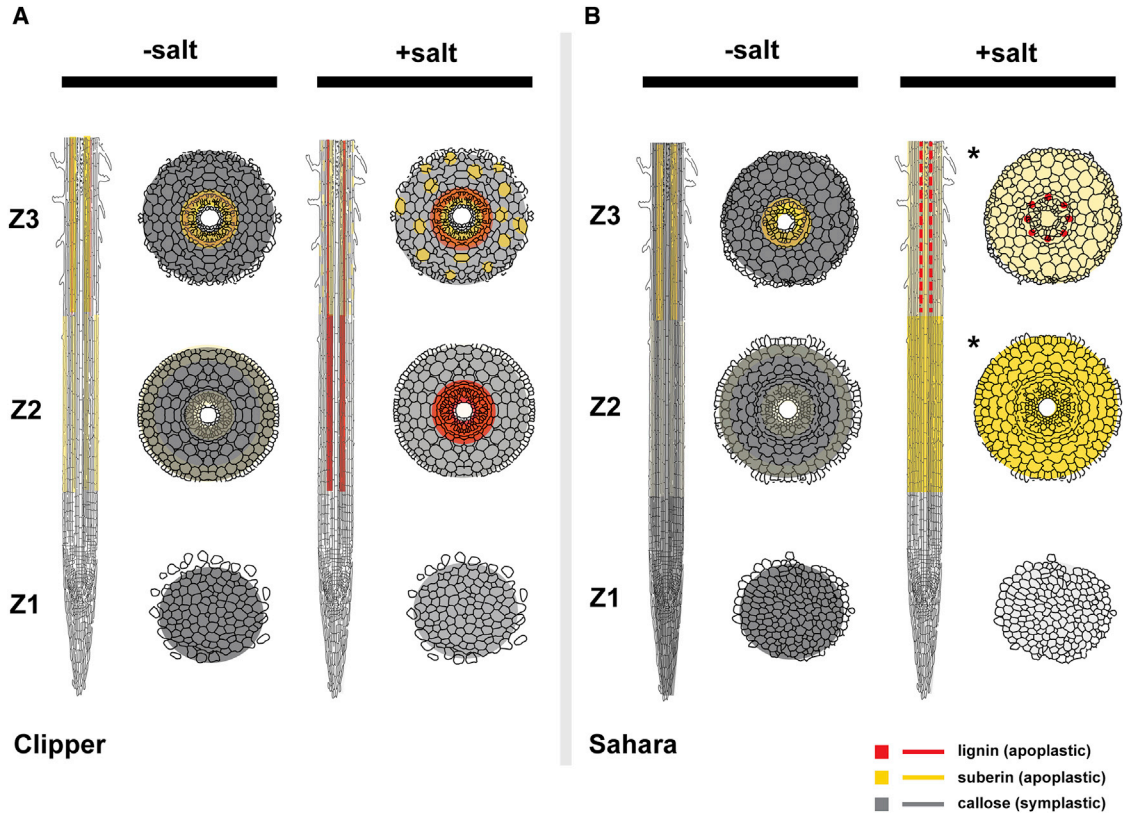

Figure 7. A Model Deciphering Cell Wall Modifications in Roots of Clipper and Sahara in Presence or Absence of Salinity Stress.
(A and B) Longitudinal and the corresponding transverse sections of different rootzones for **(A)** Clipper and **(B)** Sahara with or without salinity stress. Color intensities represent the relative levels of lignin, suberin, or callose, which were quantified based on their precursors detection through LC/GC-MS and/or RNA-seq with support of the direct detection of the compounds through histological methods. Localization of each compound was determined by the histo-/immuno-chemical stainings with proven specificity and optical filters applied for minimizing any autofluorescence or background signals. Red broken line (Sahara Z3, +salt) represents lignin deposition at only vasculature. Asterisks indicate rootzones at cortical, endodermal, and stelic regions with concurrent localizations of suberin and callose (where callose deposition was suppressed after the salt stress and only the intense suberin deposition is shown in this rootzone for clarity).

Intriguingly, our transcriptomic data shows that genes involved in suberin production were downregulated in Clipper Z2 relative to Sahara Z2 (Supplemental Figure 5H–5J). Reduced Fluorol Yellow staining in Clipper Z2 verifies this result and shows a decline in suberin levels throughout the whole rootzone after salinity treatment (Supplemental Figure 8B and 8H). The exodermis is a specialized outermost layer of the cortex in which Casparian strip development is inducible by salt and is found only in wild relatives of barley, such as *Hordeum marinum* (Byrt et al., 2018). In the absence of the exodermis in *Hordeum vulgare* L. genotypes, such as Clipper and Sahara, low suberization of cell layers surrounding the endodermis of Clipper Z2 would therefore imply that there is no additional barrier to sodium ion entry into its root epidermis and cortex under salt stress. Consistent with this hypothesis, whole seminal roots of Clipper were shown to have higher

Figure 6. Selected Modules of Weighted Coexpression Correlation Networks Showing Abundance Profiles of Transcripts and Metabolites.
(A) The abundance profile unique to Clipper.
(B–I) The abundance profiles unique to Sahara.
(J–O) The abundance profiles significantly contrast between the two barley genotypes.
Profiles showing either positive or negative correlations by clustering abundance into differently colored modules through weighted correlation networks. Additional profiles with less obvious differentiation between the two genotypes can be found in Supplemental Figure 11. The color of each module is consistent with Supplemental Figure 10. The most representative trend or centroid of each module represented by solid lines are determined by k-mean clustering (distance method: Pearson) with optimal number of clusters calculated from within-group sum of square method (Madsen and Browning, 2009). Second, the most representative centroid (if any) is indicated by a dotted line. Only expression profiles within 99th percentile are shown for clarity. Annotation of each co-expression clusters are determined by means of the statistical enrichment of GO categories below the cutoff (adjusted $p \leq 0.05$) and specific biological role of each module specified here is designated by manual curation of the enrichment outcomes. Asterisks denote the clusters with no significant over-representation and annotations assigned to these clusters are the GO categories with the highest possible level of significance (Supplemental Data 8). Annotated lists of members for each module with significant match (E < 1.00E-4) against TAIR10 genome release (version: June 2016) ranked in descending order according to kME of members can be found in Supplemental Data 9.

accumulation of sodium ions than Sahara when grown under the same salinity strength (Shelden et al., 2013).

Plasmodesmatal conductivity is known to be regulated by the controlled buildup of callose at the plasmodesmatal neck (De Storme and Geelen, 2014). In our study, immunochemical detection showed substantial callose deposition throughout Clipper Z2 independent of the salt stress (Supplemental Figure 9B and 9E). Assuming our observed callose deposition contributed to modulating the aperture size of the symplastic channels, this may suggest a persistent restriction of symplastic flow and hence accumulation of salt in cell walls of the epidermal and cortical regions. Indeed, the interwoven network of the cellulose microfibrils and pectin (such as homogalacturonan, rhamnogalacturonan I and II) is one of the major factors contributing to the cell wall strength with homogalacturonan chain interaction modulated by calcium ions (O'Neill et al., 2004). While barley root cell walls have also been suggested to be a "sodium ion trap" for restricting ionic movement from the root to the shoot (Flowers and Hajibagheri, 2001), the salt-tolerant varieties have shown to possess up to two-fold greater capacity of ion adsorption than sensitive ones, suggesting that the excessive amounts of sodium ions in the apoplast might displace calcium ions and thus weaken pectin chain calcium ion cross bridges (Ravanat and Rinaudo, 1980). To the best of our knowledge, there is no compelling evidence to support the presence of an active exclusion mechanism for the removal of an excess of sodium ions from the apoplast of Clipper. Assuming the root cell wall was under an optimal pH required for the interaction of sodium ions and uronic acids of pectin, presence of such a high level of apoplastic sodium ions would in turn weaken the cell wall strength of roots of Clipper, implying a shortcoming of this tolerance strategy for supporting the long-term development of this genotype.

Notably, production of lignin G-units was detected in both Z2 and Z3 of Clipper, but S-unit precursors of lignin were found only in Z2, not in Z3 under salt stress (Supplemental Figure 6N and 6O). Lignin G-units are known to be a major component of tracheary elements (Higuchi, 1990), which are the key components of xylem vessels that provide mechanical resistance in plants against the negative pressure associated with the transport of minerals and water to the aerial tissues in the rising sap (Turner, 1997). The continuance of synthesis of these units in Clipper Z3 is likely to reinforce and waterproof these cells. This suggests that the vital function for preventing the root structures from collapse and maintaining the hydro-mineral sap distribution to the whole plant served by the tracheary elements likely be independent of the salt stress. Taken together, roots of Clipper seedlings could adopt a "growth-sustaining" strategy, which maximizes root growth to increase the likelihood of overcoming the unfavorable saline conditions, but with the trade-off of developing a less effective epidermal or cortical barrier with suberin for preventing the subsequent salt accumulation in the root cortex (Figure 7A).

Salinity-Induced Flavonoids and Suberin Production to Shield Roots of Sahara

Unlike in Clipper Z2, our integrated pathway analysis also suggests that lignin production in Z2 of Sahara was not triggered by salinity. Instead, the building blocks of phenylpropanoids in this rootzone were in part diverted to suberin, and in part to the production of flavonoids, implying suppression of root cell elongation (Figure 5B).

Under normal growth conditions, suberization of root cells occurs in the endodermis subsequent to Casparian strip formation (Geldner, 2013). These wall modifications restrict the apoplastic uptake of water and solutes into endodermal cells. Under osmotic stress, increased numbers of suberized endodermal cells were observed at the late elongation zone of barley roots (Kreszies et al., 2018b). Under salinity stress, cereal crops, such as maize would, however, further expand their apoplastic diffusion barrier by inducing the suberization of cell walls in the entire root cortex to limit water loss from the cell layers and salt entry into xylem vessels (Andersen et al., 2015). In this study, we detected high levels of suberin synthesis-related gene expression and localization of suberin throughout Z2 of Sahara, but not of Clipper (Supplemental Figures 5H–5J, 8N, and 8T). This suggests that Sahara responds similarly as other cereal crops under salt stress by restricting apoplastic transport in Z2 via suberin deposition. Consistently, our global co-expression correlation study indicates high level of salt-induced *AGD2* transcripts, a factor known for supressing callose deposition (Rate and Greenberg, 2001) (Supplemental Table 2: Sahara, AZ). Such inhibition across the subepidermal regions of Sahara Z2 (especially at the stele and endodermal regions) was confirmed by the immunochemical detection (Supplemental Figure 9H and 9K). Callose deposition is known to be crucial for regulating the closure of plasmodesmata (De Storme and Geelen, 2014). In the heavily suberized and cortical cells of Sahara Z2 with restricted apoplastic movement of nutrients taken up from the rhizosphere, inhibition of the callose deposition at plasmodesmata thus reduces the symplastic transport barrier allowing sharing and distribution of resources via the symplastic passages.

Furthermore, irrespective of the salt treatment, production of suberin (Supplemental Figure 5H–5J) and G-units of lignins (Supplemental Figure 6N) persisted in Sahara Z3, inferring the vital importance of these precursors in the maturation of the Casparian strip and tracheary elements, respectively. Notably, unlike the untreated control, Basic Fuchsin staining of Sahara Z3 showed an intense deposit of lignin around the meta- and proto-xylemic cell walls, accompanied by a small amount of lignins laid at the endodermis and pericycle after salt treatment (Supplemental Figure 7C and 7D). In the absence of the widespread salt-induced suberization of cells in epidermal and cortical layers observed in Sahara Z2 after salt stress (Supplemental Figure 8T and 8S), this lignin deposition at the stele of Sahara Z3 could serve as the last barrier of salt ions carried by apoplastic flows. Furthermore, similar to the response of the Sahara Z2, a boost in production of flavonoids was also observed in Sahara Z3 after the salt treatment (Supplemental Figure 6P). This implies that a comprehensive salt- or osmotic-induced growth restriction was triggered in both the zones of elongation (Z2) and maturation (Z3), which is consistent with the previous physiological data (Shelden et al., 2013). Taken together, seedling roots of Sahara appear to implement a "salt-shielding" strategy. Such a strategy restricts salt from being imported into the roots and minimizes water

loss from root cells under the unfavorable salinity conditions, but at the expense of the rate of growth (Figure 7B).

Understanding the distinctive salt tolerance mechanisms adopted by seedling roots of different barley genotypes may help in designing plants to cope with the predicted increase in salinity stress, which will impact our ability to maintain yield in important food and feed crops in the near future.

METHODS

Plant Materials

Genotypes of barley (*Hordeum vulgare* L.) were sourced from the University of Adelaide. Two genotypes of barley, the domesticated malting cultivar Clipper (Australia) and the landrace Sahara 3771 (North Africa), were used for the transcript, primary metabolite, lipid, and phenylpropanoid analyses in this study, and were selected based on previously reported physiological diversity in salt tolerance (Widodo et al., 2009; Shelden et al., 2013).

Growth Conditions and Sample Preparation

For study of the transcriptomes (RNA sequencing [RNA-seq]), we re-analyzed the raw sequencing reads of four biological replicates for each sample totaling 48 samples obtained from the previous study (Hill et al., 2016). No additional sample collection or extraction was performed. For study of the primary metabolomes and lipidomes gas chromatography-triple quadruple-mass spectrometry [GC-QqQ-MS] for sugar and organic acid quantification, liquid chromatography-triple-quadrupole-mass spectrometry [LC-QqQ-MS] for amine quantification, GC-quadruple-MS [GC-Q-MS] for fatty acid methyl ester quantification, and LC-QqQ-MS for lipid analysis), we made use of the exact same set of root material obtained from our large-scale root collection for the transcriptomics studies to side-by-side extract the corresponding metabolomes and lipidomes from three out of the four replicates totaling 36 samples. For detection of the phenylpropanoids, three biological replicates were prepared for each sample in three independent experiments with a total of 36 samples. All dissected seminal roots were collected into pre-chilled 1.5-ml tubes, immediately snap-frozen in liquid nitrogen, weighed, and then stored at –80°C until extraction of RNA, primary metabolites, lipids, and phenylpropanoids.

RNA Isolation and Sequencing

RNA isolation and sequencing were described previously (Hill et al., 2016). In short, the total RNA was extracted from 50 mg root tissue separately per genotype, treatment, and rootzone using the QIAGEN RNeasy kit following the manufacturer's protocol. All RNA-seq libraries were constructed and paired-end sequenced (100-bp) on an Illumina HiSeq 2000 system at the Australian Genome Research Facility (Melbourne, Australia). Four lanes were used for each genotype, and all 48 samples were run on a single flow cell. The RNA was sequenced to a depth of approximately 31 million read-pairs per sample per lane, giving a total of 1.48 billion reads (749 million read-pairs).

Metabolite and Lipid Quantification

Metabolites (sugars, organic acids) were quantified as described in (Dias et al., 2015). Amines and amino acids were quantified as described in (Boughton et al., 2011). Fatty acids were quantified as described in (Eder, 1995). Lipids were quantified as described in (Natera et al., 2016). Phenylpropanoids were extracted from three biological replicates of root tissues (10 mg) per genotype, treatment, and rootzone from exactly the same growth settings, using 500 μl of cold methanol each. After homogenization by CryoMill (Bertin Technologies), samples were agitated for 15 min at 70°C and 10 000 rpm in a thermoshaker (Eppendorf), then allowed to cool down the extract before being centrifuged for 5 min at room temperature at 14 000 rpm. Supernatant was transferred into a clean Eppendorf tube for further clean-up process using solid-phase extraction (SPE) cartridges. For the SPE clean-up process, 60 mg Agilent Bond Elut Plexa cartridges were conditioned using 1 ml of methanol, followed by 1 ml of water. The supernatant from root extracts was loaded and washed by passing 1 ml of methanol, then metabolites were eluted using 400 μl of methanol, followed by 400 μl of 5% formic acid in methanol. Combined elute was dried down in a speed vacuum and reconstituted in 100 μl of 50% methanol:water before LC/MS analysis.

Phenylpropanoids were analyzed using an Agilent 6490 triple quadrupole mass spectrometer coupled to an ultra-high-performance liquid chromatograph (LC-QqQ-MS) (Santa Clara, CA). An Agilent Luna C18 column (2.1 × 150 mm, 3 μm) was used for compound separation. The mobile phase composition included (A) 10 mM ammonium acetate in methanol/water/acetonitrile (10/85/5, v/v/v) and (B) 10 mM ammonium acetate in methanol/water/acetonitrile (85/10/5, v/v/v) with a gradient elution: 0–10 min, 45% A; 10–20 min, 55%–100% B; 20–22 min, 100% B; 22–25 min, 55% B to equilibrate the column to initial conditions. The flow rate of the mobile phase was maintained at 0.2 ml min^{-1} and the column temperature was maintained at 50°C. The needle wash was 20% (v/v) acetonitrile in water with sample injection volume of 5 μl. Analysis was performed using Agilent MassHunter acquisition software, version 7. Compounds were quantified based on calibration curves prepared using authentic standards.

MS detection was performed using an electrospray ionization source operated in positive ion mode. The source parameters were set as: capillary voltage 4.0 kV; iFunnel high pressure RF in positive and negative mode at 130V; low pressure RF in positive and negative mode at 60V; source temperature 200°C; sheath gas temperature 400°C; gas flow 12 l min^{-1}; sheath gas flow 12 l min^{-1}; fragmentor voltage 380 V; and cell accelerator 5V. Data were collected using in-house multiple reaction monitoring developed based on individual standards. Dwell time for each compound was set as 10 ms and data were quantified using MassHunter Quant software version 7.

Histochemical and Immunochemical Microscopy

Roots of Clipper and Sahara, grown on agar medium supplemented with either 0 or 100 mM NaCl for 3 days, were fixed in 4% paraformaldehyde overnight at 4°C and then washed in phosphate-buffered saline. For lignin and suberin staining, the roots were embedded in 6% agar followed by sectioning of 80-μm-thick sections using a VT1000 S vibratome (Leica Microsystems). Sections for lignin staining were cleared using Clearsee (Kurihara et al., 2015) and stained using 0.2% (w/v) Basic Fuchsin and 0.1% (w/v) Calcofluor White (general cell wall stain) (Ursache et al., 2018). Vibratome sections for suberin staining were placed in 0.01% (w/v) Fluorol Yellow 088 (Santa Cruz Biotechnology) in polyethylene glycol 200 for 1 h at 90°C (Brundrett et al., 1991) followed by counterstaining with 0.5% aniline blue for 30 min. Roots for callose labeling were dehydrated in an ethanol series followed by infiltration and embedding in London White Resin (ProSciTech). Sections (1 μm) were cut using an Ultracut S Ultramicrotome (Leica Microsystems) and labeled with the primary (1:3)-β-glucan antibody (Biosupplies Australia) (Meikle et al., 1991) at a concentration of 1:300 (Wilson et al., 2015) followed by the secondary anti-mouse 568 Alexa Fluor antibody (Thermo Fisher) at a 1:200 dilution.

A Nikon C2 confocal microscope (Coherent Scientific, Australia) equipped with a spectral detector was used to image the cell wall fluorescence using the following settings: Basic Fuchsin, ex 561 nm, em 600–650 nm; Calcofluor White, ex 405 nm, em 425–475 nm; anti-mouse Alexa Fluor 568 antibody, ex 561 nm, em 570–650 nm. Fluorol Yellow staining was imaged using a Leica DM6000 microscope equipped with a Leica DFC450 camera using the I3 (GFP/FITC) filter. Images were analyzed using FIJI (NIH).

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

C.B.H., M.S.D., M.C.S., A.B., and U.R. designed the experimental part of the research. C.B.H. performed the salinity experiment, collected and extracted samples, processed data from mass spectrometry, and performed initial metabolite data analyses. T.R. carried out the phenylpropanoid detection and processing of mass spectrometry data. A.V.D.M. performed histological work on barley root sections. W.W.H.H. designed and implemented the bioinformatics part of the research, and performed all subsequent statistical and computational analyses, including functional annotation of barley genome with high-performance computing, GLM-based differential analyses, and omics data integration via integrated pathway analysis and global expression correlation networks. W.W.H.H. and U.R. interpreted the data and wrote the article. All authors revised, edited and approved the manuscript.

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Supplemental Information

Integrative Multi-omics Analyses of Barley Rootzones under Salinity Stress Reveal Two Distinctive Salt Tolerance Mechanisms

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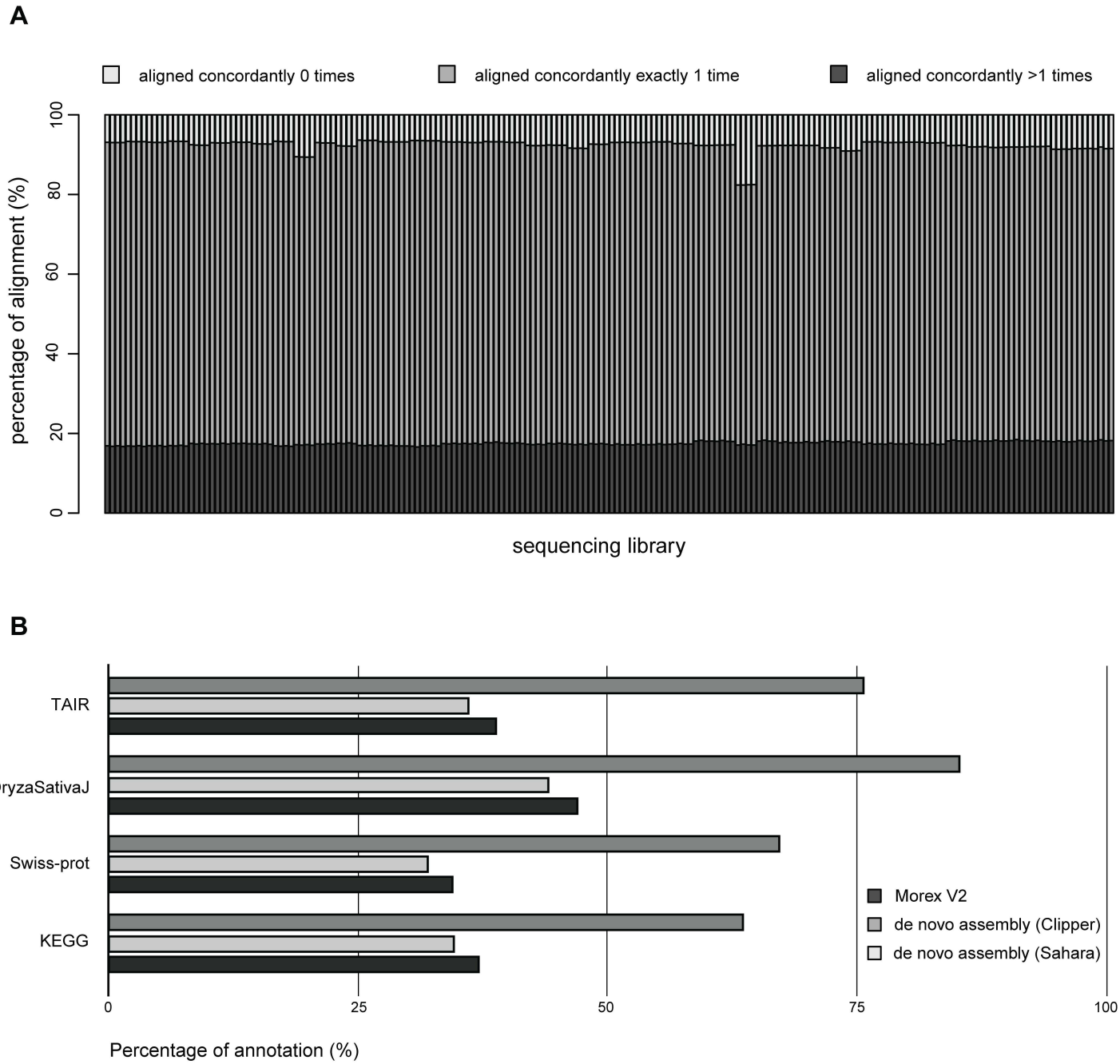
Supplemental Information

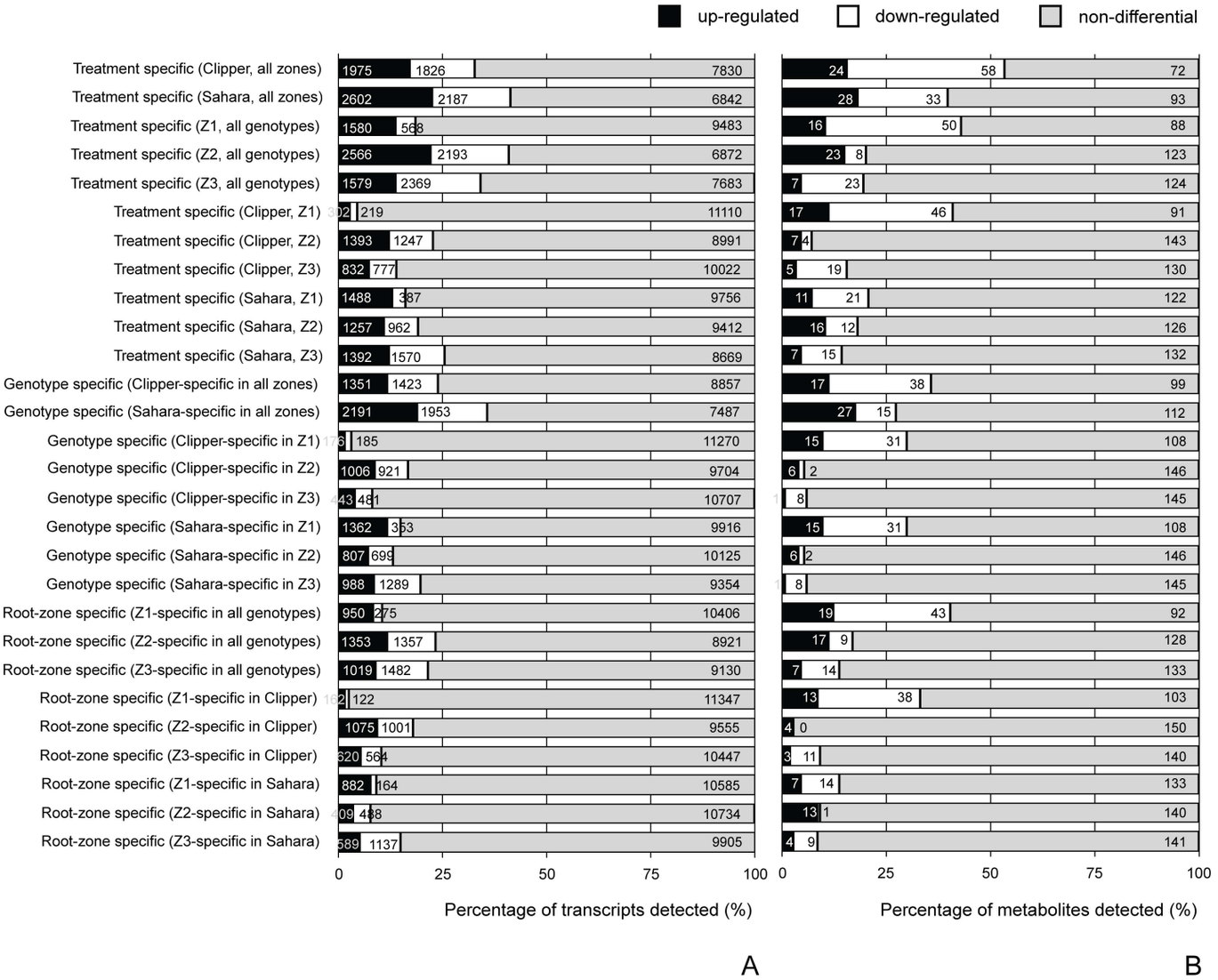
Resource Article: Integrative Multi-Omics Analyses of Barley Rootzones Under Salinity Stress Reveal Two Distinctive Salt Tolerance Mechanisms



Supplemental Figure 1. Overview for mapping and functional annotation of different barley genomes or assemblies.

(A) Mapping efficiency of the 192 RNA-seq libraries. Each stacked bar represents the rate of alignment for each technical sequencing replicate per biological sample. Average of the overall alignment rate across the 192 sequencing libraries is 95.71 ± 1.60 %.

(B) Degree of functional annotation of the latest version of barley genome (cv. Morex), and of the *de novo* Assemblies (cv. Clipper and landrace Sahara) against the four major databases of functional annotations. The four representative functional annotation databases are TAIR (Arabidopsis thaliana: model dicot), RAP-DB (Oryza sativa cv. japonica: model monocot), Swiss-Prot (manually curated protein databank across organisms), and KEGG (molecular pathway database across organisms) are used in the comparisons. Average percentage of functional annotations for Morex V2, de novo assembly for Clipper and Sahara are 74.05%, 37.39%, and 40.06%, respectively. Only matches with E-value <1.00E-4 against the databases are considered as positively annotated.

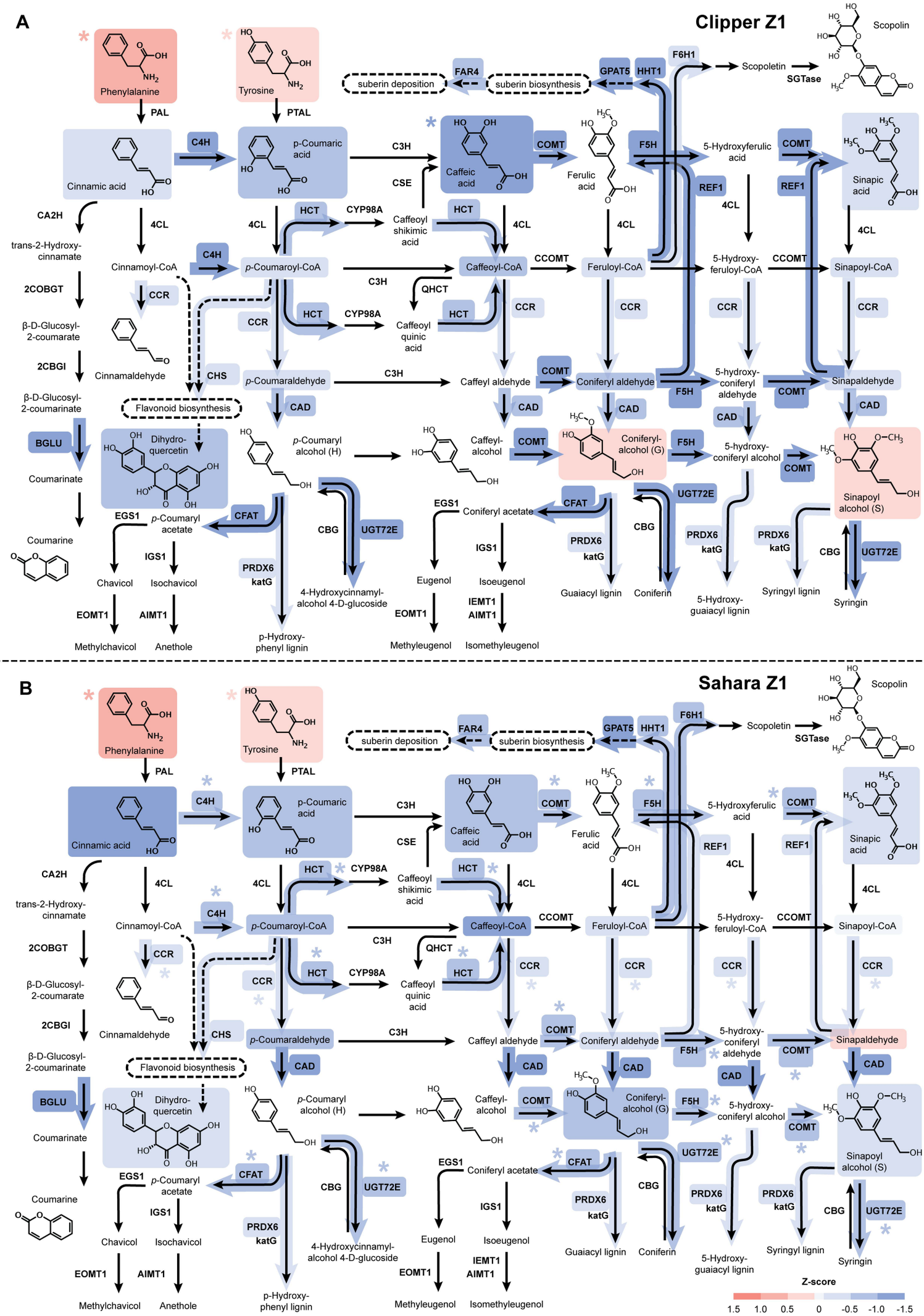


Supplemental Figure 2. Overview of the treatment-specific, genotype-specific, and rootzone-specific DEG and DAM of the two barley genotypes upon salt stress.

(A) Percentage of DEG and non-DEG with respect to the two treatments, two genotypes, and three rootzones. According to the latest version of the reference genome of barley (Mascher et al, 2017), cultivar Morex was predicted to have 85,493 of potential transcripts. However, only 11,631 transcripts were shown to have a minimal counts-per-million-reads of 10 in at least four replicates of our datasets among the predicted pool of transcripts in response to salt stress. Among the 11,631 detected transcripts, the GLM-based differential analyses revealed that the abundance of 3,801 transcripts (32.7%) changed significantly (up- or down-regulated) after the salt treatment in Clipper. In Sahara, 4,789 transcripts (41.2%) were significantly different in abundance in response to salt treatment relative to their controls, indicating the overall change in gene expression induced by salt was more pronounced in Sahara than in Clipper. From the perspective of rootzones within both barley genotypes, 2,148 (18.5%), 4,759 (40.9%), and 3,948 (33.9%) transcripts within the 11,631 quantifiable pool altered significantly after the salt treatment in Z1, Z2, and Z3, respectively. This suggests that the effect of salinity was more substantial in Z2, followed by Z3 and then Z1 at the transcript level in both genotypes. Further, among the 3,801 treatment-specific DEG in Clipper, 2,774 transcripts (73.0%) were shown to be highly specific to their genotype, compared to 4,144 (86.5%) transcripts within the 4,789 treatment-specific DEG in Sahara. In contrast, among the treatment-specific DEG in Z1 (2,148), Z2 (4,759), and Z3 (3,948), only 1,225 (57.0%), 2,710 (56.9%), and 1,501 (38.0%) transcripts were found to be highly specific to their respective rootzones. This indicates the salt-induced responses at transcript level in roots were more dependent on their genotypes than on their developmental zones.

(B) Percentage of DAM and non-DAM with respect to the two treatments, two genotypes, and three rootzones. In our experiment, 154 metabolites are within the limit of quantification of GC-QqQ-MS or LC-QqQ-MS after salt treatment. The GLM-based differential analyses showed that the abundance of 82 (53.3%) and 61 compounds (39.6%) varied significantly with salt treatment in both Clipper and Sahara relative to their controls, respectively. Across the root-zones, the abundance of 66 (42.9%), 31 (20.1%), and 30 (19.5%) compounds among the 154 quantifiable pools of metabolites changed significantly after the salt treatment in Z1, Z2, and Z3 of both genotypes, respectively. Furthermore, within the 82 treatment-specific DAM in Clipper, 55 compounds (67.1%) were shown to be highly specific to this genotype, compared to 42 compounds (68.9%) among the 61 treatment-specific DAM in Sahara. In comparison, among the treatment-specific DAM in Z1 (66 compounds), Z2 (31 compounds), and Z3 (30 compounds), 62 (93.9%), 26 (83.8%), and 21 (70.0%) compounds were found to be highly specific to their respective rootzones. The differential analyses at the primary metabolite and lipid levels suggest a higher degree of dependence of the salt-induced responses on root-zones than on genotype, compared to the transcriptional level. This implies an intriguing dynamic, where gene expression differences due to salt treatment are dominated by genotype and the downstream metabolic outcome is more influenced by rootzones.

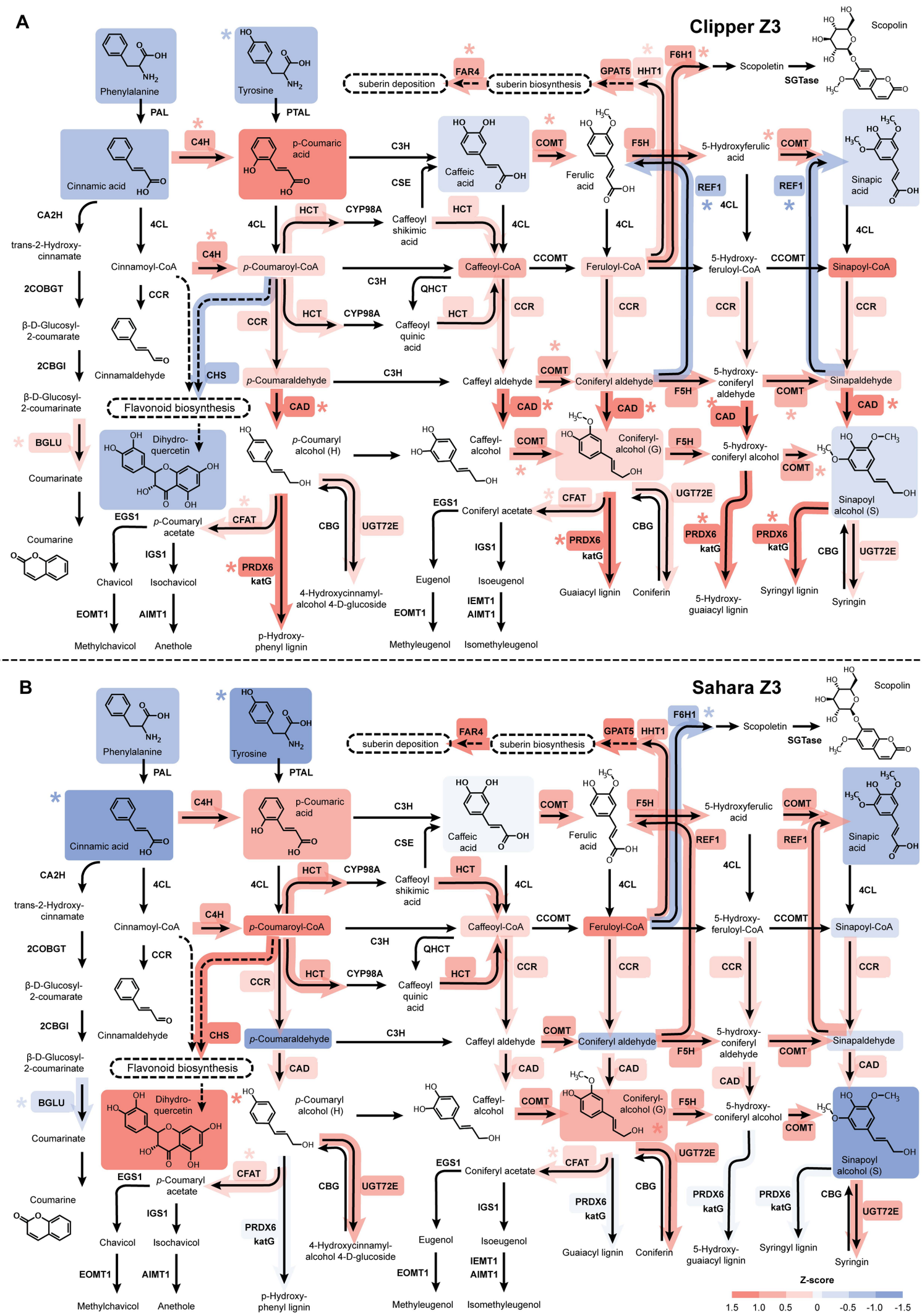
Statistically significant differential gene expression or metabolite abundance is defined at a cutoff of FDR-adjusted p-value ≤ 0.05. Up- or down-regulation is defined by expression or abundance level relative to the corresponding uninduced control. Numbering at each segment of stacked bar of (A) and (B) represents the corresponding number of transcripts detectable by RNAseq and of metabolites detectable by GC-QqQ-MS and LC-QqQ-MS, respectively. DAM, differentially abundant metabolites; DEG, differentially expressed genes; Z1, zone 1 (meristematic zone); Z2, zone 2 (elongation zone); Z3, zone 3 (maturation zone).



Supplemental Figure 3. Levels of transcripts and metabolites involved in phenylpropanoid biosynthesis at Z1 of the two barley genotypes under salt stress.

(A) The abundance of transcripts and metabolites involved in the biosynthesis at Clipper Z1.
(B) The abundance of transcripts and metabolites involved in the biosynthesis at Sahara Z1.

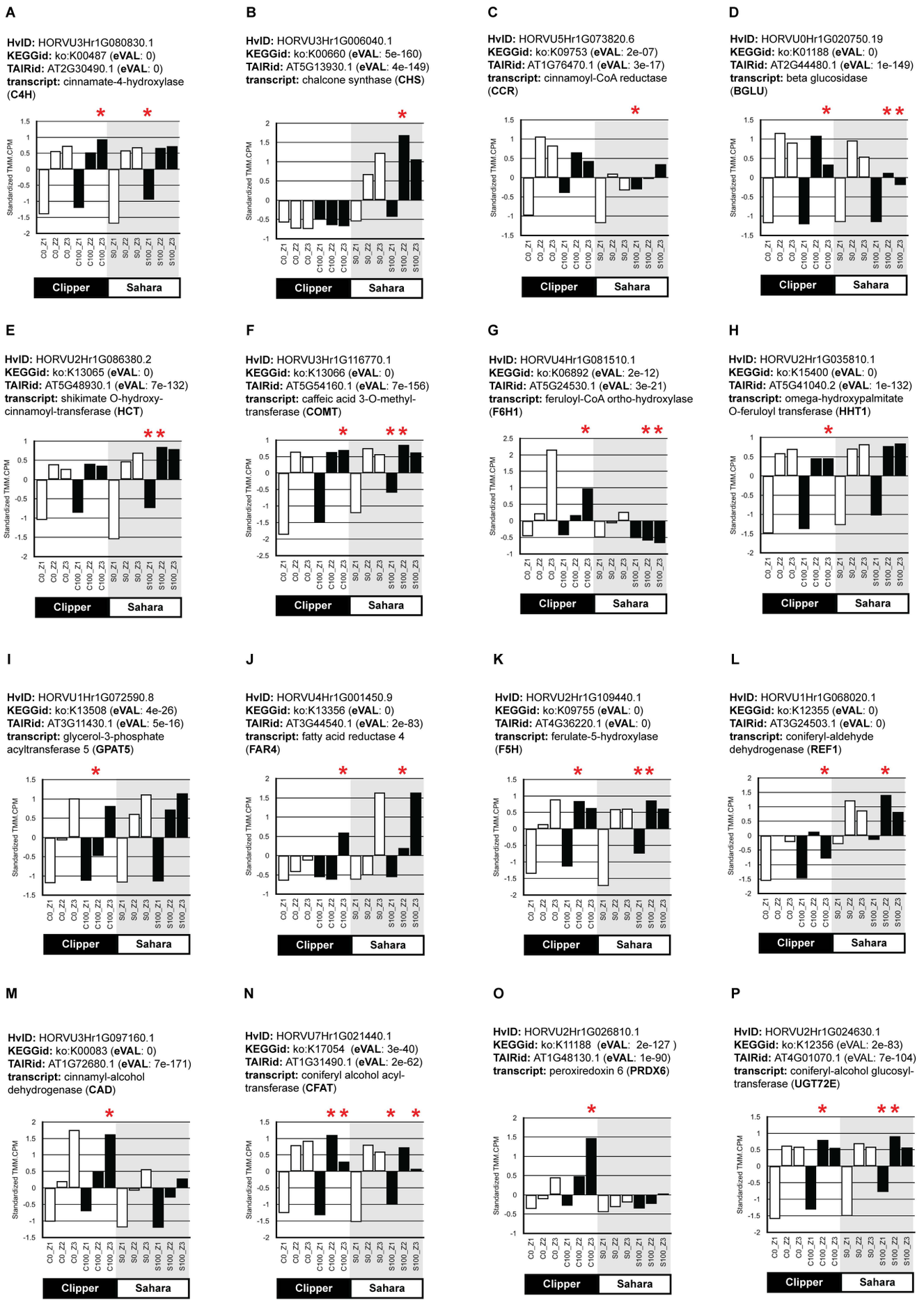
Values, color code, and full names for products of transcripts are given in **Figure 5**.



Supplemental Figure 4. Levels of transcripts and metabolites involved in phenylpropanoid biosynthesis at Z3 of the two barley genotypes under salt stress.

(A) The abundance of transcripts and metabolites involved in the biosynthesis at Clipper Z3.
(B) The abundance of transcripts and metabolites involved in the biosynthesis at Sahara Z3.

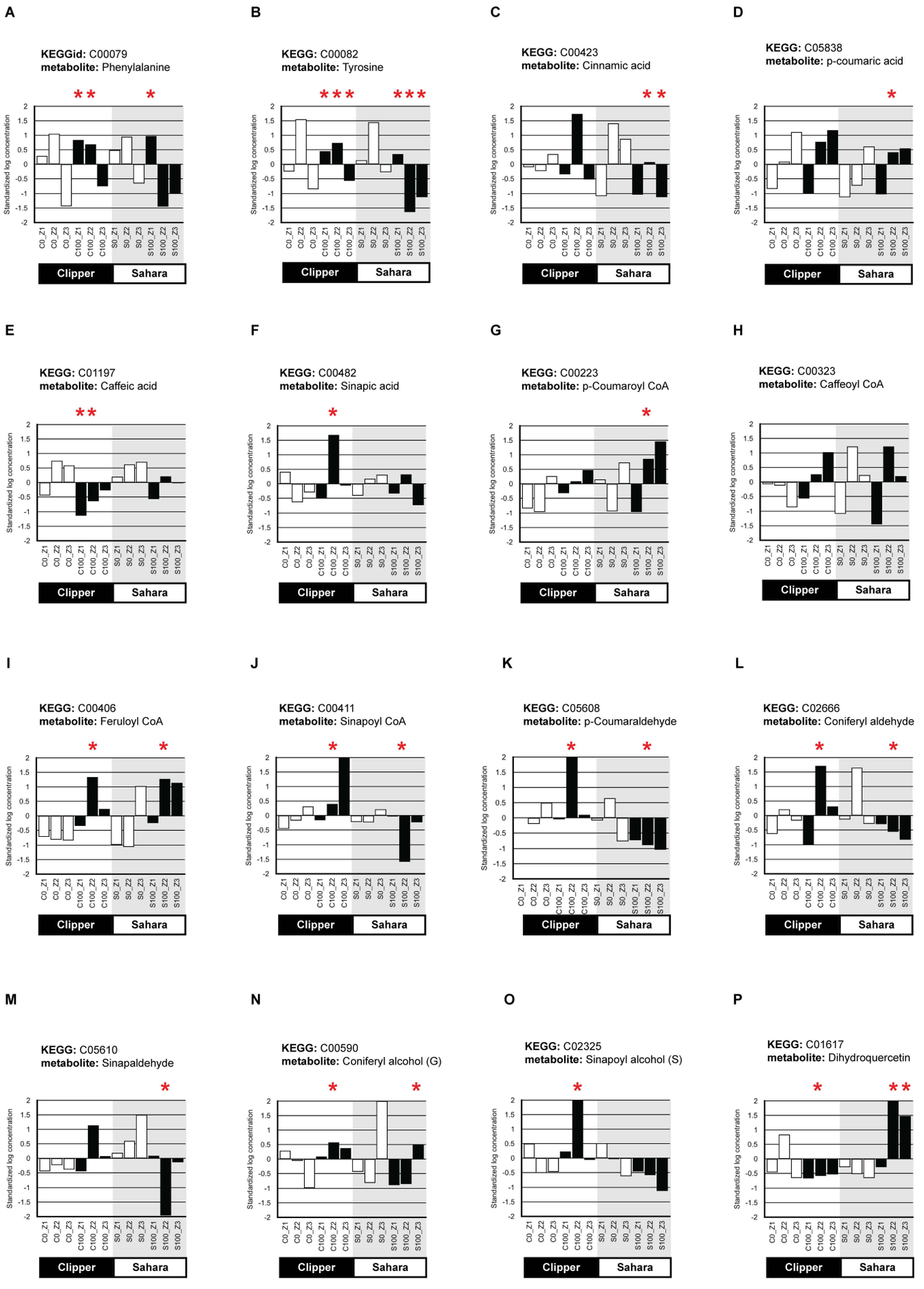
Values, color code, and full names for products of transcripts are given in **Figure 5**.



Supplemental Figure 5. Standardized abundance of transcripts involved in phenylpropanoid biosynthesis in Clipper and/or Sahara in response to salt stress.

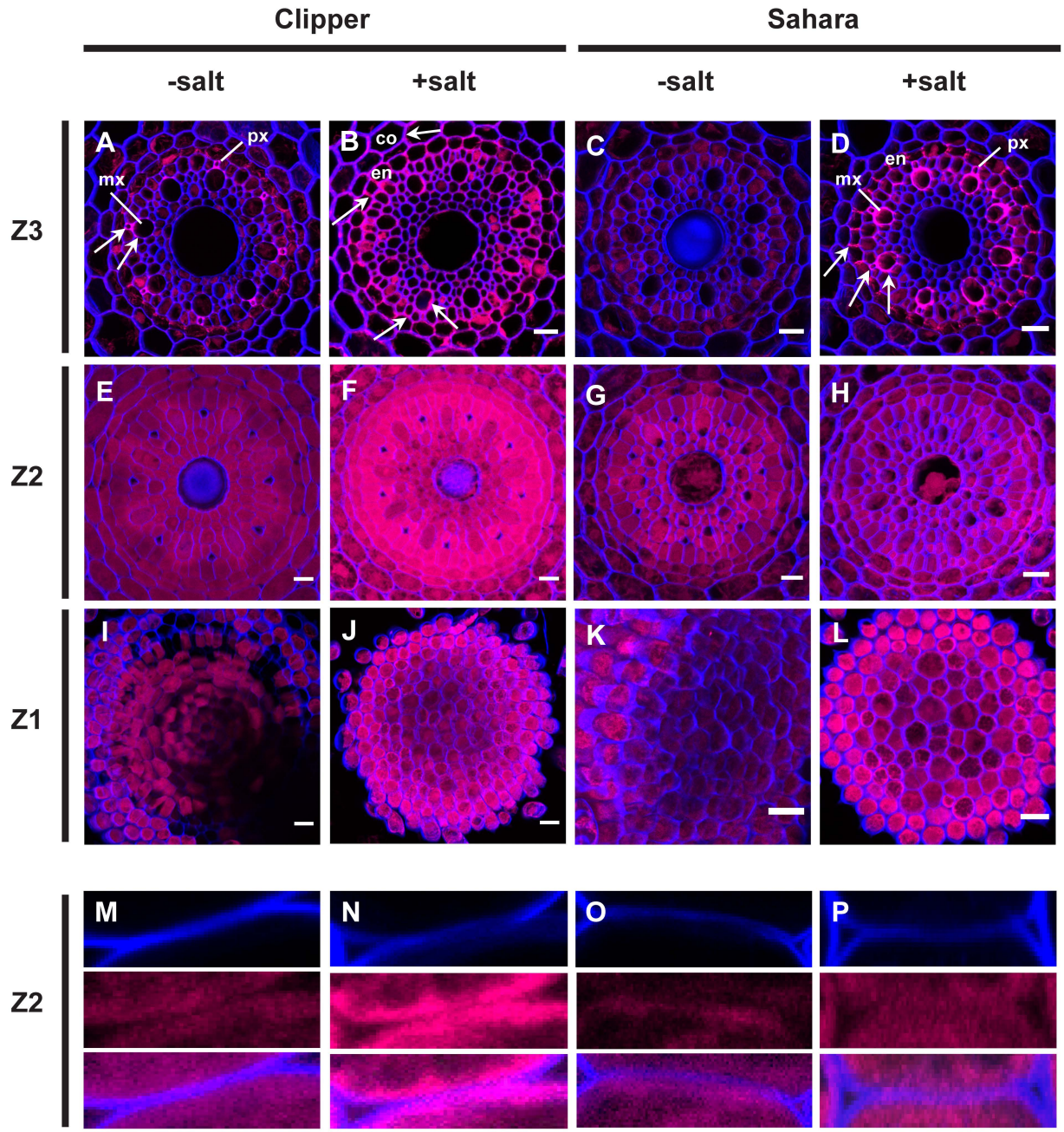
Standardized abundances of transcripts are calculated by generating the Z-score for TMM normalized CPM. Statistically significant differentiation (with Benjamini-Hochberg adjusted *p* value < 0.05) of transcript-abundance upon salt treatment are indicated by red asterisks.

C0, Clipper treated with 0mM NaCl; C100, Clipper treated with 100mM NaCl; eVAL, E-value (for the corresponding BLAST search); HvID, ID of transcripts based on the deep-sequenced genome of *Hordeum vulgare* L. (cv. Morex) (Mascher et al., 2017.); KEGGid, identifiers of Kyoto Encyclopedia of Genes and Genomes database; S0, Sahara treated with 0mM NaCl; S100, Sahara treated with 100mM NaCl; TAIRid, identifier of The Arabidopsis Information Resource; TMM.CPM, trimmed mean of M-values normalized count per millions reads; Z1, meristemic zone (zone 1); Z2, elongation zone (zone 2); Z3, maturation zone.



Supplemental Figure 6. Standardized abundance of metabolites involved in phenylpropanoid biosynthesis in Clipper and/or Sahara in response to salt stress.

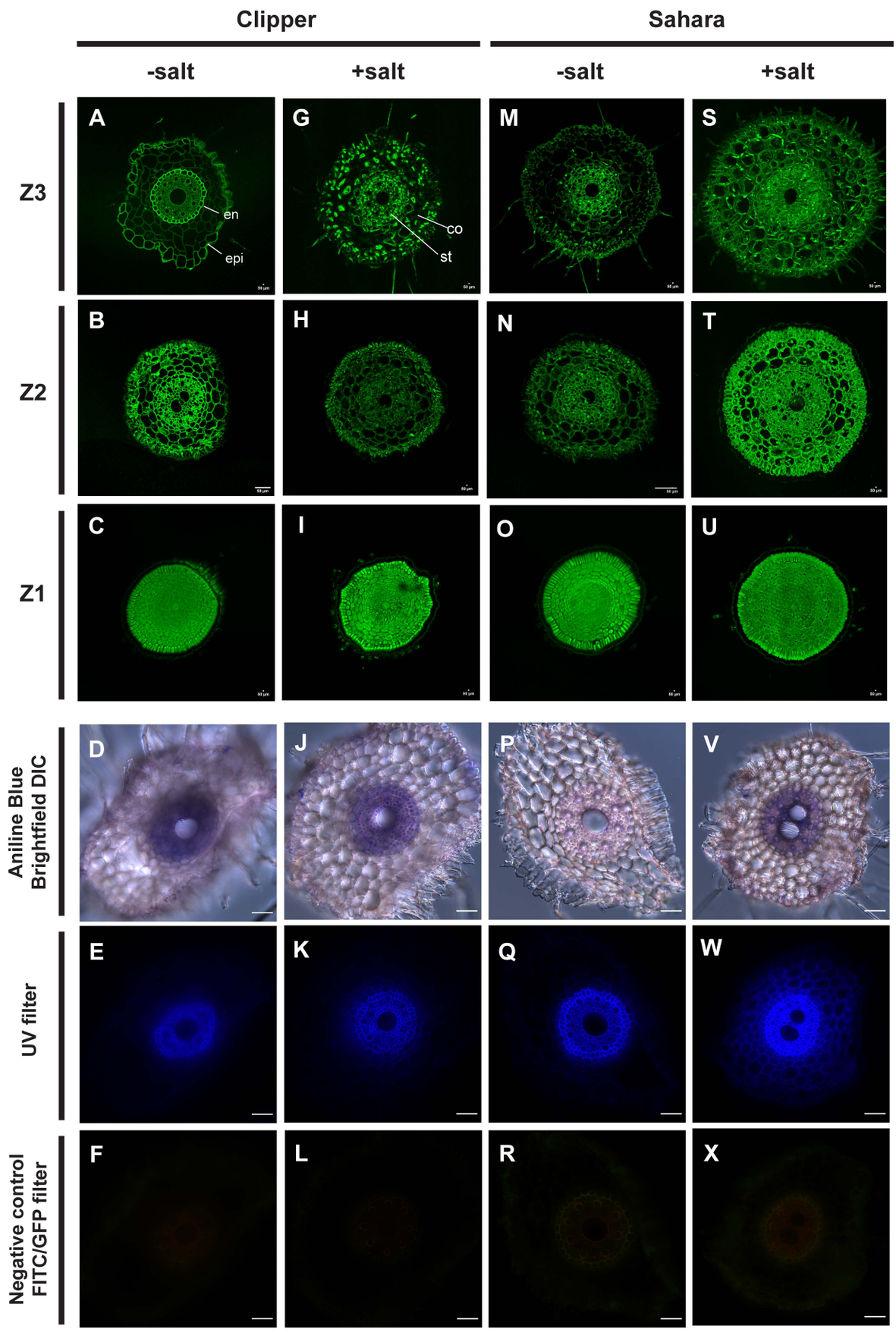
Standardized abundances of metabolites are calculated by generating the Z-score for the median-normalized concentration. Statistically significant differentiation (with Benjamini-Hochberg adjusted p value < 0.05) of metabolite-abundance upon salt treatment are indicated by red asterisks. C0, Clipper treated with 0mM NaCl; C100, Clipper treated with 100mM NaCl; eVAL, E-value (for the corresponding BLAST search); KEGGid, identifiers of Kyoto Encyclopedia of Genes and Genomes database; S0, Sahara treated with 0mM NaCl; S100, Sahara treated with 100mM NaCl; Z1, meristemic zone (zone 1); Z2, elongation zone (zone 2); Z3, maturation zone.



Supplemental Figure 7. Salinity-induced abundance and localization shift of lignin between different rootzones of the two barley genotypes upon salt stress.

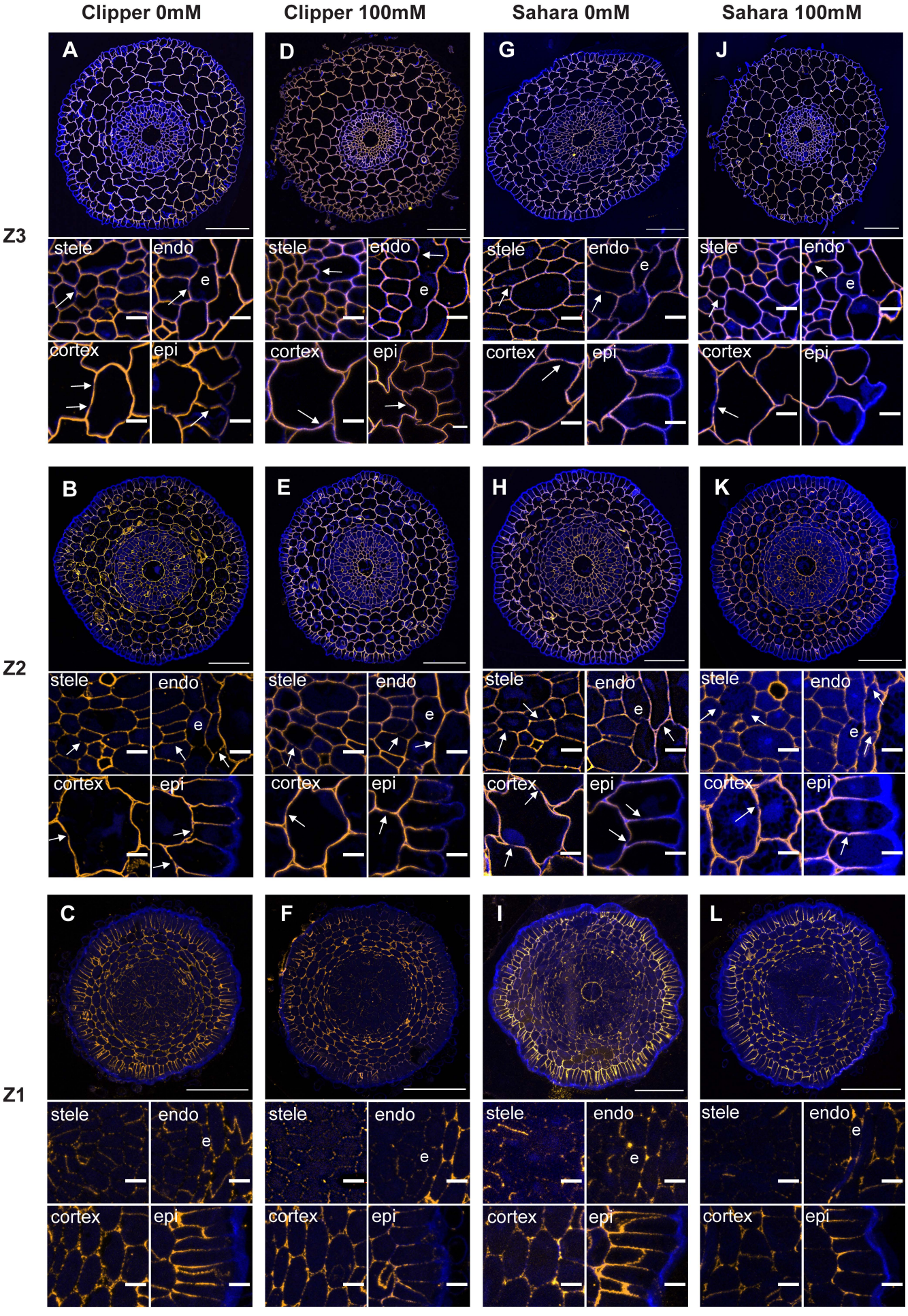
Transverse sections from different rootzones of the two genotypes grown under 0mM NaCl (Clipper: **A,E,I,M**; Sahara: **C,G,K,O**) or 100mM NaCl (Clipper: **B,F,J,N**; Sahara: **D,H,L,P**) observed using confocal microscopy with the fluorescent lignin stain Basic Fuschin (magenta) and the general cell wall stain Calcofluor White (blue). **M,N,O,P** are the magnified views (upper panel: Calcofluor White stain only; middle panel: Basic Fuschin fluorescent stain only; lower panel: overlay of the two images) to the cell walls of endodermal cells at Z2 of Clipper (-salt), Clipper (+salt), Sahara (-salt), and Sahara (+salt), respectively.

Basic Fuschin interacts with lignin but also acidic components of the cytoplasm. Lignin presence was expected when the blue and magenta signals overlapped, but not in the cytoplasm of cells. Wall lignin was most obvious in Z3 (**A-D**). While tiny amount of lignin (white arrows) is detected in xylem vessels of Clipper with no salt treatment (**A**), a remarkable amount of lignin is deposited around the proto- and meta-xylem (**px** and **mx** respectively) and in the walls of the endodermal (**en**) and cortical (**co**) cells after salt treatment (**B**). For Sahara, very little lignin was observed in Z3 of the non-salt treated roots (**C**); however, intense deposits of lignin were observed in the meta- and proto-xylemic cell walls, accompanied by a small amount of lignins laid at walls of endodermis and pericycle after salt treatment (**D**). In Z2, most of the outer stelic layers including endodermal, periclinal, and xylemic regions of Clipper Z2 show a much higher intensity of magenta (**F**) than Sahara Z2 after the salt treatment (**H**). Compared to the magnified view to the endodermal cells of Sahara Z2 (**P**), the view at Clipper Z2 also shows a more intense magenta fluorescence at cell walls (indicated by the blue fluorescence) rather than in cytoplasm (**N**), suggesting the increased lignin deposition occurred at cell walls of Clipper Z2, but not of Sahara Z2. Scale bars = 50 μm (**A, C, E, G, L, K, M, O, Q, S, U , W**) or 20 μm (**B, D, F, H, J, L, N, P, R, T, V, X**).



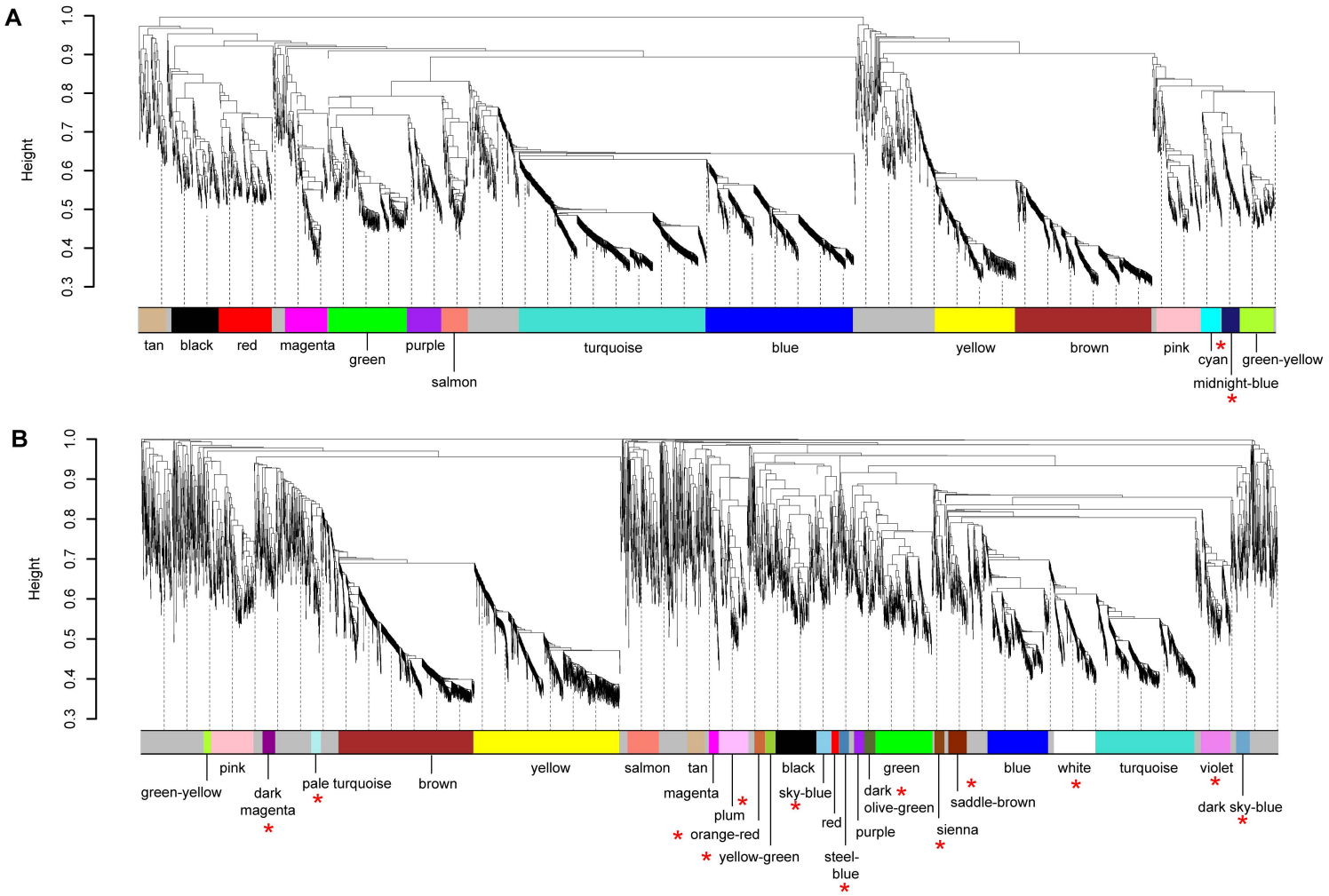
Supplemental Figure 8. Salinity-induced abundance and localization shift of suberin between different rootzones of the two barley genotypes upon salt stress.

Transverse sections from different rootzones of the two genotypes grown under 0mM NaCl (Clipper: **A-F**; Sahara: **M-R**) or 100mM NaCl (Clipper: **G-L**; Sahara: **S-X**) using confocal microscopy with Fluorol Yellow, a fluorescent suberin-specific stain, observed under FITC/GFP filter (Clipper: **A-C,G-I**; Sahara: **M-O,S-U**), or Z3 root sections (Clipper: **D-F,P-R**; Sahara: **J-L,V-X**) without staining with Fluorol Yellow observed under brightfield DIC (**D,J,P,V**), UV filter (**E,K,Q,W**), or FITC/GFP filter (**F,L,R,X**). Fluorol Yellow is a suberin-specific stain. The Aniline Blue (**D,J,P,V**) is a counterstain, which show the cytoplasmic details and structure in roots under brightfield DIC. The UV filter (**E,K,Q,W**) shows autofluorescence from lignin and other wall components such as vasculature. The negative control (**F,L,R,X**) underwent the same treatment except staining with Fluorol Yellow. Only a minimal autofluorescence can be observed under the FITC/GFP filter. Scale bars = 50µm. co, cortex; DIC, differential interference contrast; en, endodermis; epi, epidermis; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; st, stele; UV, ultraviolet.



Supplemental Figure 9. Salinity-induced abundance and localization shift of callose between different rootzones of the two barley genotypes upon salt stress.

Transverse sections from different rootzones of the two genotypes grown under 0mM NaCl (Clipper: A-C; Sahara: G-I) or 100mM NaCl (Clipper: D-F; Sahara: J-L) observed using confocal microscopy for the callose (anti-(1,3)-beta-glucan) antibodies (orange) and autofluorescence (blue). From each genotype, rootzone, and treatment, higher magnification images of cells at the central stele (stele), endodermis (endo; endodermal layer is denoted by 'e'), cortical cells (cortex) or epidermis (epi) are shown below each transverse section. Plasmodesmata are present in some cells (arrows). In Z1, callose is deposited in a punctate pattern and plasmodesmata are not obvious. Scale bars = 100 µm (for whole sections); 10 µm (for magnified cell images).

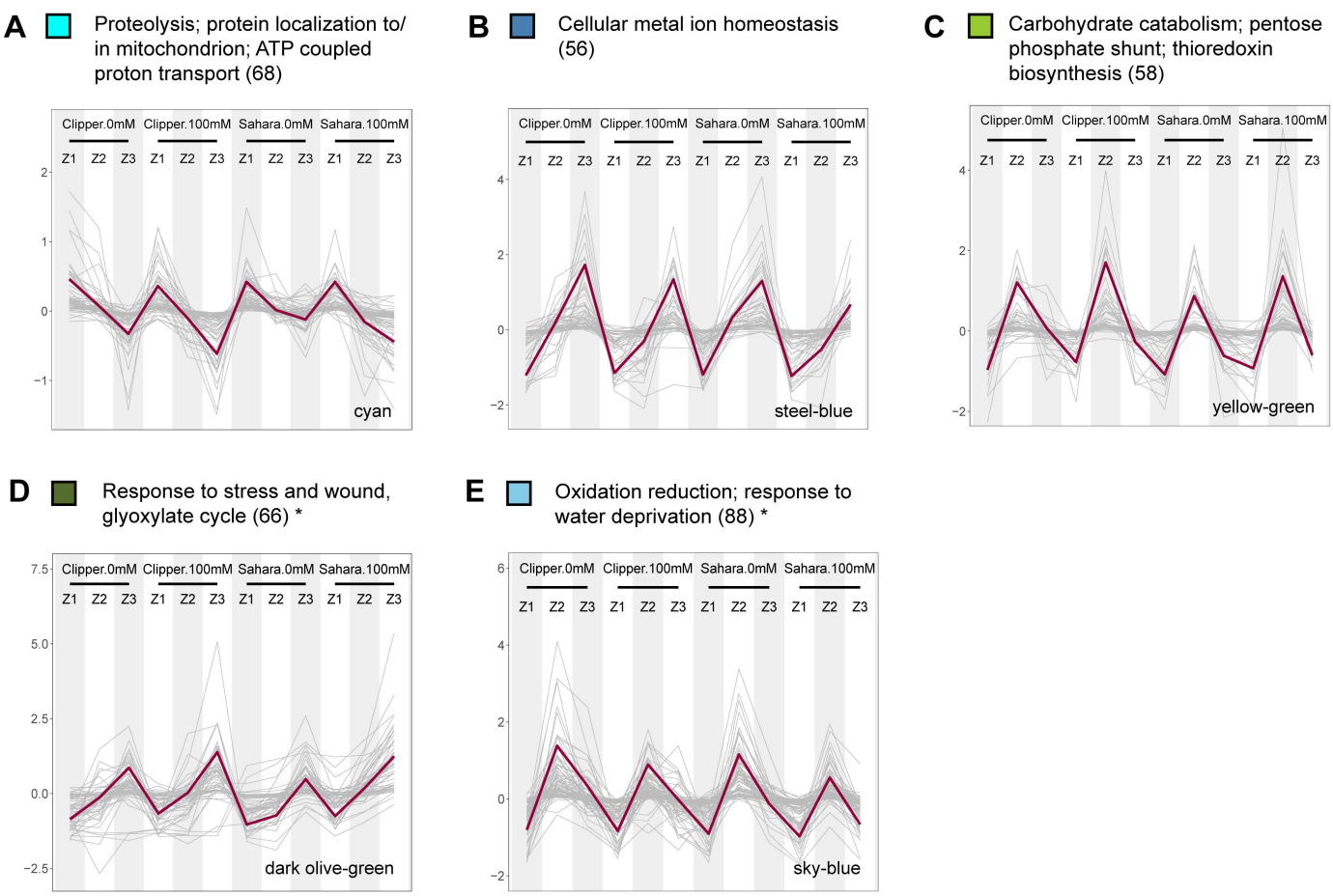


Supplemental Figure 10. Clustering dendrograms of global gene-coexpression in each barley genotype with dissimilarity based on topological overlap.

(A) Global gene-coexpression clustering of Hordeum vulgare L. cv. Clipper. Sixteen different module-colors are assigned for each coexpression cluster, which include: turquoise, blue, tan, green, grey, black, brown, yellow, magenta, red, purple, cyan, midnight-blue, salmon, pink, and greenyellow.

(B) Global gene-coexpression clustering of the landrace Sahara. Twenty-seven different module-colors are assigned for each coexpression cluster, which include: turquoise, blue, white, yellow, green, orange-red, sienna, grey, yellow-green, tan, brown, salmon, dark-magenta, red, plum, black, pink, saddlebrown, violet, pale-turquoise, dark-olivegreen, skyblue, magenta, dark skyblue, purple, greenyellow, and steel-blue.

Red asterisks beneath each assigned module-color indicate modules unique to Clipper or to Sahara. Grey-colored modules representing genes with unclustered expression pattern are not labelled for clarity.



Supplemental Figure 11. Additional modules of weighted coexpression correlation networks showing abundance profiles of transcripts and metabolites unique to either Clipper or Sahara.

(A) The abundance profile unique to Clipper or significantly contrast from Sahara.
(B-E) The abundance profiles unique to Sahara or significantly contrast from Clipper.

These modules are considered as additional or minor due to their non-apparent difference of profiles between Clipper and Sahara. Annotated lists of members for each module with signficant match (E-value < 1.00E-4) against TAIR10 genome release (version: Jun 2016) ranked in descending order according to kME of members can be found in **Supplemental Data Set 9 online**. For abbreviations and symbols, see **Figure 6**.

Supplemental Note 1

To derive the genotype-specific DEG for each root zone, we first clustered the twelve transcriptomes into four groups based on genotype (Clipper, C; Sahara, S) and treatment (untreated, U; treated, T).

Genotype	Treatment	Group
Clipper	salt-treated	CT
Clipper	untreated	CU
Sahara	salt-treated	ST
Sahara	untreated	SU

Second, DEG upon salt treatment in a genotype were determined by fitting basic GLM to test for the "contrasts" (a term for describing GLM-based comparisons as defined by (Chen et al.) and was denoted as " < > " in this work) between the treated and untreated pairs: CT < > CU and ST < > SU. For each "contrast", we designated genes that were significantly different (adjusted *p* value <0.05) in Clipper as sector A and in Sahara as sector B (Figure 2C). Third, to find DEG due to salt treatment between Clipper and Sahara, we fitted interaction GLM considering the interactions between genotypes and treatments to form "contrasts": (CT < > CU) < > (ST < > SU) and (ST < > SU) < > (CT < > CU). Genes expressed significantly differently (adjusted *p* value <0.05) between the genotypes were defined within sector C (Figure 2C). Fourth, we intersected the three sectors. Subsector A - (C ∪ B) corresponded to DEG unique to Clipper, subsector A ∩ B ∩ C corresponded to DEG significantly different in expression between the two genotypes, and subsector (A ∩ C) - B corresponded to DEG unique to Clipper and significantly differed between the two genotypes at the same time, which three subsectors together constituted the Clipper-specific DEG in a root zone. Accordingly, subsectors B - (A ∪ C), A ∩ B ∩ C, and (B ∩ C) - A represented the Sahara-specific DEG for each root zone (Figure 2C).

To derive the root zone-specific DEG for each genotype, similar to the derivation of the genotype-specific DEG, we clustered the twelve transcriptomes into six groups, namely salt-treated Z1 (group Z1T), untreated Z1 (group Z1U), salt-treated Z2 (group Z2T), untreated Z2 (group Z2U), salt-treated Z3 (group Z3T), and untreated Z3 (group Z3U).

Root-zone	**Treatment**	**Group**
Z1	salt-treated	Z1T
Z1	untreated	Z1U
Z2	salt-treated	Z2T
Z2	untreated	Z2U
Z3	salt-treated	Z3T
Z3	untreated	Z3U

Second, to find DEG upon salt treatment for each root zone, we fitted basic GLM to form "contrasts" between the treated and untreated pairs, including Z1T < > Z1U (for Z1), Z2T < > Z2U (for Z2) and Z3T < > Z3U (for Z3). Genes expressed significantly different (adjusted *p* value <0.05) in Z1, Z2, and Z3 were designated as sector D, E, and F, respectively (Figure 2D to 2F). Third, to find DEG responded differently to salt among the three root zones, we used interaction GLM considering the interactions between root zones and treatments to form "contrasts": (Z2T < > Z2U) < > (Z1T < > Z1U), and (Z3T < > Z3U) < > (Z1T < > Z1U) (for Z1 meristematic zone); (Z1T < > Z1U) < > (Z2T < > Z2U), and (Z3T < > Z3U) < > (Z2T < > Z2U) (for Z2 elongation zone); (Z1T < > Z1U) < > (Z3T < > Z3U), and (Z2T < > Z2U) < > (Z3T < > Z3U) (for Z3 maturation zone). For each "contrast", we selected genes that were significantly different (adjusted *p* value <0.05) as sector G, H, I, J, K, and L, respectively (Figure 2D to 2F). Fourth, we intersected the five sectors in each root zone. Subsectors D - (E ∪ F ∪ G ∪ H), (D ∩ G) - (E ∪ F ∪ H), and (D ∩ H) - (E ∪ F ∪ G) correspond to DEG unique to Z1; subsectors D ∩ E ∩ F ∩ G ∩ H, (D ∩ E ∩ G ∩ H) - F, (D ∩ F ∩ G ∩ H) - E corresponds to DEG with expression at Z1 significantly different from the other two root zones; subsector (D ∩ G ∩ H) - E ∪ F corresponds to DEG unique to Z1 and as well as significantly differential from Z2 and Z3 at the same time, which five subsectors together constituted the Z1-specific DEG for each genotype (Figure 2D). Accordingly, subsectors E - (D ∪ F ∪ I ∪ J), (D ∩ I) - (E

∪ F ∪ J), (E ∩ J) - (D ∪ F ∪ I); D ∩ E ∩ F ∩ I ∩ J, (D ∩ E ∩ I ∩ J) - F, (E ∩ F ∩ I ∩ J) - D; and (E ∩ I ∩ J) - (D ∪ F) indicated the Z2-specific DEG in for each genotype (Figure 2E). Subsectors F - (D ∪ E ∪ K ∪ L), (F ∩ K) - (D ∪ E ∪ L), (F ∩ L) - (D ∪ E ∪ K); D ∩ E ∩ F ∩ K ∩ L, (D ∩ F ∩ K ∩ L) - E, (E ∩ F ∩ K ∩ L) - D; and (F ∩ K ∩ L) - (D ∪ E) represented the Z3-specific DEG for each genotype (Figure 2F; for the detailed DEG lists, see Supplementary Data Set 3 online).

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Supplemental Note 2

Outcomes of Global Co-expression Correlation Network Analysis

Among the selected clusters, module A, J, K, L grouped members with stronger increase in abundance at Z2 of Clipper than Sahara upon salt stress. In which, **Module A** harboured 48 DEG and GO analysis revealed the most relevant biological processes to be multidimensional cell growth (Fig. 7A). In line with the growth-sustaining phenotype of Clipper under high salt conditions, a homolog of CELLULASE 1 (CEL1) (HORVU0Hr1G020000.1), which is a key player involved in cell elongation with endo-1,4-beta-glucanase activity, was found to be the modular member with the highest kME and known function (Shani et al., 1997) (Supplementary Data Set 8 and 9: sheet 'midnight-blue'). **Module J** comprises 138 DEG and glycine as the only DAM in this cluster. Enrichment analysis revealed a significant overrepresentation of biological processes involved in amino acid metabolism, response to stimulus, and cell wall organization (Fig. 7J). Among the GO category of response to stimulus, a barley homolog of glutamine receptor 2.7 (GLR2.7) (HORVU7Hr1G031530.1), which is known to be a member of the ligand-gated ion channel subunit family mediating cellular calcium ion homeostasis in response to abiotic stress of *Arabidopsis thaliana*, was pinpointed in this module (Kim et al., 2001). Also, a homolog of the EXPANSIN B2 (EXPB2) (HORVU1Hr1G054240.2) was found at the top of the kME list for this cluster. Although the induced abundance of this EXPB2 homolog in Clipper Z2 could not surpass its level in Sahara Z2 after salt, this could be in line with the fact that the regulatory roles of the huge family of EXPANSIN and EXPANSIN-like proteins are not limited to cell elongation only (Lee et al., 2001). As recently indicated by a study on an EXPB homolog in wheat, the EXPB2 in this cluster could rather play a common role in the two genotypes for regulating the activity of root cell wall-bound peroxidase under the oxidative pressure induced by salt (Han et al., 2015) (Supplementary Data Set 8 and 9: sheet 'black'). **Module K** represented 80 DEG upon high salinity stress. Amino acid transport, protein ubiquitination, and toxin catabolism were determined as the most relevant biological processes to this module (Fig. 7K). Coherent with the proposed involvement of its modular members in transport of amino acids, a barley transcript (HORVU5Hr1G093090.2) with sequence identical to its homolog in *Arabidopsis thaliana* or in *Oryza sativa* (E-value < 1.00E-250), which encoded for a type of cationic amino acid- / polyamine-transporters known as AMINO ACID TRANSPORTER 1 (AAT1), was

ranked fifth on the kME list of module K (Frommer et al., 1995). Polyamine is a known group of compounds involved in the regulation of redox homeostasis during salt stress in plants (Saha et al., 2015; Shu et al., 2015). Besides, GLUTATHIONE S-TRANSFERASE TAU18 (GST U18), a member of the glutathione S-transferase family belonging to the GO category of toxin catabolism, was identified in this cluster and shown to have higher transcript abundance at Z2 when comparing Clipper to Sahara. Evidence to demonstrate the importance of GSTs for minimising the damages induced by oxidative stress *in planta* were previously described in (Cummins et al., 1999; Kampranis et al., 2000; Roxas et al., 1997) (Supplementary Data Set 8 and 9: sheet 'purple'). **Module L** grouped 156 DEG with biological processes significantly enriched in cell wall loosening, auxin homeostasis and lignin biosynthesis (Fig. 7L). This cluster harboured the exact matches (E-value < 1.00E-250) of *CELLULOSE SYNTHASE 1* (CESA1) and *CELLULOSE SYNTHASE 3* (CESA3) in barley (HORVU6Hr1G013670.30, HORVU0Hr1G002350.1), with CESA1 being identified as the member with the highest kME of this module. Together with CESA6, CESA1 and CESA3 were known to form a hexameric plasma membrane complex mediating cellulose biosynthesis in primary cell wall (Desprez et al., 2007). Also, three transcripts (HORVU4Hr1G072660.1, HORVU4Hr1G072580.1, HORVU5Hr1G119680.2) encoded for homologs of EXPANSIN A11 (EXPA11), one transcript (HORVU6Hr1G063180.1) for EXPANSIN B4 (EXPB4), and one transcript (HORVU1Hr1G051640.1) for EXPANSIN A7 (EXPA7) were co-regulated in this cluster. Abundance of all five *EXPANSIN* were induced by salt and reached levels higher in Z2 of Clipper than Sahara, except *EXPA7*. Notably, besides a barley homolog of a key enzyme involved in brassinosteroid biosynthesis known as DEETIOLATED2 (DET2) (HORVU3Hr1G085400.1) was co-regulated in this cluster (Fujioka et al., 1997), three transcripts (HORVU5Hr1G035980.1, HORVU6Hr1G075650.1, HORVU4Hr1G019380.1) encoded for homologs of the TEOSINTE-BRANCHED 1/CYCLOIDEA/PCF1 (TCP) transcription factor family, namely TCP8, TCP15, and TCP23, which have their family recently been proven to involve in regulation of salicylic acid (SA) biosynthesis in response to range of plant abiotic stress responses, could also be pinpointed in this module (Lei et al., 2017; Wang et al., 2015) (Supplementary Data Set 8 and 9: sheet 'red').

In response to the salt treatment, **Module N** was the only cluster built up from members showing mostly drastic changes in abundance at Z3 of both genotypes, with a majority of the changes higher in Clipper than in Sahara. This module consisted of putrescine as the only DAM in the cluster and 107 DEG that were significantly enriched in biological processes such as cell

wall loosening, lipid storage and catabolism (Fig. 7N). Apart from a homolog of XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 13 (XTH13) (HORVU2Hr1G101150.1) known to involve in cell wall organization and biogenesis was grouped in this module (Thompson and Fry, 2001), we found two barley transcripts (HORVU3Hr1G058700.1, HORVU5Hr1G056030.1) co-regulated in this cluster with sequence well-matched to their dicot homologs, DAD1-LIKE SEEDING ESTABLISHMENT-RELATED LIPASE (*DSEL*) (E-value = 3.00E-125) and OLEOSIN 1 (*OLE1*) (E-value = 1.00E-16), respectively. Intriguingly, DSEL was shown to possess acylglycerol lipase activity and inhibits the breakdown of storage oils in seedlings, while OLE1 was known to have oilbody biogenic properties and involve in seed oil body formation for lipid accumulation in response to freezing stress in *A. thaliana* (Shimada et al., 2008; Kim et al., 2011). Transcript abundance of both *DSEL* and *OLE1* increased more strongly when compared Z3 of Clipper to Sahara after the salt treatment. In comparison, five transcripts (HORVU3Hr1G105160.1, HORVU3Hr1G073120.1, HORVU3Hr1G105180.1, HORVU7Hr1G119360.1, and HORVU2Hr1G093690.1) with sequences homologous to three paralogs of *EXPANSIN* in *A. thaliana* (namely *EXPA2*, *EXPA13*, *EXPB2*) were identified in this cluster and their abundance were boosted at Z3 of both genotypes upon salt. Except *EXPA13*, the abundance of these EXPANSIN were higher in Z3 of Sahara than Clipper and are likely to play a role in the oxidative stress tolerance as in Sahara Z2 (Supplementary Data Set 8 and 9: sheet 'magenta').

The subnetwork, including modules B, G, O, and I, was the co-expression cluster with members showing substantial decline in abundance at Z3 of both genotypes. **Module B** represented 177 DEG significantly overrepresented in nitrile biosynthesis, glycosinolate catabolism, and defence response (Fig. 7B). While abundance of the two barley homologs of *FLAVIN-DEPENDENT MONOOXYGENASE 1* (*FMO1)* (HORVU5Hr1G086710, HORVU5Hr1G086770) that was known to be a crucial component involved in the plant-type hypersensitive response were decreased to comparable levels in the two genotypes after the salt treatment (Mishina and Zeier, 2006), transcript level of four mannose-binding lectin (MBL) superfamily proteins (HORVU5Hr1G009040.2, HORVU5Hr1G009040.6, HORVU5Hr1G009040.5, HORVU5Hr1G009100.1) were consistently lower in Z3 of Sahara relative to Clipper upon salt stress. One of the well-characterized members of the MBL protein family is MYROSINASE-BINDING PROTEIN 1 (MBP1) and its presence was proven to be essential for the formation of myrosinase isoenzymes, which were found to play a key role in glucosinolate hydrolysis upon wounding in *Brassica napus* (Eriksson et al., 2002; Angelino et

al., 2015). Notably, transcripts encoding for a barley homolog of C-REPEAT BINDING FACTOR 3 (CBF3) (HORVU5Hr1G080300.1) was also found to be co-regulated in this cluster with its expression substantially suppressed in Clipper Z2 and Z3, but mostly unaffected in Sahara. CBF3 was proven to be a negative regulator of gibberellin signalling pathway through promotion of DELLA accumulation and *GIBBERELLIN 2-OXIDASE 7* (*GA2OX7*) expression (Zhou et al., 2017) (Supplementary Data Set 8 and 9: sheet 'white'). **Module G** comprised of 125 DEG and GO analysis revealed the most relevant biological processes to this cluster were lipid catabolism, phenylpropanoid biosynthesis, and cell wall modification (Fig. 7G). Among the category of lipid catabolic process, two transcripts in this cluster (HORVU3Hr1G068280.1, HORVU1Hr1G055840.1) were shown to be members of alpha/beta-hydrolases superfamily, of which HORVU1Hr1G055840.1 shared a high degree of sequence similarity with DAD1-LIKE LIPASE 1 (DALL1) in *A.thaliana* (E-value: 1.00E-112). DALL1 was shown to be capable of hydrolyzing triacylglycerols, phosphatidylcholines as well as glycolipids, and shown to act redundantly in jasmonate biosynthesis after wounding or in response to salt (Ellinger and Kubigsteltig, 2010; Ruduś et al., 2014). Higher abundance of *DALL1* induced or remained at Z1 and Z3 of Sahara than Clipper could indicate a higher accumulation of jasmonate in Sahara in response to osmotic damages caused by salt (Supplementary Data Set 8 and 9: sheet 'violet'). **Module O** was a co-expression cluster harboured 49 DEG, of which their abundance in Clipper Z3 were remained higher when compared to Sahara even after the suppression by salt stress. Based on the enrichment analysis, nicotianamine biosynthesis and phloem transport are the most significantly overrepresented biological processes (Fig. 7O). Intriguingly, four barley transcripts (HORVU4Hr1G089750.2, HORVU6Hr1G090040.4, HORVU0Hr1G017720.1, HORVU6Hr1G090180.1) encode NICOTIANAMINE SYNTHASE 3 (NAS3) and three other transcripts (HORVU4Hr1G089870.1, HORVU6Hr1G032290.1, HORVU4Hr1G087390.1) were the homologs of NICOTIANAMINE SYNTHASE 4 (NAS4) in barley (Supplementary Data Set 8 and 9: sheet 'salmon'). Previous findings showed that NAS genes in *Triticum aestivum* L. encode for enzymes that convert S-adenosyl-L-methionine to nicotianamine, which is a non-protein amino acid involved in fundamental aspects of metal homeostasis and was shown to confer higher salt tolerance to bread wheat (Bonneau et al., 2016). **Module I** harboured 44 DEG and tryptophan as the only DAM in this cluster. The most relevant biological process to this cluster is tryptophan metabolism, fatty acid metabolism, abscisic acid (ABA) signalling, and salt overly sensitive (SOS) pathway (Fig. 7I). While the abundance of barley transcripts (HORVU7Hr1G083490.7, HORVU2Hr1G117610.1) encoded for ACYL-CoA OXIDASE 3

(ACX3) and SOS3-INTERACTING PROTEIN 4 (SIP4) homologs in barley were suppressed to comparable levels between the genotypes upon salt treatment, two members of the GLYCOSYL HYDROLASE (GH) family 32 were shown to have their transcripts (HORVU7Hr1G001070.6, HORVU7Hr1G001070.19) maintained at higher levels in general for all root-zones when compared Clipper to Sahara. GH (aka. glycoside hydrolases or glycosidases) assist in the hydrolysis of glycosidic bonds in complex sugars or glucosides (such as indole-3-acetic acid-glucoside, salicylic glucoside, pyridoxine glucoside, flavone glucoside), which high concentration have shown to be strongly correlated to different abiotic stress responses in rice (Chern et al., 2005; Markham et al., 1998; Morino et al., 2005; Suzuki et al., 1986b; Suzuki et al., 1986a). Furthermore, transcripts encoded for a barley homolog (HORVU6Hr1G088460.1) of ASCORBATE PEROXIDASE 1 (APX1), which is a central component known for scavenging reactive oxygen species (ROS) in plant cells (Davletova et al., 2005), were also found to be co-regulated in this cluster. Intriguingly, transcript abundance of *APX1* was strongly induced Clipper Z2, but mostly suppressed in all root zones of Sahara (Supplementary Data Set 8 and 9: sheet 'sky-blue').

The subnetwork consisting of modules C, D, E, and F comprises members with in general stronger salinity-induced abundance for all root-zones in Sahara than in Clipper. **Module C** harboured 66 DEG and agmatine, which is a precursor of putrescine used for the production of 1,3-diaminopropane appeared only upon salt stress (Erdei et al., 1990), as the only DAM in this cluster. GO analysis suggested the biological processes most relevant to this cluster are toxin catabolism, raffinose biosynthesis, and glycine betaine biosynthesis (Fig. 7C). Barley homologs of two components (HORVU1Hr1G078350.1, HORVU2Hr1G070680.1) involved in the biosynthesis of glycine betaine via choline, namely PHOSPHOETHANOLAMINE METHYLTRANSFERASE1 (PEAMT) and ALDEHYDE DEHYDROGENASE 10A8 (ADH10A8), were found to be co-regulated in this cluster (McNeil et al., 2001; Missihoun et al., 2015). Glycine betaine is a type of known protectants *in vitro* or *in vivo* for mitigating the deleterious effect of salt stress in different plant species (Hasanuzzaman et al., 2014; Tian et al., 2017; Yildirim et al., 2015). Besides, two additional transcripts (HORVU1Hr1G060810.1, HORVU4Hr1G071300.12) encoding the barley homologs of GIBBERELLIN INSENSITIVE DWARF1C (GID1C) and ABERRANT GROWTH AND DEATH 2 (AGD2) were also identified in this co-expression cluster. GID1C and AGD2 were shown to be involved in the activation of gibberellin signalling by lifting the DELLA repressor activity (Ariizumi et al., 2008), and the induction of spontaneous cell death or suppression of callose deposition in plant

species (Rate and Greenberg, 2001), respectively (Supplementary Data Set 8 and 9: sheet 'orange-red'). **Module D** comprised of 39 DEG and significantly enriched in hemicellulose metabolism, sulphur metabolism, and transition metal ion homeostasis (Fig. 7D). Five transcripts (HORVU3Hr1G016850.1, HORVU5Hr1G048030.1, HORVU5Hr1G114540.1, HORVU4Hr1G089670.1, HORVU3Hr1G091400.1) encoded for barley homologs of XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 20 (XTH20), ESKIMO 1 (ESK1), PECTIN ACETYESTERASE 7 (PAE7), MUCILAGE-RELATED10 (MUCI10), and PLANT GLYCOGENIN-LIKE STARCH INITIATION PROTEIN 1 (PGSIP1), which were known to involve in organisation of different hemicelluloses in *A.thaliana* such as xyloglucan crosslink-formation, xylan backbone acetylation, pectin acetylation, glucomannan galactosylation, and galacturonic acid substitution of xylan, respectively (Zhong et al., 2018; Voiniciuc et al., 2015; Philippe et al., 2017; Urbanowicz et al., 2014; Miedes et al., 2013). While the abundance of *PGSIP1* were was similar for both Z2 and Z3 between the two genotypes, transcripts of *XTH20*, *ESK1*, and *PAE7* homologs reached higher abundance levels after the salt treatment in Z2 of Clipper, and in Z1, Z3 of Sahara. In contrast, abundance of *MUCI10* in all three root-zones were higher when compared Sahara to Clipper (Supplementary Data Set 8 and 9: sheet 'sienna'). **Module E** harboured 105 DEG ,and based on GO analysis indicated the most relevant biological processes in this cluster were systemic acquired resistance, regulation of endopeptidase, and response to ROS (Fig. 7E). Under the category of the acquired resistance, sequences of two transcripts (HORVU2Hr1G102050.1, HORVU2Hr1G102100.1) were found to be homologous to the *DEFECTIVE IN INDUCED RESISTANCE 1* (DIR1) (E-value = 5.00E-13; 7.00E-27) and one transcript (HORVU4Hr1G087860.1) was well-matched with its *AZELAIC ACID INDUCED 1* (AZI1) homolog in dicots (E-value = 4.00E-25). Intriguingly, both of these defence proteins were known to possess lipid binding and transfer abilities and overexpression of AZI1 was proven to increase salt tolerance of *A. thaliana* (Yu et al., 2013; Guelette et al., 2012). In addition, two transcripts (HORVU3Hr1G113120.1, HORVU5Hr1G018720.1) encoding for homologs of PATHOGENESIS-RELATED 4 (PR-4) (E-value = 2.00E-74) and RELATED TO AP2 11 (RAP2.11) (E-value = 7.00E-21) were co-regulated with members of this cluster. While transcript level of PR-4 was known to increase in response to ethylene and salt (Catinot et al., 2015; Kim et al., 2014), RAP2.11 expression was suggested to be positively feedback regulated by ethylene and ROS (Kim et al., 2012), which are commonly found in plant cells upon salinity stress (Supplementary Data Set 8 and 9: sheet 'plum'). **Module F** grouped 38 DEG and GO overrepresentation analysis suggested the metabolism of folic acid, tetrahydrofolate, and

pteridine were significantly upregulated in this cluster (Fig. 7F). Within this module, two transcripts (HORVU5Hr1G074900.1, HORVU5Hr1G074900.2) highly homologous to SARCOSINE OXIDASE (SOX) were also found to be induced to a much higher level of abundance in all root-zones of Sahara than in Clipper after salt. But unlike its homologs in *Corynebacterium* that catalyse the oxidation of sarcosine (Chlumsky et al., 1995), a non-protein amino acid known as pipecolate was determined as the endogenous substrate of SOX in plants (Goyer et al., 2004). Pipecolic acid was proven to be an endogenous mediator that orchestrates defence amplification, positive regulation of salicylic acid biosynthesis, and establishment of systemic acquired resistance In *A. thaliana* (Návarová et al., 2012). Oxidation of pipecolic acid mediated by the higher abundance of SOX in Sahara after salt could therefore imply a stronger suppression of such orchestration upon the local salinity impact (Supplementary Data Set 8 and 9: sheet 'saddle-brown').

In response to salinity stress, **Module H** was the only co-expression cluster with members highly up-regulated at Z1 and, for most cases, magnitudes of changes were higher in Sahara than Clipper. This cluster comprised 29 DEG and GO analysis revealed the most relevant biological processes were sterol biosynthesis and cell division (Fig. 7H). Among them, we found a transcript (HORVU2Hr1G011720.3) highly homologous to sequence of CYTOCHROME P450 51A2 (CYP51A2) in *A. thaliana* (E-value: 3.00E-172) with higher abundance only in Sahara Z1 in response to salinity stress. CYP51A2 is an upstream enzyme involved in the biosynthesis of brassinosteroids and modulation of membrane steroid contents, of which the membrane integrity maintained was proven to correlate to the restriction of ROS- and ethylene-mediated premature cell death (Kim et al., 2005; Kim et al., 2010). Further, three transcripts (HORVU2Hr1G073480.1, HORVU2Hr1G041950.1, HORVU6Hr1G071120.2) were homologs of CELL DIVISION CONTROL 2 (CDC2), CYCLIN P4;1 (CYCP4;1), and INDOLE-3-BUTYRIC ACID RESPONSE 5 (IBR5) and mostly up-regulated in Z1 of both genotypes upon salt, but with CDC2 and CYCP4;1 reaching higher abundance levels in Clipper, and IBR5 higher in Sahara, respectively. While IBR5 was proven to negatively regulate MAP kinase activity that control expression of cyclins in cell cycle (Johnson et al., 2015; Lee et al., 2009), CYCP4;1 and CDC2 are positive modulators of cell cycle progression and control of cell division (Torres Acosta et al., 2004; Zhao et al., 2012). On the whole, this suggests cell differentiation and division were highly constrained by salt in Z1 of Sahara, not in Clipper (Supplementary Data Set 8 and 9: sheet 'pale turquoise').

Lastly, **Module M** built up from members with abundance being predominantly induced at Clipper Z2, but substantially repressed at Z3 of both genotypes by salt. This module consists of 94 DEG that are significantly enriched in biological processes such as lipid localization, cell wall loosening, and receptor recycling (Fig. 7M). In this cluster, an exact match of UDP-D-GLUCURONATE 4-EPIMERASE 1 (GAE) known to be involved in pectin biosynthesis was also found at the top of the kME-ranked list (HORVU6Hr1G084390.1) (Gu and Bar-Peled, 2004), implying a potential role for maintaining barley root cell wall integrity played by members of this modules. Further, similar to the lipid-transporting AZI1 and DIR1 found in module E, sequences of six transcripts (HORVU7Hr1G109140.1, HORVU0Hr1G031750.1, HORVU0Hr1G025270.1, HORVU0Hr1G025250.2, HORVU2Hr1G097190.1, HORVU2Hr1G029480.1) were found to be highly homologous to members of the lipid transfer protein-related hybrid proline-rich protein (LTP-HyPRP) family, which is mostly bifunctional by exhibiting lipid-transporting and proteolytic activities (Pitzschke et al., 2014; Zhang and Schläppi, 2007). One of the most well-studied members of LTP-HyPRP is EARLY ARABIDOPSIS ALUMINIUM INDUCED 1 (EARLI1), which was shown to protect plant cells from freeze-induced damages and could improve root elongation under salt stress upon overexpressing *EARLI1* in *A. thaliana* (Xu et al., 2011; Zhang and Schläppi, 2007). Notably, a barley homolog (HORVU3Hr1G007280.2) encoded for another key enzyme involved in brassinosteroid biosynthesis known as STEROL 1 (STE1) was also found to be co-regulated in this cluster (Gachotte et al., 1995) (Supplementary Data Set 8 and 9: sheet 'pink').

Distinctive Phases of Salinity Responses Observed in Clipper and Sahara

As defined by (Julkowska and Testerink, 2015), responses of plant cells during the exposure to salinity stress can be categorized into four main phases, namely early signalling (ES) phase, quiescent (Q) phase, recovery (R) phase, and recovery extent (RE) phase. Responses induced at the ES phase, such as the salt overly sensitive (SOS) pathway (Shi, 2002) and aquaporin internalization (Prak et al., 2008), can be triggered and completed within seconds or mostly hours upon exposure to salt stress (Julkowska and Testerink, 2015). In this study, root zones of the two barley genotypes were presumably in stage of Q, R, or RE phase after three days of growth on media enriched with salt. Notably, in line with the striking growth differences observed amongst plant organs and between main and lateral roots in response to salt (Julkowska et al., 2014), our global co-expression correlation study reveals that salinity

impacts the two barley genotypes remarkably differently in terms of the phase of responses reached by their individual root zones. Implications from the molecular and hormonal clues of the study are summarized in Supplemental Table 2 (STable2) and discussed below.

Upon exposure to salt stress, inhibition of cell cycle progression restricted the cell division and differentiation processes in Sahara Z1 (STable2: Z1, Sahara). As substantial repression of the reactive oxygen species (ROS)-scavenging mechanisms in combination with the ethylene-mediated ROS accumulation were detected in this root zone, cells in Sahara Z1 were likely retained at Q phase and not RE phase in response to the salt treatment. Notably, the ROS-related activities in the apoplast mediate cell wall stiffening through crosslinking of glycoproteins and phenolic compounds, which are known to be the milestone events detected only at the Q phase upon salt stress (Tenhaken, 2014). By contrast, divisions of cells in Clipper Z1 were maintained and the corresponding biological processes for supporting rapid cell expansion, such as cellulose biosynthesis and cell wall loosening, were observed in Clipper Z2 (STable2: Z1, Clipper; Z2, Clipper). Although the positive modulation of cell divisions could indicate Clipper Z1 was in the stage subsequent to the Q phase (i.e. either R or RE phase), the significant upsurge of biosynthetic enzymes involved in brassinosteroid biosynthesis and initiation of the ROS-scavenging mechanism suggest Clipper Z2 was in R phase, and yet to be in RE phase. There are insufficient hormonal clues to help define the phase of responses for Clipper Z3 (STable2: Z3, Clipper). For Sahara Z2 and Z3, salt stress induced the expression of *C-REPEAT BINDING FACTOR 3* (*CBF3*) (STable2: Z2, Sahara; Z3, Sahara). In the presence of CBF, GIBBERELLIN 2-OXIDASE 7 (GA2OX7) specifically deactivates the bioactive C-20 gibberellins (GA) (Zhou et al., 2017). Assuming the amount of bioactive GA was minimal under the action of GA2OX7 in barley, GA signalling and thus its growth-promoting function was restricted in response to salinity stress, implying Sahara Z2 and Z3 was retained at Q phase after the three days of salt treatment.

Strengthening the conclusion drawn from the integrated pathway analysis, our global correlation study indicates that the Z2 of Clipper proceeded to R phase for restoration of its growth rate, while all root zones of Sahara remained at a prolonged Q phase in response to the extreme salinity conditions.

Furthermore, in addition to diverting the resources for maintenance of root growth, a range of known downstream salt tolerance mechanisms, such as polyamine transport and toxin

catabolism (Frommer et al., 1995; Roxas et al., 1997), were also activated in Z2 of Clipper in order to cope with the salinity stress (STable2: Z2, Clipper). Notably, the majority of the tolerance mechanisms triggered were different between Z2 and Z3 of Clipper, where biological processes including seed oil body formation, glucosinolate hydrolysis, and nicotianamine biosynthesis (Bonneau et al., 2016; Eriksson et al., 2002; Shimada et al., 2008) were either induced or maintained in Z3 of Clipper, but not in Z2 (STable2: Z3, Clipper). Only hydrolysis of glucosides (Markham et al., 1998) has widespread up-regulation in all root zones of Clipper (STable2: AZ, Clipper), suggesting the salt tolerance strategies adopted by this genotype are mostly root zone-dependent; a mechanism that can only be explicitly revealed by the spatial multi-omics approach described here.

In contrast, with only two salt-induced biological processes, membrane steroid modulation and inhibition of cell cycle progression, with members up-regulated or maintained at high abundance in Sahara Z1 (STable2: Z1, Sahara), seven out of seventeen processes in Sahara were shared among two root zones (STable2: asterisks). Members involved in the eight processes remained such as biosynthesis of glycine betaine, modulation of GA signalling, and LTP-mediated tolerance response in all root zones of Sahara were found to be induced or maintained at higher abundance than in Clipper (STable2: AZ, Sahara). This finding suggests the tolerance mechanisms triggered in Sahara were mostly root zone-independent. Such independence is also consistent with our viewpoint that all root zones of Sahara are in the same phase of the salinity response.

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Supplemental Note 3

Functional Annotation of the New Barley Reference Genome

To further enrich the functional annotations of the mapping base for RNA-seq, the latest version of the new barley reference genome sequences (cv. Morex v2) and the genome structural annotation files were obtained from IPK Barley server of the International Barley Sequencing Consortium (IBSC) (Mascher et al., 2017). The total population of coding sequences of the genome was extracted by the gffread utility of Cufflinks (Trapnell et al., 2012) and refined using the degapseq script of EMBOSS 6.6.0.0 (Rice et al., 2000). The latest version of Basic Local Alignment Search Tool (BLAST) was obtained from the FTP server of the National Center for Biotechnology Information (NCBI) (Altschul et al., 1990), and a local BLAST pipeline was constructed in eight NeCTAR Research Cloud instances in Ubuntu 16.04 LTS (Xenial) environment (Li et al., 2018). The total population of translated coding sequences of the barley genome were BLASTx searched against three protein sequence databases, i.e. TAIR10 (Lamesch et al., 2012), UniProtKB/Swiss-Prot (The UniProt Consortium, 2017), RAP-DB (Sakai et al., 2013), and two ontology databases i.e. Gene Ontology (GO) (Ashburner et al., 2000) and KEGG Ontology (KO) (Kanehisa and Goto, 2000). The latest version of InterProScan-5 and Panther models 10.0 were obtained from the FTP server of the European Bioinformatics Institute (EMBL-EBI) and the getorf script of EMBOSS was applied to make InterProScan-5 to be compatible to nucleotide inputs. Scanning of InterPro protein domains databases was performed according to the user manual (Jones et al., 2014). Only the top hits of each coding sequence with the lowest e-values were listed in the functional annotation list and considered for biological interpretation.

Read Processing, and Mapping

Paired-end libraries of raw reads from the RNA-seq were verified and converted using FASTQ Groomer (Blankenberg et al., 2010) and sequence quality was validated using FastQC (Andrews). Based on the outcomes of the read quality assessment, threshold was defined (q=20; minimum read length: 24; Illumina TruSeq Adaptor primers removed; singletons discarded) and Trimmomatic was applied to trim reads for quality (Bolger et al., 2014). Mapping or paired-read alignment was performed via HISAT2 (Kim et al., 2015) and the sorted BAM files were

subjected to HTSeq code (Anders et al., 2015) for generation of the counting matrix using the genome structural annotation available from IBSC (Mascher et al., 2017).

DEG Determination and Enrichment Analysis of Gene Ontologies

To prepare for DEG determination, we filtered the lowly expressed genes from the matrix were filtered based on a minimum CPM threshold of 11.5 present in at least four samples, which corresponds to an average read count of 10-15 across the 192 libraries, to minimise the multiple testing burden when estimating false discovery rates (Robinson et al., 2010). TMM normalization was applied to the transformed CPM matrix to eliminate composition biases between libraries (Robinson and Oshlack, 2010). Multidimensional scaling of the TMM-normalized matrix explicitly revealed one biological replicate of Clipper control at Z3 as an outlier and was therefore excluded from all subsequent analyses. Variation of library sizes, sample-specific quality weighting, and mean-variance dependence of the data matrix were addressed by the voom transformation workflow available in limma package (v.3.7) (Ritchie et al., 2015). Detailed procedures for estimating group mean and gene-wise variances, as well as fitting of basic and interaction GLM to test for differential expression were detailed in (Smyth et al., 2002). Notably, as discussed by (Zhang and Cao, 2009), assumptions required for fold-change filtering and *t*-statistic adopted in DEG determination were contradictory, therefore only the *t*-statistic-based adjusted *p* value was applied as a cutoff in this study.

For enrichment analysis of GO, BiNGO was applied to determine the overrepresented GO terms in each DEG list focusing only on the GO Biological Processes category (Maere et al., 2005). Unless otherwise specified, the analyses were performed using the hypergeometric test with the whole barley annotation as a reference set and Benjamini-Hochberg FDR correction with *q* value cutoff at 0.05. Each enrichment list was summarized by REVIGO with small (0.5) allowed similarity (Supek et al., 2011) and enrichment networks resulted were visualized in Cytoscape (v.3.4.0) (Shannon, 2003).

DAM Determination and Metabolite Set Enrichment Analysis

Data matrices corresponding to each type of primary metabolomes and phenylpropanoids were standardized by sample weights to achieve unit-conformity across different extraction and detection workflows. To reduce systemic bias during sample collection and impact of the large

feature (metabolite) values, log-transformed matrices were normalized by median across samples and mean-centred, respectively (van den Berg et al., 2006). Each normalized matrix was individually evaluated for unwanted variances by means of relative log adjustment - within group (RLAwg), principal component analysis (PCA), and hierarchical-clustering (HCR) (Xia and Wishart, 2011), which unambiguously indicated one out of four of the biological replicates in the primary metabolome detection as an outlier which was therefore excluded from all subsequent analyses. Potential batch effects attributed to sample degradation and/or instrumentation platform differences were evaluated and adjusted using the RUV-R method (Livera et al., 2015). For determination of DAM, a limma-based linear modelling algorithm fitted with moderate statistics (simple Bayesian model) developed by (Livera and Bowne, 2014) was adopted to construct the basic and interaction GLM contrasts required for determination of DAM. MBROLE (v.2.0) with use of the full database as reference set, but selected only the functional roles that are non-ambiguous and can be found in the *Plantae*, were utilized to detect the enrichment of metabolite sets of each list of DAM (López-Ibáñez et al., 2016).

Integrated Pathway Analysis

To integrate the omics datasets at the pathway level, coding sequences of DEGs identified from the differential analyses of the twelve transcriptomes upon salt treatment were translated and BLASTx searched against the *Arabidopsis* genome release (TAIR10, version: Jun 2016) and KEGG pathway repository (version: May 2017). Only matches with E-value < $1.00E^{-4}$ (or smallest possible E-value in the case of multiple hits for the same gene) against either or both databases were retained and corresponding K numbers in the KEGG repository were fetched for the subsequent integration step. For primary metabolites, the C numbers of DAMs detected in each LC/GC-MS-based quantification were determined by comparison of their chemical structures, formulae, molecular weights, and/or IUPAC nomenclatures between the reference standards used and the KEGG compound repository (May 2017). KEGG mapping of the K and C numbers acquired was performed against the pathway repository of *Arabidopsis thaliana*, which is the most comprehensive and representative pathway collection among all plant species within the KEGG database, following the procedures as stated previously (Aoki and Kanehisa, 2005). Generic outputs from the KEGG mapper (including: ath01100 Metabolic pathways, ath01110 Biosynthesis of secondary metabolites) were defined as outputs from the KEGG mapper common to any kind of inputs and were therefore excluded from the ranking process. Only pathways statistically enriched in terms of GO categories (as determined by BiNGO) and

of metabolite sets (as determined by MBROLE2) were ranked in descending order according to the number of significant DEG and DAM matches.

Correlation Network

Abundance matrices of the total population of DEG and DAM from each barley genotype were concatenated as individual inputs for the Weighted Correlation Network Analysis (WGCNA). Processing of the matrices and network comparisons were performed as described (Langfelder and Horvath, 2008). In brief, matrices were evaluated for missing value using the goodSamplesGenes function and any outliers were determined by hierarchical clustering. Scale-free topology and mean connectivity of each network were plotted against the soft thresholding power to derive the optimal adjacency or dissimilarity. Two coexpression correlation networks (also known as hierarchical clustering of transcript and metabolite abundance) specific to Clipper and Sahara were built based on dissimilarity-based topological overlap matrix (TOM). Modules of each network were defined by dynamicTreeCut and modules unique to each network were determined the matchModules function. Comparability of the two matrices was confirmed by verifying the correlation of ranked expression and ranked connectivity between the two datasets. Module preservation between the independent coexpression-correlation networks of Clipper (as 'reference' set) and Sahara (as 'test' set) were calculated by the 'modulePreservation' function of the WGCNA package v1.61, which outputted the 'Zsummary.pres' value for each module based on preservation-statistics and module quality-statistics (including quality, preservation, accuracy, reference separability, and test separability). $Z>10$ (including modules brown, turquoise, yellow, blue, greenyellow, and green), $5<Z<10$ (including black, purple, red, cyan, pink), and $Z<5$ (including magenta, tan, salmon) indicate high preservation, moderate preservation, and low preservation or modules with significant contrast, respectively. Modules with Z-score <10, excluding module 'tan', which was determined as noise, are defined as weakly preserved modules or modules with significant contrast between the two barley genotypes. Parallel plots for showing either positive or negative correlation of different abundance clusters (within 99^{th} percentile) were generated using the ggplot package of R software. The most representative trend or centroid of each module represented by purple solid lines was determined by k-mean clustering (distance method: Pearson) with optimal number of clusters calculated using the within-group sum of square method (Madsen and Browning, 2009). Module memberships (kME) of genes and

metabolites harboured among module or cluster unique to either network or significantly different to the other network were calculated by signedKME function of the WGCNA package.

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Supplemental Table 1. Quantitative Assessment of Module Preservation Between the Genotype-specific Correlation Networks.

Module	Size of Modules	Zsummary.pres
brown	381	26.0347534
turquoise	400	21.5008584
yellow	330	21.2736166
blue	400	18.3925979
grey	400	14.6007575
greenyellow	176	11.6411528
green	243	10.4537462
black	173	9.0217124
gold	100	8.6063239
purple	126	8.2068145
red	222	6.7381024
cyan	59	5.9966218
pink	136	5.6553828
magenta	184	2.9000761
tan	33	1.0179650
salmon	106	0.8221007

The grey module contains uncharacterized genes and the gold module contains random genes as determined in a permutation test with 30 permutations. Zsummary.pres represents the Z-score summary statistics of module preservation. In general, the higher the value of “Zsummary.pres”, the more preserved the module is between the datasets ($Z<5$ suggests low preservation, $5<Z<10$ indicates moderate preservation, and $Z>10$ indicates high preservation). Grey and gold modules contains uncharacterized and random genes respectively.

Supplemental Table 2. Summary of the global coexpression correlation analyses of the two barley genotypes under salinity.

	Clipper			Sahara		
	Biological Process	**Module**	**RMM**	**Biological Process**	**Module**	**RMM**
Z1	- positive modulation of cell division	H	*CYCP4;1, CDC2*	- ROS-scavenging mechanism (suppression) *	I	*APX1*
				- lipase-mediate JA biosynthesis *	G	*DALL1*
				- xyloglucan crosslink-formation *	D	*XTH20*
				- xylan backbone acetylation *	D	*ESK1*
				- pectin acetylation *	D	*PAE7*
				- membrane steroid modulation	H	*CYP51A2*
				- inhibition of cell cycle progression	H	*IBR5*
Z2	- cell elongation	A	*CEL1*	- cell wall-bound peroxidase *	J	*EXPB2*
	- calcium ion homeostasis	J	*GLR2.7*	- suppression of GA biosynthesis and signalling *	B	*CBF3*
	- polyamine transport	K	*AAT1*			
	- toxin catabolism	K	*GSTU18*			
	- cellulose biosynthesis	L	*CESA1, CESA3*			
	- cell wall loosening	L	*EXPA11, B4, A7*			
	- brassinosteroid biosynthesis	L	*DET2*			
		M	*STE1*			
	- regulation of SA biosynthesis	L	*TCP8, 15, 23*			
	- ROS-scavenging mechanism	I	*APX1*			
	- crosslinking of xyloglucan	D	*XTH20*			
	- xylan backbone acetylation	D	*ESK1*			
	- pectin acetylation	D	*PAE7*			
	- pectin biosynthesis	M	*GAE*			
	- lipid transport and proteolysis	M	*LTP-HyPRP*			
Z3	- cell wall organization	N	*XTH13*	- cell wall-bound peroxidase *	N	*EXPA2, A13, B2*
	- seed oil body formation	N	*DSEL, OLE1*	- suppression of GA biosynthesis and signalling *	B	*CBF3*
	- glucosinolate hydrolysis	B	*MBP1*			
	- nicotianamine biosynthesis	O	*NAS3, NAS4*	- lipase-mediated JA biosynthesis *	G	*DALL1*
				- ROS-scavenging mechanism (suppression) *	I	*APX1*
				- xyloglucan crosslink-formation *	D	*XTH20*
				- xylan backbone acetylation *	D	*ESK1*
				- pectin acetylation *	D	*PAE7*
AZ	- hydrolysis of glucosides	I	*GH*	- biosynthesis of glycine betaine	C	*PEAMT, ADH10A8*
				- modulation of GA signalling	C	*GID1C*
				- suppression of callose deposition	C	*AGD2*
				- glucomannan galactosylation	D	*MUCI10*
				- LTP-mediated defense/ salt tolerance response	E	*DIR1, AZI1*
				- salinity-/ ethylene-responsive	E	*PR-4*
				- ethylene-/ ROS-responsive	E	*RAP2.11*
				- suppression of defense amplification	F	*SOX*

Only biological processes with modular members being induced or remained at higher abundance after the salt treatment in different root zones of the two barley genotypes are listed. Asterisks indicate biological processes that shared between two root zones. AZ, all zones (including zone 1, 2, and 3); GA, gibberellin; JA, jasmonic acid; LTP, lipid transfer protein; RMM, representative modular member(s); ROS, reactive oxygen species; SA, salicylic acid; Z1, zone 1 (meristematic); Z2, zone 2 (elongation); Z3, zone 3 (maturation).