Canadian Science Publishing
NRC Research Press journals | Publishing Services

Genome / Génome

Transcript analysis in two alfalfa salt tolerance selected breeding populations relative to a non-tolerant population

Journal:	*Genome*
Manuscript ID	gen-2016-0111.R1
Manuscript Type:	Article
Date Submitted by the Author:	26-Jul-2016
Complete List of Authors:	Gruber, Margaret; Saskatoon Research Centre Xia, Jianguo; McGill University, Department of Animal Science Yu, Min; Agriculture and Agri-Food Canada Steppuhn, Harold ; Saskatoon Research Centre Wall, Ken; Semiarid Prairie Agricultural Research Centre Messer, Duaine; Swift Current Research and Development Centre Sharpe, Andrew; 2NRC Plant Biotechnology Institute Acharya, Surya; Agriculture and Agri-Food Canada Lethbridge Research Centre, Sustainable Production Systems Wishart, David; University of Alberta, Departments of Computing Science and Biological Sciences Johnson, David; Alforex Seeds Miller, Don; Alforex Seeds Taheri, Ali; Saskatoon Research Centre, Molecular Genomics; Tennessee State University, Agricultural and Environmental Sciences
Keyword:	Alfalfa, RNA-seq, Salinity

Transcript analysis in two alfalfa salt tolerance selected breeding populations relative to a non-tolerant population.

[1,2,14]Gruber MY, [2,3,4]Xia J, [1]Yu M, [5,13]Steppuhn H, [5]Wall K., [5]Messer D, [6,7]Sharpe A, [8]Acharya SN, [2,9]Wishart DS, [10, 11]Johnson D, and [12]Miller DR, and [1,13*]Taheri A,
[1]Saskatoon Research Centre, Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon, Saskatchewan, S7J 0X2, Canada.
[2]Department of Computing Science, University of Alberta, 2-21 Athabasca Hall, Edmonton, Alberta, T6G 2R3, Canada.
[3]new address: Institute of Parasitology, McGill University, Macdonald Campus, 21111 Lakeshore Road, Sainte-Anne-de-Bellevue, Quebec, H9X 3V9, Canada
[4]new address: Department of Animal Science, McGill University, Macdonald Campus, 21111 Lakeshore Road, Sainte-Anne-de-Bellevue, Quebec, H9X 3V9, Canada
[5]Semiarid Prairie Agricultural Research Centre, Agriculture and Agri-Food Canada, PO Box 1030, Swift Current, SK, S9H 3X2, Canada
[6]National Research Council, 110 Gymnasium Pl, Saskatoon, SK, S7N 0W9, Canada.
[7]new address: Global Institute for Food Security, 110 Gymnasium Pl, Saskatoon, SK, S7N 0W9, Canada.
[8]AAFC Lethbridge Research Centre, Agriculture and Agri-Food Canada, 5403 - 1st Avenue S., Lethbridge, AB, T1J 4B1, Canada.
[9]Department of Biological Sciences, University of Alberta, 11455 Saskatchewan Drive, Edmonton, Alberta, T6G 2R3, Canada
[10]Former address: Alforex Seeds, an affiliate of Dow AgroSciences, N4505 CTH M, West Salem, WI, 54669, USA.
[11]New address: 3640 Old Vineyard Rd. La Crosse, WI 54601, USA
[12]Alforex Seeds, an affiliate of Dow AgroSciences, Longbranch Station, Suite 203, 16 12th Ave. S, Nampa, ID, 83651, USA.
[13]New address: Department of Agricultural and Environmental Sciences, Tennessee State University, 3500 John A Merritt Blvd., Nashville, Tennessee, 37209, USA
[14]Retired.
*corresponding author:
Tel: +1 615-963-6056
Email: Ataheri1@tnstate.edu

Abstract

With the growing limitations on arable land, alfalfa (a widely cultivated, low-input forage) is now being selected to extend cultivation into saline lands for low-cost biofeedstock purposes. Here, minerals and transcriptome profiles were compared between two new salinity-tolerant North American alfalfa breeding populations and a more salinity-sensitive Western Canadian alfalfa population grown under hydroponic saline conditions. All three populations accumulated two-fold higher sodium in roots than shoots as a function of increased electrical conductivity. At least 50% of differentially expressed genes ($p < 0.05$) were down-regulated in the salt-sensitive population growing under high salinity, while remained unchanged in the saline-tolerant populations. In particular, most reduction in transcript levels in the salt-sensitive population were observed in genes specifying cell wall structural components, lipids, secondary metabolism, auxin and ethylene hormones, development, transport, signalling, heat shock, proteolysis, pathogenesis-response, abiotic stress, RNA processing, and protein metabolism. Transcript diversity for transcription factors, protein modification, and protein degradation genes was also more strongly affected in salt-tolerant CW064027 than in salt-tolerant Bridgeview and salt-sensitive Rangelander, while both saline-tolerant populations showed more substantial up-regulation in redox-related genes and B-ZIP transcripts. The report highlights the first use of bulked genotypes as replicated samples to compare the transcriptomes of obligate out-cross breeding populations in alfalfa.

Key words: Salinity, Alfalfa, RNA-Seq, Transcriptome, Abiotic stress,

Introduction

Salinity is of great concern in arid and semi-arid regions of the world, where soil salt concentration is often excessive, and precipitation is insufficient for leaching. Historic assessments of the distribution of world saline lands (3.23M km² on the FAO/UNESCO Soil Map of the World) showed 84.7M ha in Australia, 69.5M ha in Africa, 59.4M ha in Latin America, 53.1M ha in the Near/Middle East, 20.7 M ha in Europe, and 19.7M ha in Asia and the far East (Brinkman, 1980). In some areas, land has become salinized due to the replacement of native vegetation with shallow-rooted crops or pastures and adoption of European farming methods (*http://www.environment.gov.au/land/pressures/salinity/index.html*). In Australia, salinization was expected to rise over 40 years (Clark et al., 2002). Within the Great North American Prairies, root-zone salinity exceeds 10M ha in the United States (personal communications, North Dakota State Office, Natural Resources Conservation Service, USDA 2011), with saline land extending from the Montana prairies down into California and the American deserts through into Mexico. Moreover, 20M ha (30%) of total Canadian prairie lands are also extensively salinized (Steppuhn, 1996; Wiebe et al., 2007).

Alfalfa (*Medicago sativa*) is the world's most predominant and valued forage, due to its high protein content and N fixation capability. It is also being considered for its potential as a low-input biofuel crop due to its long tap root (to find water) and low fertilizer inputs, among other features. The North American prairie has large areas of alfalfa production in Canada (*http://www.fao.org/ag/agp/AGPC/doc/Counprof/Canada/Canada.html*) and the USA, the latter of which is the world's highest alfalfa producer for export (*http://alfalfa.ucdavis.edu/IrrigatedAlfalfa/pdfs/UCAlfalfa8287ProdSystems_free.pdf*). Recently, interest in developing (and understanding the basis of) salt-tolerant perennial legumes (such as alfalfa) has risen, since they have the potential to play a large role in improving degraded ecosystems. They could also expand the land base available for cultivating low-input biomass feedstocks for livestock and biofuel.

Alfalfa has been the subject of a number of biochemical and genomics studies to begin uncovering mechanisms that may provide tolerance to elevated salt levels. These studies include the development of a suppressive subtractive alfalfa seedling cDNA library (Jin et al., 2010) and the ensuing discovery of several useful genes (Chen et al., 2012; Chao et al., 2013), a salinity-responsive alfalfa root cDNA library (Postikovno et al., 2013), and proteomic analysis of germinating osmo-primed alfalfa seeds (Yacoubi et al., 2013). Alfalfa salinity research is also

benefiting from salinity transcriptome and metabolome studies in the related model legume *M. truncatula* (Li et al., 2011; Bell et al., 2001). Birdsfood trefoil legume forage (*Lotus corniculatus*), its related species *L. tenuis*, the model legume *L. japonicus*, and the extremophile *L. creticus*, have also been the subject of metabolome, ionome, and transcriptome investigations (Sanchez et al., 2008a,b; 2011).

Each of these legume studies above has utilized NaCl, while the North America drylands mainly feature alkaline soils laden with Na_2SO_4 and $MgSO_4$ interspersed with other ions or minerals (said references; and Daniels, 1987; Steppuhn, 2012). Conifers have been treated with sulphate-based and chloride-based sodium solutions (Nguyen et al., 2006), and metabolite profiles have been compared in lettuce seedlings treated with either NaCl or Na_2SO_4 (Mahmoudi et al., 2010).

Earlier, we compared germination rate, seedling vigor, and young and mature forage yields between eight salinity-selected (tolerant) alfalfa breeding populations from Canada and the USA and Rangelander (a more salinity-sensitive dormant Western Canadian prairie cultivar) using hydroponic ion cocktails that mimic the saline seeps found in the North American Great Plains and Canadian Prairies (Steppuhn et al., 2012). At this time, we present the mineral profiles and transcriptomes of two of these salt-tolerant breeding populations (CW064027 and Bridgeview) to determine patterns that distinguish them from each other and from a more salt-sensitive population, Rangelander. The report highlights stress-related transcripts and mineral profiles that likely contribute to greater plant health (and therefore greater biomass) under salt stress in specific salinity selected populations.

Materials and Methods

Alfalfa populations and sample tissue preparation:

Alfalfa forage samples were collected in July, 2009 from the final re-growth harvest (4th cut) of three alfalfa populations (two salinity-tolerant selected populations and one non-selected more salt sensitive population), which had been grown together with other populations (as a part of a larger population assessment) in hydroponic sand tanks situated in a greenhouse facility at the Canadian Salt Lab at Swift Current, SK, Canada (Figure 1A,B). Growth and salinity test conditions were set-up as published in Steppuhn et al. (2012), in which seeds for each

population had been placed 13 mm deep into the sand bed and positioned 40 mm apart within and between the rows, resulting in 641 seed m^{-2}. Upon completion of emergence (after 1 month), the surviving plants had been thinned to 25 genotypes per tank quadrant. Each tank held three randomly chosen salinity-tolerant populations (i.e., three quadrants) and the remaining quadrant (randomized for within-tank position) held 25 genotypes of a more salt-sensitive AC Rangelander population (Figure 1C). Quadrants were designated using small plastic sticks, and three electrical conductivity (EC) test levels formulated together with hydroponic growth solutions were used to irrigate the tanks: 1.53 dSm^{-1} as the no-salt control solution, and 8.03 and 15.61 dSm^{-1} as salt-supplemented solutions containing increased concentrations of sulphate-based sodium, calcium, and magnesium salts (composition fully defined in Steppuhn et al., 2012). Each EC level was replicated three times, and the two salinity-tolerant populations were replicated four times among the tanks, such that a total of 100 genotypes per salt-tolerant population were in the experiment. This covered the entire genotypic variation within each outcrossed alfalfa population at each EC level, and the salt-sensitive Rangelander genotypes were included in every tank as a population and tank control (Figure 1D). Since growth was retarded in all populations irrigated at 15.61 dSm^{-1}, high-EC tanks were also positioned at an outer edge so that small plants would not be over-shadowed by vigorously growing plants at the other EC levels (Figure 1D). Sand tanks were irrigated 4 times (for 5 min each) over a 24 h cycle with the hydroponic solutions. Harvest date was determined when the 4th-regrowth forage was at 10% flowering in populations growing at 1.53 dSm^{-1}.

Populations tested in this report included: (A) Rangelander (non-selected dormant-type, western Canadian cultivar as a more salt-sensitive population control); (C) Bridgeview (Canadian variety, selected and described in Acharya and Steppuhn, 2012); (C) and (F) CW064027 (Alforex Seeds, USA, selected for germination, seedling, and mature plant tolerance to NaCl). Each of these populations was positioned within the hydroponic tanks as illustrated in Figure 1D).

Forage shoots (4th-cut mixed leaves and stems) for ionome and nutritional analysis were harvested at 3 cm above the sand bed and processed into samples after placing the fresh shoots into paper bags. All shoots per genotype (but one shoot saved on dry ice for transcriptome analysis) were bulked from 25 genotypes in one rep-bag); then each bag was dried at ~40^{o} C for 7 days in a forced air drying oven. This drying process aimed to simulate hot summer outdoor drying conditions normally in place for field-grown harvested alfalfa hay. Dried

forage (shoots) was then ground in a Wiley mill (until it passed through a 1 mm sieve), shaken to achieve uniformity, and dispensed as dry evenly-mixed powder in covered jars. Powder portions were then submitted to the Swift Current Analytical Lab (Agriculture and Agri-Food Canada) for ion and mineral analysis. Biomass was not taken on the 4th cut, having been measured in 3 previous harvests and published in Table 7 of Steppuhn et al. (2012). Root samples were processed similarly to shoots, but roots were first thoroughly and carefully washed to remove sand particles and then sub-sampled in small quantities to avoid over-sampling, so that individual population genotypes could be returned live (as contracted) to their plant breeders for further alfalfa population development.

Ionome Analysis on Shoots and Roots

Na was analyzed by Atomic Absorption Spectroscopy with flame atomization on a Hitachi polarized Zeeman Z8200 spectrometer using selective wavelengths. S, Fe, Mn, Ca, Mg, and Zn were analyzed in perchloric acid digests in a Questron Technologies inductively coupled plasma spectrophotometer (Hitachi, 1987; Messer, 2008). Although statistically significant means (n=4; $p<0.05$) were detected for concentrations of 11 ions and minerals, higher variability (standard deviations) was discovered for a number of ions and minerals ((Supplementary Table S1). The sources of these higher deviations are not understood in all cases, since all analytical methods were conducted on the same samples sourced from the same tanks, and the tissues from 25 genotypes per rep were carefully ground and mixed for each sample. Likely, the high variability in several samples for Fe resulted from metal contamination arising from the sample grinder, since this contamination was found across most of the sample reps.

Initial principal component (PC) analysis across the entire suite of analyses accounted for 4 factors: Electrical Conductivity (EC) (3 levels of salinity @1.53, 8, 15.6 dSm^{-1}), population (called cultivar; over 6 alfalfa populations), Tank, and Tank Location. Several statistical methods were used to test the rigor of the data and to explore different data normalization methods. Tank and Tank Location showed no significant differences of the means at $p<0.05$ and did not contribute to the overall profiles; hence, we ignored these factors in the final analysis. Log & glog normalizations were similar and equally suitable, since all were positive numbers (data not shown). Autoscaling and Pareto scaling also showed similar effects based on PCA cluster patterns with regard to the two main effects (EC and population).

Transcriptome analysis using RNA sequencing: Fresh-frozen forage was selected for RNA sequencing from the two salinity tolerant populations (Bridgeview and CW064027) and the non-selected more salinity-sensitive control population var. Rangelander. Fourth-cut forage harvested from three reps (25 unique genotypes within each rep) for each of the three EC levels were sub-sampled for the transcriptome analysis by selecting and freezing one entire shoot per genotype. The 25 frozen shoots (representing 24 genotypes) were frozen on dry ice in one Ziploc rep-bag (as one rep per population and EC level) and stored at -80^{o} C. Traditional transcriptome experiments use replicates comprised of tissues from individual genetically pure plant lines or from one individual genotype, but obligate out-cross pollinating breeding populations, such as alfalfa, are composed of many individual sibling genotypes derived from multiple intercross-pollinated parents (rather than genetically pure lines). Hence, we chose 100 individual genotypes (divided into four independent reps) to represent each alfalfa population at one EC level (and tested 300 genotypes across the three EC levels). This limited the analysis costs while maintaining sufficient replication (4 reps) to cover the entire within-population genotypic variation. Each of the frozen bulked 25 shoots per rep-bag was then sub-sampled on dry ice from the shoot top so that leaves and stem lengths were equally represented within a frozen bulked shoot sub-sample (as one biological rep) for RNA extraction; then each frozen sub-sample was finely ground (and mixed) in liquid N_2. Bulked sampling was also successfully used to assess amplified fragment length polymorphism in alfalfa across multiple populations (Segovia-Lerma et al., 2003; Keivanil et al., 2010). RNA extractions were conducted using a standard procedure and then developed into 27 cDNA libraries (i.e. 3 populations x 3 EC levels x 3 biological reps for each population-EC treatment) using an Illumina mRNA sequencing kit (Illumina, CA, USA). The libraries were constructed with a paired-end method; then sequencing was conducted from both ends on an Illumina Genome Analyzer IIx in Dr. Andrew Sharpe's Laboratory at the National Research Council, Saskatoon, SK, Canada. Between two to four million short reads with read length of 80 was generated for each sample.

Transcriptome Bioinformatics: Two publicly available alfalfa EST databases (one comprised of 60,290 ESTs described in Postinkova et al., 2013 and the other comprised of 124,025 ESTs in Alfalfa Gene Index 1.0, (Yang, 2011) were combined and duplicate transcripts removed using the CD-HIT suite (Ying et al., 2010) to create an assembled alfalfa reference EST database (comprised of 136,281 ESTs). The alfalfa RNAseq data was trimmed and mapped to the combined EST database using TopHat software from Tuxedo suite (Trapnell et al., 2010); then ESTs with the closest identity to each read were used to query the closest model genome (from

M. truncatula) for functional annotation of each EST. This mapping strategy allowed for only ~30% annotation of the alfalfa reads, but was unavoidable due to the lack of an alfalfa genome. The aligned RNAseq reads were then assembled into transcripts and their relative abundance estimated using the Cufflinks package (Tuxedo suite). Statistical analysis (using four biological RNA reps per population and EC level described above) was then conducted using a t-test with side-by-side treatment comparisons of transcript intensity for each individual gene per population and EC level (treatment 1) relative to the population control (Rangelander) grown at the no-salt control level (1.53 dSm^{-1}) (treatment 2) using the CuffDiff package in Cufflinks (Tuxedo suite). Relative expression data without significantly different means ($p<0.05$) were filtered out; then the remaining statistically significant data was organized into 35 metabolism categories (Bins) by importing the differentially expressed ESTs into MapMan software (Thimm et al. 2004). Since some of the transcripts had no known function, MapMan could not map some of these transcripts into functional metabolism categories. For example at the 15.6 dSm^{-1} EC level, Mapman could map 2380 out of 2948 Bridgeview transcripts (80%) and 2338 out of 2985 CW064027 transcripts (78%), but only 4626 out of 6020 Rangelander transcripts (77%). Single nucleotide polymorphism (SNP) analysis was not conducted due to the lack of an alfalfa genome and polyploid level of this species, which made SNP calling a challenging task using an EST database as a reference. *De novo* assembly of these RNA seq data using software programs such as Trinity or Oases were also avoided due to the polyploid nature of alfalfa and the short read-length fragments (100 bp) obtained in our analyses. In hexaploid wheat, homeologous transcripts were shown to collapse into undesirable chimeric contigs using Velvet/Oases (60-80% chimeric sequences) or Trinity (50% chimeric sequences) software (Schreiber et al. 2012). Our short RNA fragments would not be mapped to such chimeric contigs and would ultimately result in an even greater increase in unmapped sequences.

The generated reads and de novo assemblies were deposited into Gene Expression Omnibus at NCBI with accession number GSE84825.

Results

Alfalfa ions and minerals:

Forage ions and minerals that would impact on livestock health, productivity, and product quality were assessed to determine population differences and whether these feed components changed as a function of increased salinity. Ions were also evaluated to determine how salinity

affected ion distribution between shoots and roots. Drying methods were not expected to impact ion concentrations compared with fresh frozen tissues. PC analysis indicated that EC (i.e., salinity) had the largest effect (PC1 38.5%) on forage ions and minerals, with population ranking as a 2nd somewhat minor component (PC2 19.5%) (Supplementary Figure S1). Cluster analysis indicated three patterns (data not shown; Supplementary Table S1): Cluster1 (forage NO_3-N, total K, and total P): high at EC 1.53 dSm^{-2} and 8 dSm^{-2}, with a decrease at EC 15.6 $dS.m^{-2}$; Cluster 2 (Mn, Ca, and Mg): higher at EC 1.53 dSm^{-2}, with a decrease at EC 8 dSm^{-2} and 15.6 dSm^{-2}; Cluster 3 (Zn, Na, S, total Kjehldal N, and Fe): relatively low at EC 1.53 dSm^{-2}, with an increase at EC 8 and 15.6 dSm^{-2}. Alfalfa populations (or cultivars) showing the highest concentrations of individual forage ions and minerals did not substantially change from these three main clustering patterns (Supplementary Table S1). All three populations accumulated sodium and zinc in a linear pattern in their forage, although Na was accumulated at 100-fold higher than Zn (Fig. 2). Variation between populations was particularly noticeable in forage Mg, Zn, S, and Fe (Fig. 2; Supplementary Table S1). Principal component 1 (salinity) and 2 (population) were even higher for roots (50.3% and 30.5%, respectively) than for forage (38.5% and 19.5%, respectively) (Supplementary Figure S1). Total accumulated Na was 2-fold higher in roots than in forage for all populations (Fig. 2). The four ions mentioned above, plus Mn, also accumulated at higher levels in roots, although Fe and Mg levels dipped at the moderate conductivity level and then rose to their original levels (Fig. 3; Supplementary Table S1). These data indicated that the differences for salinity and population were more pronounced in roots than in shoots.

Overall Impact of salinity and population on the forage transcriptome:

The global forage transcriptomes of the two salt-tolerant alfalfa populations yielding high biomass at 15.6 dSm^{-1} (CW064027 from the USA and Bridgeview from Canada) were compared with the more salt-sensitive low-yielding control population Rangelander. Mapman analysis of RNAseq transcripts at 1.53, 8.0 and 15.6 dSm^{-1} gave us a broad scope (34 metabolism categories) within which to view the overall expression responses within forage. Both salinity-tolerant populations showed raised numbers of changed transcripts at the no-salt level (i.e., EC 1.53 dSm^{-1}), with CW064027 having greater differences (527 ESTs down-regulated and 685 up-regulated) than Bridgeview (139 ESTs down-regulated and 368 up-regulated) relative to salt-sensitive Rangelander at the same salt level (Supplementary Figures S2 and S3). At 8.0 dSm^{-1} (i.e., modest salt supplementation), salt-sensitive Rangelander transcript numbers responded

with an increase in transcript diversity (342 down-regulated and 501 up-regulated relative to Rangelander at the No Salt level) (Supplementary Figure S4). However, this response was much less than the response by salt-tolerant Bridgeview (1045 ESTs down-regulated and 375 up-regulated) and salt-tolerant CW064027 (949 ESTs down-regulated and 537 up-regulated) (Supplementary S5 and S6).

At the high salt level (15.6 dSm^{-1}) the pattern of response between the three populations changed dramatically. The intensity and diversity of gene response was much stronger and completely reversed compared with the 8.0 dSm^{-1} pattern, with 10 major categories of changed metabolic transcripts for 15.6 dSm^{-1} (listed in Supplementary Table S2 worksheets). Here, salt-sensitive Rangelander shoot response was very extreme in 10 of the 34 MapMan metabolism categories (3357 ESTs down-regulated and 1255 up-regulated) relative to Rangelander at 1.53 dSm^{-1} (Figure 4; Supplementary Figure 7). Transcript levels for categories 10 (cell wall), 11 (lipid metabolism), 16 (secondary metabolism), 17 (hormone metabolism), 31 (cell organization), 33 (development), and 34 (transport) were more modestly affected, in Rangelander at this high EC level, while transcripts for categories 20 (stress), 26 (miscellaneous), 27 (RNA processing), 29 (protein metabolism), 30 (signalling), and 35 (unknown/not assigned) were extremely affected, mainly showing down-regulated ESTs. In contrast, salt-tolerant Bridgeview and salt-tolerant CW064027 populations were 50% less affected in shoot transcript diversity and intensity at 15.6 dSm^{-1} (1516 ESTs and 1196 down-regulated and 843 and 1129 up-regulated, respectively) within the same categories affected in Rangelander at the same high salt level (Figure 4; Supplementary Figures S8; S9).

Hormone and Stress-related Genes

The forage transcriptome for salt-tolerant Bridgeview and salt tolerant CW064027 at 15.6 dSm^{-1} also showed reduced numbers of changed transcripts coding for auxin and ethylene genes, heat shock, proteolysis, and-pathogenesis responsive genes compared with Rangelander (Supplementary Table S1, worksheets 6,7,10), while the numbers of up-regulated abiotic stress-related genes (worksheet 6) in the two salt tolerant populations was limited to only 1/3 of those up-regulated in salt-sensitive Rangelander at the high salinity level (Figure 5). This huge number of Rangelander stress-responsive transcripts were mainly down-regulated (as were Bridgeview stress-responsive genes), while CW064027 genes were frequently up-regulated. Here, stress signalling transcript changes were also 1/2-1/3 lower in salt-tolerant CW064027 (391) and salt-tolerant Bridgeview (210) compared with salt-sensitive Rangelander (643) (data

not shown). Moreover, redox-related genes were more substantially down-regulated in Rangelander compared with CW064027 and Bridgeview, but the overall numbers of changed redox genes in all three populations was relatively low even at the highest EC level compared with other gene classes (Figure 5). In contrast, the stress transcriptome at 8.0 dSm^{-1} was more down-regulated in the two salt-tolerant populations compared with Rangelander at the same EC level (Supplementary Figures S10; S11).

Transcription Factors, Protein Modification, and Protein Degradation Genes

Transcript diversity for transcription factors, protein modification factors, and protein degradation factors was strongest in CW064027 than for the other two populations at 8.0 dSm^{-1} and 15.6 dSm^{-1} (Figure 6; Supplementary Figure S12; Supplementary Table S1, worksheets 9,11,13), such that 563 of these transcript types were down-regulated, and 169 were up-regulated. B-ZIP transcripts in CW064027 were much higher, and heat shock protein transcripts were reduced by two-fold (47 ESTs) at 15.6 dSm^{-1} in the latter population. Gene changes specifying secondary metabolism were also observed at 8.0 dSm^{-1} and 15.6 dSm^{-1} for the two salt-tolerant populations in Mapman sub-bins 16-5 (phenylpropanoids), 16-7 (lignin/lignans), 16-8 (chalcones), 16-9 (isoflavonoids), 16-10 (dihydroflavonols), and 16-12 (mevalonic acid) (Figure 7; Supplementary Figure 12, Supplementary Table S1, worksheet 5), although similar changes in phenylpropanoids, lignan/lignins, and dihydroflavonols were also seen in Rangelander tissues at the high EC (salinity) level. Less dramatic increases in numbers of dihydroflavonol ESTs (sub-bin 16-10) and carotenoid ESTs (16-14) were also noted all three populations.

Transport Genes

Since forage ion contents were changed in the salt-tolerant alfalfa populations, we evaluated the transcript diversity and intensity of ion transport genes in addition to a host of other transport genes. Transcripts representing Ca-related channel genes, a sodium symporter, nitrate and K+ channels, a P translocator, and four other metal transporters were strongly decreased or undetectable at 15.6 dSm^{-1} in salt-sensitive Rangelander, but maintained at the no-salt level in salt-tolerant Bridgeview and CW064027 (Table 1; Supplementary Table S1, worksheet 15). This correlated with changes for these ions in the three populations. Moreover, salt-sensitive Rangelander showed strong increases in two sulphate transporters (Table 1).

Since Bridgeview and CW064027 also showed greater biomass at 15.6 dSm^{-1} than Rangelander at the high EC level relative to Rangelander at the No Salt level, we also examined

cell wall gene expression profiles in more detail. Here, transcripts for cellulose synthases, ligins, cell wall proteins, cell wall degradation genes specifying mannose, xylan, arabinose, and fucose, as well as pectate lyases, polygalacturonases, and pectin esterases, and other miscellaneous carbohydrate hydrolases and transferases were either maintained or up-regulated in Bridgeview and CW064027 at the high EC level, while transcripts for these same genes were completely undetectable in Rangelander under the high salt level (Tables 5,6; Supplementary Table S1, worksheets 3,8; Figure 6, sub-bin 8). Transport transcripts specifying amino acids, sugars, and oligopeptides were also substantially reduced in Rangelander at the high EC level (Table 1; Supplementary Table S1, worksheet 15).

Discussion

Excessive salinity causes both osmotic stress and ion toxicity, disrupting the integrity of cellular membranes, chloroplast function and photosynthesis, the up-take and transport of other ions, activities of various enzymes, and many other processes that affect growth and development in plants (Parida and Das, 2005; Gupta and Huang, 2014). Plant stress also causes the production of reactive oxygen species (ROS) (Dixon and Paiva, 1995; Hernandez et al., 2001), which results in additional tissue injury (Türkan and Demiral, 2009). A robust defense system is critical for plant survival under high saline conditions. This includes Na^+ exclusion or vacuolor uptake (Hauser and Horie, 2010), an effective antioxidant system, reduction of cellular osmotic potential by net accumulation and transport of inorganic ions (particularly K^+ and Ca^{2+}), as well as accumulation of compatible osmoprotecting solutes (Munns and Tester, 2008; Türkan and Demiral, 2009). It also includes specific hormone increases (abscisic acid, jasmonate, salicylic acid, and brassinosteroids) to allow water uptake for cell turgor maintenance, stomatal opening, photosynthesis and cell expansion, as well as a signaling mechanism featuring an increase in intracellular Ca^{+2} (which protects cell membranes) and induction of three salt overly sensitive (SOS) proteins (which include an Na+/H+ antiporter that increases Na+ efflux and restores ion homeostasis) (Tester and Davenport, 2003; Munns and Tester, 2008; Parida and Das, 2005; Gupta and Huang, 2014).

In spite of the widely diverse genetic sources within the three alfalfa breeding populations, our data clearly showed that CW064027 and Bridgeview (both salt-tolerant) and Rangelander (salt-sensitive) handled salinity similarly by accumulating sodium at a two-fold higher level in their roots compared to their shoots. All three populations also accumulated greater amounts of or did not change levels of Zn, S, Fe, and Mg in their shoots with increased salinity, whereas roots

accumulated a greater amount of these minerals in addition to maintaining greater amounts of Mn than shoots. In contrast, transcript levels for transport genes specifying many of these ions declined dramatically in salt-sensitive Rangelander at the high EC level compared to the two salt-tolerant tester populations, suggesting that Rangelander may be less capable or has a different mechanism of managing ion changes than the two salt-tolerant populations.

Transcripts specifying the transport of sugars, amino acids, and oligopeptides were maintained at the No Salt level for Bridgeview and CW064027growing under the high EC level, whereas these types of transcripts strongly decreased in salt-sensitive Rangelander. This suggests that transport genes in these two salt-tolerant populations were less sensitive to high electrical conductivity than salt-sensitive Rangelander. Unfortunately, the lack of an alfalfa genome standard and the depth of RNA-seq data used precluded the evaluation of SNPS at this time.

In an earlier report, alfalfa root amino acids were shown to increase (but free organic acids and free sugars decrease) when 100-150 mM NaCl was applied to salt-selected alfalfa, while roots from unselected alfalfa cv. Europe showed strong changes in lactate, asparagine, proline, and sucrose (Fougere et al., 1991). Moreover, proline accumulation was higher in a more saline-sensitive alfalfa cv. Defi compared with more salt-tolerant cv. Zhongmu 1 treated with NaCl, which suggested that proline may be be an osmoprotective signal in response to salinity in alfalfa (Wang and Han, 2009). Salinity tolerance in NaCl-treated Zhongmu 1 also included increased seedling antioxidant enzyme activity (superoxide dismutase, catalase, and polyphenol oxidase) and less malonaldehyde accumulation than in saline-susceptible cv. Defi seedlings (Wang and Han, 2009), and transcripts for some of these enzymes and processes were altered in our treated populations. Most recently, shoot N, P, Mg, and S were increased and total antioxidant levels were maintained in four non-dormant commercial alfalfa cultivars tested with up to 30 dS^{-1} in chloride/sulphate-based saline solutions (Ferriera et al., 2015). Although these reports measured compounds instead of transcripts, they show the diversity of responses to salinity within other alfalfa populations from germplasm sources which differed from ours.

The changes to forage ion and mineral profiles observed in our alfalfa populations exposed to sulfate salts were modest, but could have an impact on livestock nutrition in the future if any these populations are planted in saline soils surrounding the extreme saline seeps on the North American prairies. Sodium sulphate supplementation in ruminants can improve performance (meat, wool, and milk production) by enhancing bacterial protein synthesis in the rumen and

improving amino acid balance (reviewed in Breytenbach, 2014). Moreover, sodium, zinc, and iron are often lacking in animal diets. Hence, maintaining (or even increasing) these metabolites and ions in alfalfa growing on saline land must be considered if alfalfa forage is expected to provide these necessary nutrients to livestock (Salt Institute, ARS, USDA, 2014). Mineral status in alfalfa (eg. C:N ratios, Mg, N, P, and S concentrations) can also impact on aphid population levels in alfalfa fields (de Almeida e Silva et al., 2005).

The differential changes between the expressed genomes for salt-tolerant CW064027 and Bridgeview populations suggest that a broad spectrum of growth processes could (potentially) be negatively impacted in salt-sensitive Rangelander. These transcript changes point to metabolic processes, suggesting that salt-tolerant CW064027 and Bridgeview populations may be more physiologically and biochemically fit under saline conditions. In fact, cell wall carbohydrate transcripts (which are indicative of growth) were highly depressed in salt-sensitive Rangelander forage at the high EC level compared to the salt-tolerant populations, correlating with growth differences between these populations. Rangelander could potentially have had large reductions in proteins specifying the functional areas arising from these large transcript reductions. Gene expression changes correlating with increased salinity tolerance in *L. creticus* also included large changes in transcription factors, RNA processing, transport, protein modification and degradation, and stress responses (Sanchez et al., 2008).

Conclusion

Two unique alfalfa populations selected for tolerance to NaCl were tested for their tolerance to sulphate-based sodium and magnesium salts prevalent in the North American plains and prairies. These salinity-tolerant populations and the non-selected salt-sensitive Rangelander control appeared to have the same mechanism of handling Na^+, that of accumulating two-fold larger amounts in roots than in shoots. In salt-tolerant Bridgeview and salt-tolerant CW064027, greater tolerance to salinity also correlated with increased transcription of redox (antioxidant) proteins and maintenance of the transcript levels for a broad suite of functions that are down-regulated in salt-sensitive Rangelander at a high EC level. The report highlights the first use of bulked replicated samples to compare the ionomes and transcriptomes of obligate out-crossed populations of alfalfa in a cost-effective manner that includes the entire spectrum of genotypic variation inherent in each individual population. Expansion of the transcriptome analysis to roots and expansion of forage analysis to include metabolome, physico-chemical structural components and additional forage quality parameters are important next steps towards fully

understanding the impact of selection for salt-tolerance on these unique populations. Researchers interested in studying the *M. trunculata* homologues for these alfalfa genes or obtaining partial or full-length alfalfa genetic sequences to confirm function (especially for transcription factors and protein factors) will benefit from the extensive annotation of these identified *M. sativa* genes in Supplementary Table S2.

In this study, we compared salt-treated samples of different populations with the same control, i.e. Rangelander population without salt treatment. Additional information can be revealed by comparing the same population before and after salt treatment.

Acknowledgements

Funding for metabolome and transcriptome analysis was provided by a network grant from the Agricultural Bioproducts Innovation Program of Agriculture and Agri-Food Canada to the Canadian Cellulosic Biofuel Network. Funding for ion and forage nutritional analysis was provided by AAFC A-base grants. Dr. Xiang Li, Ms. Jennifer Holowachuk, and Mr. Wesley Wizniuk are acknowledged for sample collection and processing. We thank Cathy Coutu for preparing gene expression data and uploading them into NCBI database.

Supplementary Files

Supplementary Table S1. Accumulated ions and minerals in forage of three saline-selected alfalfa populations

Supplementary Table S2 (Worksheets 1-15). Lists of individual genes affected by EC 15.6 dSm-1 in salt-tolerant Bridgeview and salt-tolerant CW064027 compared with salt-sensitive Rangelander. Worksheets are derived from MapMan Bins 2 (worksheet 1), 3 (sheet 2),10 (sheet 3), 11 (sheet 4), 16 (sheet 5), 17 (sheet 6), 20 (sheet 7), 26 (sheet 8), 27 (sheet 9), 28 (sheet 10), 29 (sheet 11), 30 (sheet 12), 31 (sheet 13), 33 (sheet 14), and 34 (sheet 15) described in the legend of Figure 3.

Supplementary Figure S1. Principal component analysis for ions and minerals.

Supplementary Figure S2. Heat map (general metabolism) for EST shoot transcriptome changes (after Mapman analysis) for salt-tolerant Bridgeview (at 1.53 dSm^{-1}) relative to salt-sensitive Rangelander (at 1.53 dSm^{-1}) (control).

Supplementary Figure S3. Heat map (general metabolism) for EST shoot transcriptome changes (after Mapman analysis) for salt-tolerant CW064027 (at 1.53 dSm^{-1}) relative to salt-sensitive Rangelander (at 1.53 dSm^{-1}) (control).

Supplementary Figure S4. Heat map (general metabolism) for EST shoot transcriptome changes (after Mapman analysis) for salt-sensitive Rangelander (at 8 dSm^{-1}) relative to Rangelander (at 1.53 dSm^{-1}) (control).

Supplementary Figure S5. Heat map (general metabolism) for EST shoot transcriptome changes (after Mapman analysis) for salt-tolerant Bridgeview (at 8 dSm^{-1}) relative to salt-sensitive Rangelander (at 1.53 dSm^{-1}) (control).

Supplementary Figure S6. Heat map (general metabolism) for EST shoot transcriptome changes (after Mapman analysis) for salt-tolerant CW064027 (at 8 dSm^{-1}) relative to salt-sensitive Rangelander (at 1.53 dSm^{-1}) (control).

Supplementary Figure S7. Heat map (general metabolism) for EST shoot transcriptome changes (after Mapman analysis) for salt-sensitive Rangelander (at 15.6 dSm^{-1}) relative to Rangelander (at 1.53 dSm^{-1}) (control).

Supplementary Figure S8. Heat map (general metabolism) for EST shoot transcriptome changes (after Mapman analysis) for salt-tolerant Bridgeview (at 15.6 dSm^{-1}) relative to salt-sensitive Rangelander (at 1.53 dSm^{-1}) (control).

Supplementary Figure S9. Heat map (general metabolism) for EST shoot transcriptome changes (after Mapman analysis) for salt-tolerant CW064027 (at 15.6 dSm^{-1}) relative to salt-sensitive Rangelander (at 1.53 dSm^{-1}) (control).

Supplementary Figure S10. Shoot stress transcriptome at 8 dSm^{-1} (after Mapman analysis) for salt-tolerant CW064027, salt-tolerant Bridgeview, and salt-sensitive Rangelander relative to Rangelander (at 1.53 dSm^{-1}) (control)

Supplementary Figure S11. Shoot stress transcriptome at 1.53 dSm^{-1} (after Mapman analysis) for salt-tolerant CW064027 and salt-tolerant Bridgeview relative to salt-sensitive Rangelander (at 1.53 dSm^{-1}) (control).

Supplementary Figure S12. Shoot transcriptome at 8 dSm^{-1} for secondary metabolite genes, transcription factors, and protein factors (after Mapman analysis) for salt-tolerant CW064027, salt-tolerant Bridgeview, and salt-sensitive Rangelander relative to Rangelander (at 1.53 dSm^{-1}) control.

References

Acharya, S.N, and Steppuhn, H, 2012. "Bridgeview alfalfa." Canadian Journal of Plant Science, 92: 203-206. doi : 10.4141/CJPS2011-114.

Almeida, A.D., Varanda, E.M., and Primavesi, A.C. 2005. Effect of the inherent variation in the mineral concentration of alfalfa cultivars on aphid populations. Brangantia 64: 233-239. Online version ISSN 1678-4499.

Asada, K. 1999. The water-water cycle in chloroplasts: scavenging of active oxygen species and dissipation of excess photons. Annu. Rev. Plant Physiol. Plant. Mol. Biol. 50: 601–639.

Bell, C.J., Richard, A., Dixon, R.A., Farmer, A.D., et. al. 2001. The Medicago Genome Initiative: a model legume database. Nucleic Acids Res. 29:114-117.

Bohnert, H.J., Nelson, D.E., and Jensen, R.G. 1995. Adaptations to environmental stresses. The Plant Cell: 7: 1099-1111.

Breytenbach, S. 2014. Sulphur in ruminant nutrition. Dairy Cattle/Technical Articles/Nutrition. *http://en.engormix.com/MA-dairy-cattle/nutrition/articles/sulphur-ruminant-nutrition-t77/p0.htm*. Aug. 3.

Brinkman, R. 1980. Saline and sodic soils. In: Land reclamation and water management. International Institute for Land Reclamation and Improvement (ILRI). Wageningen, The Netherlands. p. 62-68.

Chao, Y., Yang, Q., Kang, J., Zhang, T,. Sun, Y., and Gruber, M.Y. 2013. Expression of the alfalfa CCCH-Type zinc finger protein gene MsZFN delays flowering time in transgenic *Arabidopsis thaliana*. Plant Sci. 215-216: 92-99.

Chen, T., Yang, Q., Gruber, M., Kang, J., Sun, Y., Ding, D., Zhang, T., and Zhang, X. 2012. Expression of an alfalfa (*Medicago sativa* L.) ethylene response factor gene *MsERF8* in tobacco plants enhances resistance to salinity. Mol. Biol. Reports 39: 6067-6075.

Clarke, J., George, R.J., Bell, R.W., and Hatton, T.J. 2002. Dryland salinity in south-western Australia: its origins, remedies, and future research directions. Australian Journal of Soil Research. 40: 93-113.

Daniels, R.B. 1987. Saline seeps in the northern great plains of the USA and the southern prairies of Canada. In: Wolman, M.G. and Fournier, F.G.A.. Land Transformation in Agriculture. SCOPE (John Wiley & Sons Ltd). Etobicoke, ON. Canada. pp. 381-406.

Ferriera, J.F.S., Cornacchione, M.V., Liu, X., and Suarez, D.L. 2015. Nutrient composition, forage parameters, and antioxidant capacity of alfalfa (*Medicago sativa*, L.) in response to saline irrigation water. Agriculture 5: 577-597.

Fougere, .F, Le Rudulier, D. and Streeter, R.G. 1991. Effects of salt stress on amino acid, organic acid, and carbohydrate composition of roots, bacteroids, and cytosol of alfalfa (*Medicago sativa* L.). Plant Physiol. 96: 1228-1236.

Foyer, C.H., and Shigeoka, S. 2011. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol. 155: 93-100.

Gupta, B., and Huang, B. 2014. Mechanism of salinity tolerance in plants: Physiological biochemical, and molecular characterization. Int. J. Genomics. Article ID 701596. pp. 1-18.

Hamm, J.W., Radford, F.G., and Halstead, F.H. 1970. The simultaneous determination of nitrogen, phosphorus and potassium in sodium bicarbonate extracts of soils. Technicon International Congress. Advances in automatic analysis. Vol. 2. Industrial Analysis Futura Publ. Co Mt. Kisco, NY 65-69.

Hauser, F. and Horie, T. 2010. A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K+/Na+ ratio in leaves during salinity stress. Plant, Cell Environ. 33: 552–565.

Hernandez, J.A. and Almansa, M.S. 2002. Short-term effects of salt stress on antioxidant systems and leaf water relations of pea plants. Physiol. Plantar. 115: 251-257.

Hitachi Ltd. 1987. Analysis Guide for Polarized Zeeman Atomic Absorption Spectrometry (Flame Atomization Method).

Keivani, M., Ramezanpour, S.S., Soltanloo, H., Choukan, R., Naghavi, M. and Ranjbar, M. 2010. Genetic diversity assessment of alfalfa (*Medicago sativa* L.) populations using AFLP markers. Aust. J Crop Sci. 4:491-497.

Messer, D.E. 2008. Methods Manual. Semi-Arid Prairie Agriculture Research Centre. Analytical Chemistry Laboratory.

Janz, D., Behnke, K., Schnitzler, J.P., Kanawati, B., Schmitt-Kopplin, P. and Polle, A. 2010. Pathway analysis of the transcriptome and metabolome of salt sensitive and tolerant poplar species reveals evolutionary adaption of stress tolerance mechanisms. BMC Plant Biol 10: 150, pp17.

Jayaraman, K.S. and Das Gupta, D.K. 2015. Drying of fruits and vegetables.In: Mujumdar, A.S. (Editor). Handbook of Industrial Drying. 4th edition. CRC Press (Taylor and Francis group). pp. 61-638.

Jin, H., Sun, Y., Yang, Q., Chao, Y., Kang, J., Jin, H., Li, Y., amd Margaret G. 2010. Screening of genes induced by salt stress from alfalfa. Mol. Biol. Rep. 37: 745-753.

Kaldy, M.S., Hanna, M.R. and Smoliak, S. 1979. Influence of drying methods on protein content and amino acid composition of three forage legumes. Can. J. Plant Sci. 59: 707-712.

Knudson, L.L., Tibbitts, T.W. and Edwards, G.E. 1997. Measurement of ozone injury by determination of leaf chlorophyll concentration, Plant Physiol. 60: 606-608.

Li, D., Zhang, Y., Hu, X., Shen, X., Ma, L., Su, Z., Wang, T. and Dong, J. 2011. Transcriptional profiling of *Medicago truncatula* under salt stress identified a novel CBF transcription factor MtCBF4 that plays an important role in abiotic stress responses. BMC Plant Biol. 2011, 11:109.

Lugan, R., Niogret, M.F., Leport, L., Guegan, J.P., Larher, F.R., Savoure, A., Kopka, J. and

Bourchereau, A. 2010. Metabolome and water homeostasis analysis of *Thellungiella salsuginea* suggests that dehydration tolerance is a key response to osmotic stress in this halophyte. Plant J. 64: 215-229.

Munns, R. and Tester, M. 2008. Mechanisms of salinity tolerance. Ann. Rev. Plan. Biol. 59: 651-681.

Parida, A.K. and Das, A.B. 2005. Salt tolerance and salinity effects on plants: A review. Ecotox. & Environ. Safety 60: 324–349.

Postnikova, O.A., Shao, J. and Nemchinov, L.G. 2013. Analysis of the alfalfa root transcriptome in response to salinity stress. Plant Cell Physiol. 54: 1041-1055.

Ratcliffe, R.G. and Shachar-Hill, Y. 2005. Revealing metabolic phenotypes in plants: Inputs from NMR analysis. Biol. Rev. 80: 27-43.

R Development Core Team. 2011. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at *http://www.R-project.org*.

Sanchez, D.H., Lipopld, F., Redstig, H., et al. 2008a. Integrative functional genomics of salt acclimatization in the model legume *Lotus japonicus*. Plant J. 53: 973-987.

Sanchez, D.H., Redstig, H., Kramer, U., et al. 2008b. Metabolome-ionome-biomass interactions. What can we learn about salt stress by multiparallel phenotyping? Article Addendum. Plant Signaling & Behavior 3: 598-600.

Sanchez, D.H., Pieckenstain, .L., Escaray, F., et al 2011. Comparative ionomics and metabolomics in extremophile and glycophytic *Lotus* species under salt stress challenge the metabolic pre-acaptation hypothesis. Plant, Cell and Environment 34: 605-617.

Sakakibara, H., Honda, Y., Nakagawa, S., Ashida, H. and Kanazawa, K. 2003 Simultaneous determination of all polyphenols in vegetables, fruits, and teas. J. Agric. Food Chem. 51(3): 571–581.

Salt Institute. 2014. Salt and trace minerals in animal nutrition and agriculture. *http://www.goatworld.com/articles/nutrition/salt.shtml*. Aug. 3.

Schreiber, A.W., Hayden, M.J., Forrest, K.L., Kong, S.L., Langridge, P. and Baumann, U. 2012. Transcriptome-scale homoeolog-specific transcript assemblies of bread wheat. BMC Genomics: 13: 492.

Segovia-Lerma, A., Cantrell, R.G., Conway, J.M. and Ray, I.M. 2003. AFLP-based assessment of genetic diversity among nine alfalfa germplasms using bulk DNA templates. Genome 46: 51-58.

Shahbaz, M. and Ashraf, M. 2013. Improving salinity tolerance in cereals. Crit. Rev. Plant Sci. 32: 237-249.

Steppuhn H 1996. What is soil salinity? In: Proc. Soil Salinity Workshop. Alberta Agriculture, Lethbridge, AB, Canada, pp. 1-5.

Steppuhn, H. 2013. Principles and crop yield response to root-zone salinity. Prairie Crops and Soils 6: 40-51. *e-publication. http://www.prairiesoilsandcrops.ca/*

Steppuhn, H. and Wall, K. 1999. Canada's salt tolerant testing laboratory. Can. Agric. Eng. 4: 185-189.

Steppuhn, H., Acharya, S.N., Iwaasa, A.D., Gruber, M.Y. and Miller, D.R. 2012. Inherent responses to root-zone salinity in nine alfalfa populations. Can. J. Plant Sci. 92(2): 235-248. doi: 10.4141/CJPS2011-174

Szabados, L., Karács, H., Zilberstein, A. and Boucherlau, A. 2011. Plants in extreme environments. Importance of protective compounds in stress tolerance. In: Turkan I, Kader J-C, and Delseny M. Advances in Botanical Research. Plant Responses to Drought and Salinity Stress. Developments in a Post-Genomic era. Academic Press (Elsevier), Oxford UK. Vol 57 pp. 105-150.

Tester, M. and Davenport, R. 2003. Na+ tolerance and Na+ transport in higher plants. Annals of Bot. 91; 503-527.

Thimm, O., Blaesing, O., Gibon, Y., et al. 2004. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant

J. 37(6): 914-39.

Turkan, I. and Demiral, T. 2009. Recent developments in understanding salinity tolerance. Env. Exp. Bot. 67: 2-6.

Wiebe, B., Ellers, R.G., Eilers, W.D. and Brierley, J.A. 2007. Application of a risk indicator for assessing trends in dryland salinization risk on the Canadian prairies. Can.J. Soil Sci. 87: 213-224.

Xia, J., Mandal, R., Sinelnikov, I.V., Broadhurst, D. and Wishart, D.S. 2012. MetaboAnalyst 2.0: a comprehensive server for metabolomic data analysis. Nucleic Acids Res. 40 (Issue W1): W127-W133 doi:10.1093/nar/gks374.

Yacoubi, R., Job, C., Belghazi, M., Chaibi, W. and Job, D. 2013. Proteomic analysis of the enhancement of seed vigour in osmoprimed alfalfa seeds germinated under salinity stress Seed Science Research. Available on *CJO 2013. doi:10.1017/S0960258513000093*.

Yang, S.S., Tu, Z.J., Cheung, F., Xu, W.W., Lamb, J.F., Jung, H.J., Vance, C.P., and Gronwald, J.W. 2011. Using RNA-Seq for gene identification, polymorphism detection and transcript profiling in two alfalfa genotypes with divergent cell wall composition in stems. BMC Genomics. 12:199. doi: 10.1186/1471-2164-12-199.

Ying, H., Beifang, N., Ying, G., Limin, F. and Weizhong, L. 2010. CD-HIT Suite: a web server for clustering and comparing biological sequences. Bioinformatics: 26: 680.

Zhang, H., Han, B., Wang, T., Chen, S., Li, H., Zhang, Y. and Dai, S. 2011b. Mechanisms of plant salt response: Insights from proteomics. J. Proteome Res. 11: 49-67.

Figure Captions:

Figure 1. Experimental set-up for measuring salinity responses in alfalfa populations using hydroponic sand tanks at the Canadian Salt Lab. A. Tanks in greenhouse with alfalfa populations growing just prior to 4th-cut harvest. B. Harvesting. C. Distribution of three salt-tolerant alfalfa populations and the salt-sensitive Rangelander population control within four quadrants in one tank. Small plastic (coffee) stir sticks divided each tank into four quadrants, each holding the 25 genotypes representing one population rep. D. Schematic illustration of the population, electrical conductivity (EC), and tank replication and randomization. The Rangelander control population was included in each tank to screen for between tank variability unrelated to population and electrical conductivity.

Figure 2. Forage minerals (Na, Mn, Zn, S, Fe) as a function of increased electrical conductivity for saline-selected alfalfa populations and one non-selected population. Statistical tests are found on Supplementary Table S1. Error bars represent significant differences of the means at $p<0.05$.

Figure 3. Root minerals (Na, Mn, Zn, S, Fe, Mg) as a function of increased electrical conductivity for saline-selected alfalfa populations and one non-selected population. Statistical tests are found on Supplementary Table S1. Error bars represent significant differences of the means at $p<0.05$.

Figure 4. Forage transcriptome diversity (numbers of genes) for 34 Mapman general metabolism categories in salt-tolerant Bridgeview, salt-tolerant CW064027, and salt-sensitive Rangelander at 15.6 dSm^{-1} relative to Rangelander at 1.53 dSm^{-1} (control).

Figure 5. Mapman heatmap cartoon of forage stress transcriptome diversity (numbers of genes) in salt-tolerant Bridgeview, salt-tolerant CW064027, and salt-sensitive Rangelander at 15.6 dSm^{-1} relative to Rangelander at 1.53 dSm^{-1} (control). Red/blue colour intensity in heat maps equals negative/positive $\log_2$ change in significantly different transcript levels ($p<0.05$) relative to Rangelander at 1.53 dSm^{-1} (control). Maximum colour represents all transcript relative levels above that number.

Figure 6. Forage transcriptome diversity for transcription factors and protein factors (based on Mapman heat map analysis) for salt-tolerant CW064027, salt-tolerant Bridgeview, and salt-sensitive Rangelander at 15.6 dSm^{-1} relative to the Rangelander shoot transcriptome at 1.53 dSm^{-1} (control). Red/blue colour intensity in heat maps equals negative/ $\log_2$ change in significantly different transcript levels ($p<0.05$) relative to Rangelander at 1.53 dSm^{-1} (control). Maximum colour represents all transcript relative levels above that number. Blue box, transcription factors. Red box, protein modification. Green box, protein degradation.

Figure 7. Mapman heatmap cartoon of forage transcriptome diversity for secondary metabolite factors (after Mapman analysis) for salt-tolerant CW064027, salt-tolerant Bridgeview, and salt-sensitive Rangelander at 15.6 dSm^{-1} relative to the Rangelander shoot transcriptome at 1.53 dSm^{-1} (control).

Table 1. **Alfalfa Transport Genes** in salt-tolerant Bridgeview (Br), salt-tolerant CW064027 (CW), and salt-sensitive Rangelander (R) with extremely different expression at 15.6 dSm-1 relative to Rangelander at 1.53 dSm^{-1}. ***±1000*** shows the relative intensity where either numerator (***-1000***) or denominator (***+1000***) transcript numbers are equal to zero for individual target genes. Less dramatic relative gene changes are shown in Supplementary Table S2.

Sub-Bin Name	ID	Alfalfa popul.	Description
		-1000	
ABC transporters and multidrug resistance	m.s.attc7271	R	AT3G62700 | ATMRP10; ATPase, coupled to transmembrane movement of substances
"	m.s.atf7snd4004iyggj	R	AT1G15520 | ATPDR12 | PDR12 (ATPase coupled to movement of substances
"	m.s.atnode_26116_length_129_cov_3.372093	R	AT2G40090 | ATATH9; transporter
"	m.s.atnode_79986_length_130_cov_3.584615	R	AT5G58270 | ATM3 | STA1 (STARIK 1); ATPase, coupled to transmembrane movement
"	m.s.atnode_84823_length_172_cov_3.279070	R	AT3G59140 | ATMRP14; ATPase, coupled to transmembrane movement
"	m.s.atnode_86902_length_61_cov_5.475410	R	AT1G59870 | ATPDR8 | PEN3 (PENETRATION 3); ATPase, Cd transmembrane movement
Amino acids	m.s.attc6230	R	AT1G47670 | amino acid transporter family protein
"	m.s.atnode_74252_length_125_cov_5.320000	R	AT1G47670 | "
"	m.s.atnode_78168_length_150_cov_2.260000	R	AT1G08230 | "
"	m.s.atnode_82435_length_128_cov_2.835938	R	AT3G30390 | "
"	m.s.atnode_83852_length_139_cov_3.604316	R	AT3G03720 | CAT4 (CATIONIC AMINO ACID transmembrane TRANSPORTER 4)
Ammonium	m.s.atf7snd4004iz5t6	R	AT4G13510 | ATAMT1;1 | AMT1;1 (AMMONIUM transmembrane TRANSPORTER 1;1)
ATPases (vacuolar and periplasmic)	m.s.attc5510	R	AT1G13210 | ACA.I (autoinhibited Ca2+/ATPase II); transmembrane movement of ions
Cations (unspecified)	m.s.atnode_66056_length_337_cov_7.587537	R	AT1G10830 | sodium symporter-related
Cyclic nucleotide or calcium regulated channels	m.s.attc3852	R	AT3G17700 | ATCNGC20 | CNBT1 (CYCLIC NUCLEOTIDE-BINDING TRANSPORTER 1)
"	m.s.atnode_51899_length_129_cov_20.992249	R	AT5G53130 | CNGC1 (CYCLIC NUCLEOTIDE GATED CHANNEL 1); calmodulin binding
"	m.s.atnode_65974_length_161_cov_6.391304	R	AT5G53130 | "
Major Intrinsic Proteins.PIP	m.s.atnode_76979_length_126_cov_2.182540	R	AT4G00430 | TMP-C, PIP1E | PIP1;4 (PLASMA MEMBRANE INTRINSIC PROTEIN 1;4); water channel
"	m.s.at50317715	R	AT4G18910 | Symbols: NIP1;2, NLM2 | NIP1;2 (NOD26-LIKE INTRINSIC PROTEIN 1;2);

			arsenite transmembrane transporter/ water channel
Metabolites (envelope membrane)	m.s.atf7snd4003fnmpg	R	AT3G17430 | phosphate translocator-related
Metabolites (mitochondrial membrane)	m.s.atf7snd4003gqh60	R	AT2G47490 | mitochondrial substrate carrier family protein
"	m.s.atf7snd4003gt4me	R	AT2G47490 | "
"	m.s.atnode_47665_length_187_cov_6.000000	R	AT5G48970 | "
Metal	m.s.at50316794	R	Unknown Protein (AHRD V1); contains domain(s) IPR006121 Heavy metal transport/ detoxification protein chr01_pseudomolecule_IMGAG_V3.5 28995548-28994308 E EGN_Mt100125 20100825
"	m.s.atnode_50069_length_123_cov_5.512195	R	AT1G63440 | HMA5 (HEAVY METAL ATPASE 5); ATPase, transmembrane ion movement
"	m.s.atnode_63971_length_154_cov_7.727273	R	AT1G63440 | "
"	m.s.atnode_85976_length_138_cov_5.253623	R	AT1G16310 | cation efflux family protein
Miscellaneous	m.s.atf7snd4004if4v9	R	AT1G19800 | TGD1 (TRIGALACTOSYLDIACYLGLYCEROL 1); lipid transporter
"	m.s.atnode_47302_length_61_cov_24.836065	R	AT5G47730 | SEC14 cytosolic factor, putative / polyphosphoinositide-binding protein
"	m.s.atnode_64071_length_148_cov_8.918919	CW	AT1G26690 | emp24/gp25L/p24 family protein
"	m.s.atnode_85127_length_132_cov_3.113636	R	AT2G27810 | xanthine/uracil permease family protein
Nitrate	m.s.atnode_23421_length_260_cov_9.526923	R	AT1G69850 | ATNRT1:2 (ARABIDOPSIS THALIANA NITRATE TRANSPORTER 1:2); calcium ion binding / transporter
"	m.s.atnode_39550_length_192_cov_3.864583	R	AT1G69850 | "
"	m.s.atnode_41416_length_157_cov_6.006370	R	AT1G69850 | "
"	m.s.atnode_70813_length_186_cov_7.715054	R	AT1G69850 | "
Nucleotide	m.s.atnode_54229_length_128_cov_10.664062	R	AT1G28220 | ATPUP3; purine transmembrane transporter
"	m.s.atnode_63706_length_151_cov_17.231789	R	AT1G28220 | ATPUP3; purine transmembrane transporter
Peptides and oligopeptides	m.s.atnode_16167_length_61_cov_14.836065	R	AT1G59740 | proton-dependent oligopeptide transport (POT) family protein
"	m.s.atnode_18692_length_61_cov_27.393442	R	AT1G59740 | proton-dependent oligopeptide transport (POT) family protein
"	m.s.atnode_36358_length_166_cov_2.933735	R	AT1G32450 | NRT1.5 (NITRATE TRANSPORTER 1.5); nitrate transmembrane transporter
"	m.s.atnode_45056_length_173_cov_15.843930	R	AT2G37900 |proton-dependent oligopeptide transport (POT) family protein
"	m.s.atnode_68083_length_190_cov_4.021053	R	AT5G62680 | "
"	m.s.atnode_77946_length_146_cov_3.479452	R, Br	AT2G37900 | "
"	m.s.atnode_82242_length_139_cov_6.503597	R	AT1G52190 | "
Potassium	m.s.atf7snd4003fl7mc	R	AT5G11800 | KEA6; potassium ion transmembrane transporter / potassium:hydrogen antiporter

Sugars	m.s.attc7351	R	AT4G16480 | INT4 (INOSITOL TRANSPORTER 4); carbohydrate transmembrane transporter/ myo-inositol:hydrogen / sugar:hydrogen symporter
"	m.s.atnode_22061_length_101_cov_11.188119	CW	AT1G75220 | integral membrane protein, putative
"	m.s.atnode_29562_length_67_cov_16.194031	R	AT5G26340 | STP13 | MSS1; carbohydrate transmembrane transporter/ hexose:hydrogen symporter/ high-affinity hydrogen:glucose symporter
"	m.s.atnode_34159_length_131_cov_36.854961	R	AT3G18830 | ATPLT5 (POLYOL TRANSPORTER 5); D-ribose / D-xylose transmembrane transporter/ carbohydrate / galactose / glucose / glycerol /mannose transmembrane transporter/
"	m.s.atnode_47950_length_228_cov_7.245614	R	AT4G16480 | INT4 (INOSITOL TRANSPORTER 4); carbohydrate transmembrane transporter/ myo-inositol:hydrogen and sugar:hydrogen symporter
"	m.s.atnode_56648_length_82_cov_14.158537	R	AT5G16150 | GLT1, PGLCT (PLASTIDIC GLC TRANSLOCATOR); carbohydrate transmembrane transporter/ sugar:hydrogen symporter
"	m.s.atnode_67134_length_128_cov_4.742188	R	AT5G19850 | hydrolase, alpha/beta fold family protein
"	m.s.atnode_81651_length_165_cov_5.860606	R	AT1G16390 | ATOCT3 (ORGANIC CATION/CARNITINE TRANSPORTER 3); carbohydrate transmembrane transporter/ sugar:hydrogen symporter
"	m.s.atnode_74165_length_188_cov_3.234043	R	AT5G17850 | cation exchanger, putative (CAX8)

1000

ABC transporters multidrug resistance systems	m.s.atnode_78044_length_172_cov_6.168605	R, CW	AT1G04120 | ATMRP5; ATPase, coupled to transmembrane movement of substances / sulfonylurea receptor
"	m.s.atf7snd4003gua5j	Br	AT2G36910 | PGP1, ABCB1 (ATP BINDING CASSETTE SUBFAMILY B1); ATPase, coupled to transmembrane movement of substances / auxin efflux transmembrane transporter/ calmodulin binding
Amino acids	m.s.atnode_40729_length_123_cov_5.260163	R	AT1G47670 | amino acid transporter family protein
"	m.s.atnode_44733_length_350_cov_4.208571	R,Br, CW	AT2G39130 | amino acid transporter family protein
"	m.s.atf7snd4004jhm5u	CW	AT1G31830 | amino acid permease family protein
"	m.s.atnode_51260_length_141_cov_2.950355	Br, CW	AT2G39130 | amino acid transporter family protein
"	m.s.atnode_62762_length_61_cov_24.344263	CW	AT2G38120 | WAV5, PIR1, MAP1 | AUX1 (AUXIN RESISTANT 1); amino acid transmembrane transporter/ auxin binding / auxin influx transporter
Anions (unspecified)	m.s.atnode_7994_length_118_cov_29.101694	R,B	AT3G62270 | anion exchange family protein
"	m.s.atnode_57849_length_118_cov_24.296610	Br	AT3G62270 | "
"	m.s.atnode_66344_length_124_cov_5.314516	Br, CW	AT5G25430 |anion exchanger
"	m.s.atnode_66639_length_261_cov_4.467433	Br	AT4G10310 | HKT1 (HIGH-AFFINITY K+ TRANSPORTER 1); sodium ion transmembrane transporter
"	m.s.atnode_86208_length_127_cov_3.582677	CW	AT5G25430 | anion exchanger

ATPases (vacuolar and periplasmic)	m.s.atnode_37650_length_175_cov_5.725714	R	AT3G27870 | haloacid dehalogenase-like hydrolase family protein
Metabolite transporters at the envelope membrane	m.s.atnode_27374_length_105_cov_17.028572	CW	AT1G61800 | GPT2; antiporter/ glucose-6-phosphate transmembrane transporter
Miscellaneous	m.s.atnode_29995_length_61_cov_5.721312	R	AT2G20840 | secretory carrier membrane protein (SCAMP) family protein
Nucleotides	m.s.atnode_41854_length_112_cov_18.741072	Br, CW	AT1G28220 | ATPUP3; purine transmembrane transporter
Peptides and oligopeptides	m.s.atnode_22358_length_162_cov_2.969136	Br, CW	AT1G22540 | proton-dependent oligopeptide transport (POT) family protein
"	m.s.atnode_25973_length_173_cov_5.202312	CW	AT1G27040 | nitrate transporter, putative
Sugars	m.s.atf7snd4004jrte6	R	AT1G30220 | INT2 (INOSITOL TRANSPORTER 2); carbohydrate transmembrane transporter/ sugar:hydrogen symporter
"	m.s.atf7snd4004ith23	Br	AT1G30220 | "
Sulphate	m.s.atnode_40271_length_133_cov_3.872180	R, CW	AT5G10180 | SULTR2;1 |sulfate transmembrane transporter
"	m.s.atnode_44388_length_326_cov_4.217792	R,Br, CW	AT5G10180 | SULTR2;1 | AST68; sulfate transmembrane transporter

Table 2. **Alfalfa Non-lignin Cell Wall Genes** in salt-tolerant Bridgeview (Br), salt-tolerant CW064027 (CW), and salt-sensitive Rangelander (R) with extremely different expression at 15.6 dSm-1 relative to Rangelander at 1.53 dSm^{-1}. ***±1000*** shows the relative intensity where either numerator (***-1000***) or denominator (***+1000***) transcript numbers are equal to zero for individual target genes. Less dramatic relative gene changes are shown in Supplementary Table S2.

Sub-Bin Name	ID	Alfalfa popul. ***-1000***	
major CHO metabolism.degradation. sucrose.Susy	m.s.atnode_67862_length_149_cov_5.832215	R	AT1G73370 | SUS6 (SUCROSE SYNTHASE 6); UDP-glycosyltransferase/ sucrose synthase
cell wall.precursor synthesis	m.s.atnode_11730_length_161_cov_3.826087	R	AT5G18070 | DRT101 (DNA-DAMAGE-REPAIR/TOLERATION 101); intramolecular transferase, phosphotransferases / magnesium ion binding
cell wall.precursor synthesis.UGE	m.s.atnode_53229_length_133_cov_5.984962	R	AT4G10960 | UGE5 (UDP-D-glucose/UDP-D-galactose 4-epimerase 5); UDP-glucose 4-epimerase/ protein dimerization
cell wall.cellulose synthesis.cellulose synthase	m.s.attc18718	R	AT5G17420 | CESA7, MUR10 | IRX3 (IRREGULAR XYLEM 3); cellulose synthase
"	m.s.attc19173	R	"
"	m.s.atnode_39164_length_171_cov_5.538012	R, Br	AT5G64740 | IXR2, E112, PRC1 | CESA6 (CELLULOSE SYNTHASE 6); cellulose synthase/ transferase, transferring glycosyl groups
"	m.s.atnode_44627_length_95_cov_41.305264	R	AT5G17420 | CESA7, ATCESA7, MUR10 | IRX3 (IRREGULAR XYLEM 3); cellulose synthase
"	m.s.atnode_67080_length_166_cov_4.728916	R	AT3G03050 | KJK | CSLD3 (CELLULOSE SYNTHASE-LIKE D3); cellulose synthase/ transferase, transferring glycosyl groups
"	m.s.atnode_78982_length_127_cov_3.574803	R	AT4G23990 | CSLG3 | ATCSLG3; cellulose synthase/ transferase/ transferase, transferring glycosyl groups
cell wall.cellulose synthesis.COBRA	m.s.atnode_27969_length_163_cov_25.024540	R	AT3G29810 | COBL2 (COBRA-LIKE PROTEIN 2 PRECURSOR)
"	m.s.atf7snd4004icn62	R	AT5G60490 | FLA12
"	m.s.atnode_19910_length_61_cov_16.295082	R	AT3G11700 | FLA18 (FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 18 PRECURSOR)
"	m.s.atnode_45779_length_61_cov_32.180328	R	AT5G60490 | FLA12
"	m.s.atnode_83967_length_61_cov_13.524590	R	AT5G03170 | FLA11
cell wall.cell wall proteins.LRR	m.s.atf7snd4003hjgci	R	AT3G19320 | leucine-rich repeat family protein
"	m.s.atnode_10504_length_107_cov_85.943924	R	AT4G18670 | protein binding / structural constituent of cell wall

"	m.s.atnode_36238_length_181_cov_15.038674	R	AT4G18670 | protein binding / structural constituent of cell wall
"	m.s.atnode_48891_length_61_cov_62.557377	R	AT3G19320 | leucine-rich repeat family protein
cell wall.degradation. mannan-xylose-arabinose-fucose	m.s.atnode_45202_length_123_cov_10.008130	R	AT1G78060 | glycosyl hydrolase family 3 protein
"	m.s.atnode_5250_length_126_cov_12.015873	R	AT2G20680 | glycosyl hydrolase family 5 protein / cellulase family protein
"	m.s.atnode_64015_length_156_cov_8.762820	R	AT1G78060 | glycosyl hydrolase family 3 protein
cell wall.degradation. pectate lyases and polygalacturonases	m.s.atnode_9498_length_151_cov_142.748337	Br	AT1G70370 | BURP domain-containing protein / polygalacturonase, putative
"	m.s.atnode_44844_length_154_cov_7.311688	Br	AT1G70370 | BURP domain-containing protein / polygalacturonase, putative
"	m.s.atnode_51712_length_177_cov_31.819208	R	AT1G70370 | "
"	m.s.atnode_71828_length_123_cov_3.569106	R	AT4G33440 | glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein
"	m.s.atnode_81927_length_161_cov_2.763975	R	AT5G06860 | PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN 1); protein binding
"	m.s.atnode_85741_length_122_cov_6.393443	R	AT4G13710 |pectate lyase family protein
"	m.s.atnode_87314_length_147_cov_5.074830	R	AT1G70370 | BURP domain-containing protein / polygalacturonase, putative
"	m.s.atnode_9303_length_235_cov_24.476595	R	AT5G06860 | PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN 1); protein binding
cell wall.modification	m.s.attc7401	R	AT4G25810 | XTR6 (XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6); hydrolyzing O-glycosyl compounds / xyloglucan:xyloglucosyl transferase
"	m.s.atnode_39955_length_166_cov_7.578313	R	AT4G25810 | XTH23 | XTR6 (XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6); acting on glycosyl bonds / hydrolase, hydrolyzing O-glycosyl compounds / xyloglucan:xyloglucosyl transferase
"	m.s.atnode_689_length_245_cov_11.567347	R	AT4G25810 | "
"	m.s.atnode_79526_length_141_cov_3.304965	R	AT4G25810 | "
cell wall. pectin*esterases.PME	m.s.atf7snd4004ix1pl	R	AT1G11580 | PMEPCRA (METHYLESTERASE PCR A); enzyme inhibitor/ pectinesterase
"	m.s.atnode_51998_length_151_cov_9.218543	R, CW	AT3G14310 | ATPME3; pectinesterase
cell wall. pectin*esterases.acetyl esterase	m.s.atnode_18483_length_61_cov_21.836065	R	AT4G19420 | pectinacetylesterase family protein

		+1000	
cell wall.cellulose synthesis	m.s.atnode_73279_length_153_cov_6.895425	CW	AT5G22740 | ATCSLA2 | ATCSLA02; mannan synthase/ transferase, transferring glycosyl groups
cell wall.cellulose synthesis.cellulose synthase	m.s.atnode_41971_length_125_cov_5.248000	R, Br, CW	AT2G32540 | ATCSLB4 | ATCSLB04; cellulose synthase/ transferase/ transferase, transferring glycosyl groups
"	m.s.atnode_60559_length_127_cov_4.637795	CW	AT2G32540 | "
cell wall.hemicellulose synthesis.xyloglucan.XXXG galactose Transferase	m.s.atnode_50474_length_146_cov_4.993151	R, CW	AT2G20370 | KAM1, MUR3 (MURUS 3); catalytic/ transferase, transferring glycosyl groups
cell wall.hemicellulose synthesis.glucuronoxylan	m.s.atnode_45990_length_207_cov_9.347826	CW	AT4G33330 |PGSIP3 | transferase, transferring glycosyl groups
cell wall.cell wall proteins.AGPs.AGP	m.s.atnode_66859_length_61_cov_12.770492	Br, CW	AT5G03170 | FLA11
cell wall.cell wall proteins.LRR	m.s.atnode_69568_length_123_cov_3.731707	CW	AT4G06744 | leucine-rich repeat family protein / extensin family protein
cell wall.degradation.mannan-xylose-arabinose-fucose	m.s.atnode_37156_length_129_cov_3.643411	R	AT1G09010 | glycoside hydrolase family 2 protein
cell wall.degradation.pectate lyases and polygalacturonases	m.s.atnode_61916_length_76_cov_19.697369	CW	AT1G23760 |JP630; polygalacturonase
"	m.s.atnode_86833_length_143_cov_3.608392	CW	AT1G60590 | polygalacturonase, putative / pectinase, putative
cell wall.modification	m.s.atnode_40911_length_168_cov_2.535714	CW	AT4G17030 | ATEXPR1, ATHEXP BETA 3.1 | ATEXLB1 (ARABIDOPSIS THALIANA EXPANSIN-LIKE B1)
"	m.s.atnode_44603_length_202_cov_11.000000	CW	AT2G36870 | xyloglucan:xyloglucosyl transferase, putative / xyloglucan endotransglycosylase/transferase, putative

Table 3. **Alfalfa Miscellaneous Genes** in salt-tolerant Bridgeview (Br), salt-tolerant CW064027 (CW), and salt-sensitive Rangelander (R) with extremely different expression at 15.6 dSm-1 relative to Rangelander at 1.53 dSm^{-1}. ±1000 shows the relative intensity where either numinator or denominator was equal to zero for that target gene. Remainder of relative gene changes are shown in Supplementary Table 1. ***±1000*** shows the relative intensity where either numerator (***-1000***) or denominator (***+1000***) transcript numbers are equal to zero for individual target genes. Less dramatic relative gene changes are shown in Supplementary Table 1.

Sub-Bin Name	ID	Alfalfa popul.	***-1000***
misc2	m.s.atnode_17159_length_124_cov_7.088710	R	AT4G31970 | CYP82C2; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
"	m.s.atnode_39004_length_142_cov_3.464789	R	AT4G31940 | CYP82C4; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
"	m.s.atnode_51057_length_189_cov_4.142857	R	AT4G31940 | "
"	m.s.at50318404	Br	AT5G06900 | CYP93D1; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
cytochrome P450	m.s.attc5512	R	AT2G27690 | CYP94C1; fatty acid (omega-1)-hydroxylase/ oxygen binding
"	m.s.atnode_13245_length_145_cov_6.117241	R	AT4G37330 |CYP81D4; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
"	m.s.atnode_17159_length_124_cov_7.088710	R	AT4G31970 | CYP82C2; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
"	m.s.atnode_19642_length_72_cov_34.861111	R	AT4G31500 | SUR2, RNT1, RED1, ATR4 | CYP83B1 (CYTOCHROME P450 MONOOXYGENASE 83B1);
"	m.s.atnode_27268_length_211_cov_4.895735	R	AT2G45550 | CYP76C4; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
"	m.s.atnode_30469_length_154_cov_5.701299	R	AT3G14690 | CYP72A15; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
"	m.s.atnode_36464_length_145_cov_5.351724	R	AT2G27690 | CYP94C1; fatty acid (omega-1)-hydroxylase/ oxygen binding
"	m.s.atnode_42098_length_146_cov_5.801370	R	AT2G27690 | "
"	m.s.atnode_64057_length_177_cov_3.016949	R	AT3G14690 | "
"	m.s.atnode_80681_length_184_cov_6.505435	R	AT5G24910 | CYP714A1; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
"	m.s.atnode_81339_length_155_cov_4.709677	R	AT4G19230 | CYP707A1; (+)-abscisic acid 8'-hydroxylase/ oxygen binding
"	m.s.atnode_88232_length_151_cov_2.543046	R	AT5G52400 | CYP715A1; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
dynamin	m.s.attc5902	R	AT1G60500 | dynamin family protein
"	m.s.atnode_33506_length_138_cov_4.652174	R	AT5G42080 | ADL1A, AG68, DRP1A | ADL1 (ARABIDOPSIS DYNAMIN-LIKE PROTEIN); GTP binding / GTPase/ protein binding

gluco-, galacto- and mannosidases	m.s.atf7snd4004j0esi	R	AT5G13980 | glycosyl hydrolase family 38 protein
"	m.s.atnode_50790_length_126_cov_3.460317	R	AT1G26560 | BGLU40 (BETA GLUCOSIDASE 40); catalytic/ cation binding / hydrolase, hydrolyzing O-glycosyl compounds
gluco-, galacto- and mannosidases.beta-galactosidase	m.s.atnode_36747_length_138_cov_9.405797	R	AT2G28470 | BGAL8 (beta-galactosidase 8); beta-galactosidase/ catalytic/ cation binding / sugar binding
"	m.s.atnode_44673_length_61_cov_2.885246	R	AT2G28470 | "
"	m.s.atnode_62341_length_140_cov_7.214286	R	AT2G28470 | "
"	m.s.atnode_80185_length_190_cov_5.278947	R	AT1G45130 | BGAL5 (beta-galactosidase 5); beta-galactosidase/ catalytic/ cation binding
β-1,3 glucan hydrolases	m.s.atnode_31104_length_259_cov_14.077220	R	AT2G05790 | glycosyl hydrolase family 17 protein
β-1,3 glucan hydrolases.glucan endo-1,3-beta-glucosidase	m.s.atnode_20357_length_149_cov_7.818792	R	AT2G27500 | glycosyl hydrolase family 17 protein
glutathione S transferases	m.s.attc6284	R	AT3G09270 |ATGSTU8 (GLUTATHIONE S-TRANSFERASE TAU 8)
"	m.s.at283574890	R	AT3G03190 | ATGSTF6 | ATGSTF11 (GLUTATHIONE S-TRANSFERASE F11)
"	m.s.at50318992	R	AT3G09270 | ATGSTU8 (GLUTATHIONE S-TRANSFERASE TAU 8)
"	m.s.atnode_1175_length_133_cov_3.458647	R	AT2G29420 | GST25 | ATGSTU7 (GLUTATHIONE S-TRANSFERASE TAU 7)
"	m.s.atnode_29270_length_155_cov_11.529033	R	AT3G09270 | ATGSTU8 (GLUTATHIONE S-TRANSFERASE TAU 8)
"	m.s.atnode_83575_length_141_cov_2.971631	R	AT5G02790 | In2-1 protein, putative
GCN5-related N-acetyltransferase	m.s.atnode_28981_length_142_cov_6.422535	R	AT2G32020 | GCN5-related N-acetyltransferase (GNAT) family protein
"	m.s.atnode_40298_length_127_cov_4.700788	R	AT2G32020 | GCN5-related N-acetyltransferase (GNAT) family protein
invertase/pectin methylesterase inhibitor family protein	m.s.atnode_74219_length_273_cov_6.139194	R	AT3G17130 | invertase/pectin methylesterase inhibitor family protein
myrosinases-lectin-jacalin	m.s.atnode_44755_length_232_cov_5.939655	R	AT5G06740 | lectin protein kinase family protein
"	m.s.atnode_78521_length_146_cov_3.876712	R	AT4G04960 | lectin protein kinase, putative
nitrilases, *nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	m.s.atnode_82269_length_151_cov_5.026490	R	AT2G23620 | MES1 (METHYL ESTERASE 1); hydrolase, acting on ester bonds
O-methyl transferases	m.s.atnode_22625_length_131_cov_9.244275	R	AT3G06930 | PRMT4B | protein arginine N-methyltransferase family protein
oxidases - copper, flavone	m.s.atnode_32205_length_128_cov_8.039062	R	AT2G42490 | copper amine oxidase, putative

etc.			
"	m.s.atnode_61247_length_217_cov_9.788018	R	AT2G42490 | copper amine oxidase, putative
"	m.s.atnode_6366_length_125_cov_6.360000	R	AT5G66920 | sks17 (SKU5 Similar 17); copper ion binding / oxidoreductase
"	m.s.atnode_67483_length_115_cov_11.600000	R	AT1G23740 | oxidoreductase, zinc-binding dehydrogenase family protein
"	m.s.atnode_70387_length_61_cov_11.754098	R	AT5G16970 | AT-AER (2-alkenal reductase)
"	m.s.atnode_73574_length_184_cov_6.440217	R	AT1G62600 | flavin-containing monooxygenase family protein / FMO family protein
"	m.s.atnode_90098_length_139_cov_5.100719	R	AT1G12140 | FMO GS-OX5 (FLAVIN-MONOOXYGENASE GLUCOSINOLATE S-OXYGENASE 5)
oxygenases	m.s.atnode_76769_length_153_cov_3.019608	R	AT1G73680 | pathogen-responsive alpha-dioxygenase, putative
peroxidases	m.s.atnode_37521_length_125_cov_4.600000	R	AT4G08770 | peroxidase, putative
phosphatases (acid and others)	m.s.atf7snd4003f5zy1	R	AT3G15820 | phosphatidic acid phosphatase-related / PAP2-related
"	m.s.atf7snd4004ifgs9	R	AT1G13900 | calcineurin-like phosphoesterase family protein
"	m.s.atnode_65344_length_154_cov_5.844156	R	AT5G34850 | PAP26 (PURPLE ACID PHOSPHATASE 26); acid phosphatase/ protein serine/threonine phosphatase
protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	m.s.at136141886	R	AT2G45180 | protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
short chain dehydrogenase/reductase (SDR)	m.s.attc17423	R	AT3G51680 | short-chain dehydrogenase/reductase (SDR) family protein
"	m.s.at50321444	Br	AT3G51680 | Symbols: | short-chain dehydrogenase/reductase (SDR) family protein
UDP glucosyl and glucoronyl transferases	m.s.attc22099	R	AT1G01420 | UGT72B3 (UDP-GLUCOSYL TRANSFERASE 72B3); quercetin 3-O-glucosyltransferase/ transferase, transferring glycosyl groups
"	m.s.attc22395	R	AT3G02100 |UDP-glucoronosyl/UDP-glucosyl transferase family protein
"	m.s.atf7snd4004j1myq	R	AT4G01070 | GT72B1; UDP-glycosyltransferase/ transferase, transferring glycosyl groups
"	m.s.atnode_11183_length_152_cov_6.993421	R	AT4G01070 | "
"	m.s.atnode_13499_length_189_cov_12.973545	R	AT4G15550 | IAGLU (INDOLE-3-ACETATE BETA-D-GLUCOSYLTRANSFERASE); UDP-glycosyltransferase/ transferase, transferring glycosyl groups
"	m.s.atnode_15248_length_204_cov_10.044118	R	AT3G02100 | UDP-glucoronosyl/UDP-glucosyl transferase family protein
"	m.s.atnode_16689_length_160_cov_9.062500	R	AT2G36800 | DOGT1 (DON-GLUCOSYLTRANSFERASE 1);

"	m.s.atnode_34242_length_158_cov_13.474684	R, Br	AT3G02100 | UDP-glucoronosyl/UDP-glucosyl transferase family protein
"	m.s.atnode_35318_length_146_cov_5.698630	R	AT4G15550 | IAGLU (INDOLE-3-ACETATE BETA-D-GLUCOSYLTRANSFERASE)
"	m.s.atnode_42212_length_140_cov_4.021429	R	AT5G47780 | GAUT4 (Galacturonosyltransferase 4);
"	m.s.atnode_6615_length_140_cov_9.035714	R	AT4G15550 | IAGLU (INDOLE-3-ACETATE BETA-D-GLUCOSYLTRANSFERASE)
"	m.s.atnode_67493_length_145_cov_24.096552	R, CW	AT3G50760 | GATL2 (Galacturonosyltransferase-like 2);
"	m.s.atnode_70986_length_174_cov_5.603448	R	AT4G34131 | UGT73B3 (UDP-glucosyl transferase 73B3);
"	m.s.atnode_71653_length_149_cov_8.798657	R	AT3G02100 | UDP-glucoronosyl/UDP-glucosyl transferase family protein
"	m.s.atnode_72794_length_187_cov_2.951872	R	AT1G73880 | UGT89B1 (UDP-GLUCOSYL TRANSFERASE 89B1)
"	m.s.atnode_74984_length_125_cov_14.208000	R	AT4G01070 | UGT72B1 | GT72B1; UDP-glucosyltransferase/ transferring glycosyl groups
"	m.s.atnode_83450_length_166_cov_4.897590	R	AT4G01070 | "
"	m.s.atnode_88653_length_61_cov_1.934426	R	AT3G61130 | GAUT1 (GALACTURONOSYLTRANSFERASE 1); polygalacturonate 4-alpha-galacturonosyltransferase/ transferring glycosyl groups
		+1000	
misc2	m.s.at50321278	R	AT1G13080 | CYP71B2 (CYTOCHROME P450 71B2); electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
"	m.s.atnode_48448_length_161_cov_6.018633	R, Br	AT3G05600 | epoxide hydrolase, putative
"	m.s.atnode_10634_length_154_cov_9.240260	Br	AT4G31970 | CYP82C2; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
cytochrome P450	m.s.atf7snd4004imjqm	CW	AT2G45550 | CYP76C4; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
"	m.s.atnode_3012_length_69_cov_6.956522	CW	AT3G14690 | CYP72A15; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
"	m+A81:B83.s.atnode_10634_length_154_ cov_9.240260	Br	AT4G31970 | CYP82C2; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
"	m.s.atnode_66477_length_132_cov_4.545455	R	AT3G61040 | CYP76C7; electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding
"	m.s.atnode_84016_length_227_cov_4.629956	R	AT5G24910 |CYP714A1; electron carrier/ heme binding / iron ion binding monooxygenase/ oxygen binding
β 1,3 glucan hydrolases.glucan endo-1,3-beta-glucosidase	m.s.atnode_636_length_154_cov_3.889610	CW	AT3G55430 | glycosyl hydrolase family 17 protein / beta-1,3-glucanase, putative
"	m.s.atnode_17725_length_128_cov_12.914062	R, Br, CW	AT2G27500 | glycosyl hydrolase family 17 protein

gluco-, galacto- and mannosidases	m.s.atnode_37156_length_129_cov_3.643411	R	AT1G09010 | glycoside hydrolase family 2 protein
"	m.s.atnode_25993_length_128_cov_5.773438	CW	AT1G61810 | BGLU45 (BETA-GLUCOSIDASE 45); catalytic/ cation binding / hydrolase, hydrolyzing O-glycosyl compounds
"	m.s.atnode_73699_length_144_cov_3.333333	CW	AT1G27520 | glycoside hydrolase family 47 protein
gluco-, galacto- and mannosidases.beta-galactosidase	m.s.atf7snd4004ieoua	R	AT2G28470 | BGAL8 (beta-galactosidase 8); beta-galactosidase/ catalytic/ cation binding / sugar binding
"	m.s.atf7snd4004ieoua	Br	AT2G28470 | "
glutathione S transferases	m.s.atnode_8970_length_416_cov_54.576923	CW	AT3G03190 | ATGSTF6 | ATGSTF11 (GLUTATHIONE S-TRANSFERASE F11); glutathione transferase
nitrilases, *nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	m.s.atnode_33081_length_136_cov_11.058824	R, CW	AT2G34790 | EDA28 | MEE23 (MATERNAL EFFECT EMBRYO ARREST 23); FAD binding / catalytic/ electron carrier/ oxidoreductase
oxidases - copper, flavone etc.	m.s.atnode_21362_length_85_cov_11.905882	R, CW	AT4G12420 | SKU5; copper ion binding / oxidoreductase
peroxidases	m.s.atnode_30888_length_299_cov_6.130435	CW	AT5G05340 | peroxidase, putative
"	m.s.atnode_37183_length_151_cov_4.854305	CW	AT5G05340 | peroxidase, putative
"	m.s.atnode_40830_length_135_cov_9.414815	R, CW	AT5G05340 | peroxidase, putative
phosphatases (acid and others)	m.s.atnode_23178_length_137_cov_5.919708	CW	AT4G14930 | acid phosphatase survival protein SurE, putative
"	m.s.atnode_59805_length_198_cov_4.747475	R, CW	AT2G38600 | acid phosphatase class B family protein
"	m.s.atnode_62741_length_126_cov_3.507936	Br	AT1G13750 | calcineurin-like phosphoesterase family protein
short chain dehydrogenase/reductase (SDR)	m.s.atnode_78272_length_163_cov_4.785276	R	AT5G50130 | short-chain dehydrogenase/reductase (SDR) family protein

Figure 1. Experimental set-up for measuring salinity responses in alfalfa populations using hydroponic sand tanks at the Canadian Salt Lab. A. Tanks in greenhouse with alfalfa populations growing just prior to 4th-cut harvest. B. Harvesting. C. Distribution of three salt-tolerant alfalfa populations and the salt-sensitive Rangelander population control within four quadrants in one tank. Small plastic (coffee) stir sticks divided each tank into four quadrants, each holding the 25 genotypes representing one population rep. D. Schematic illustration of the population, electrical conductivity (EC), and tank replication and randomization. The Rangelander control population was included in each tank to screen for between tank variability unrelated to population and electrical conductivity.

191x177mm (150 x 150 DPI)

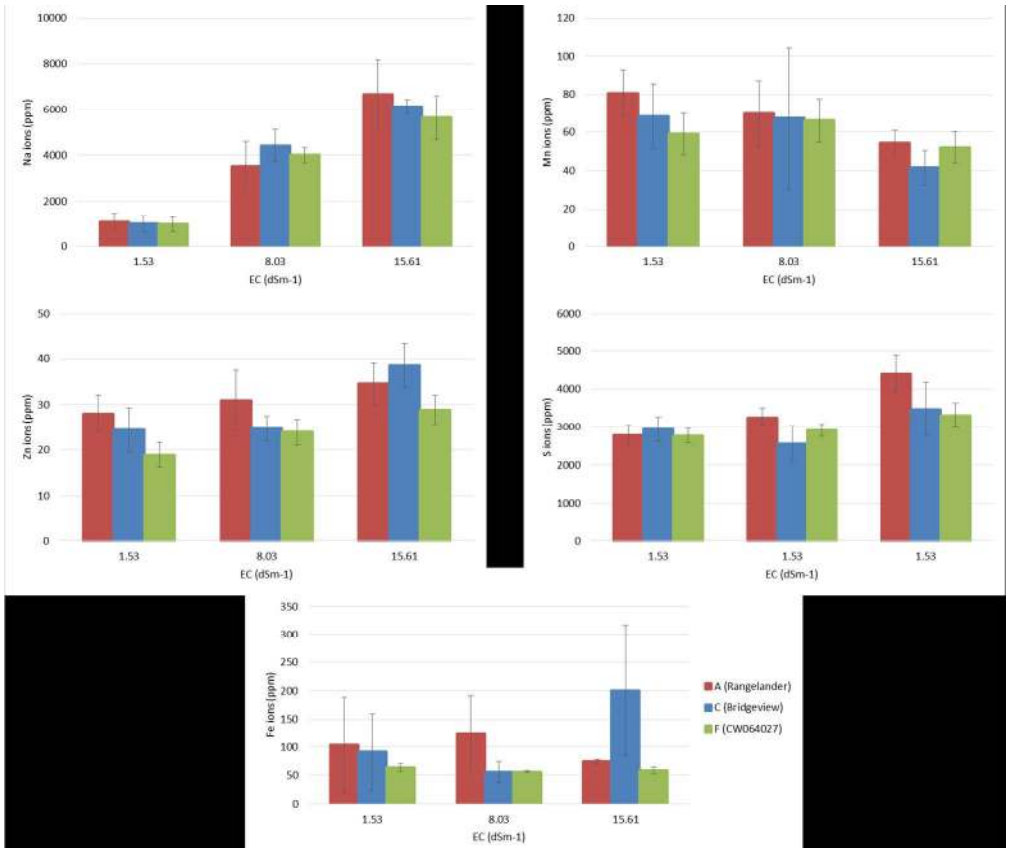


Figure 2. Forage minerals (Na, Mn, Zn, S, Fe) as a function of increased electrical conductivity for saline-selected alfalfa populations and one non-selected population. Statistical tests are found on Supplementary Table S1. Error bars represent significant differences of the means at $p<0.05$.

301x254mm (150 x 150 DPI)

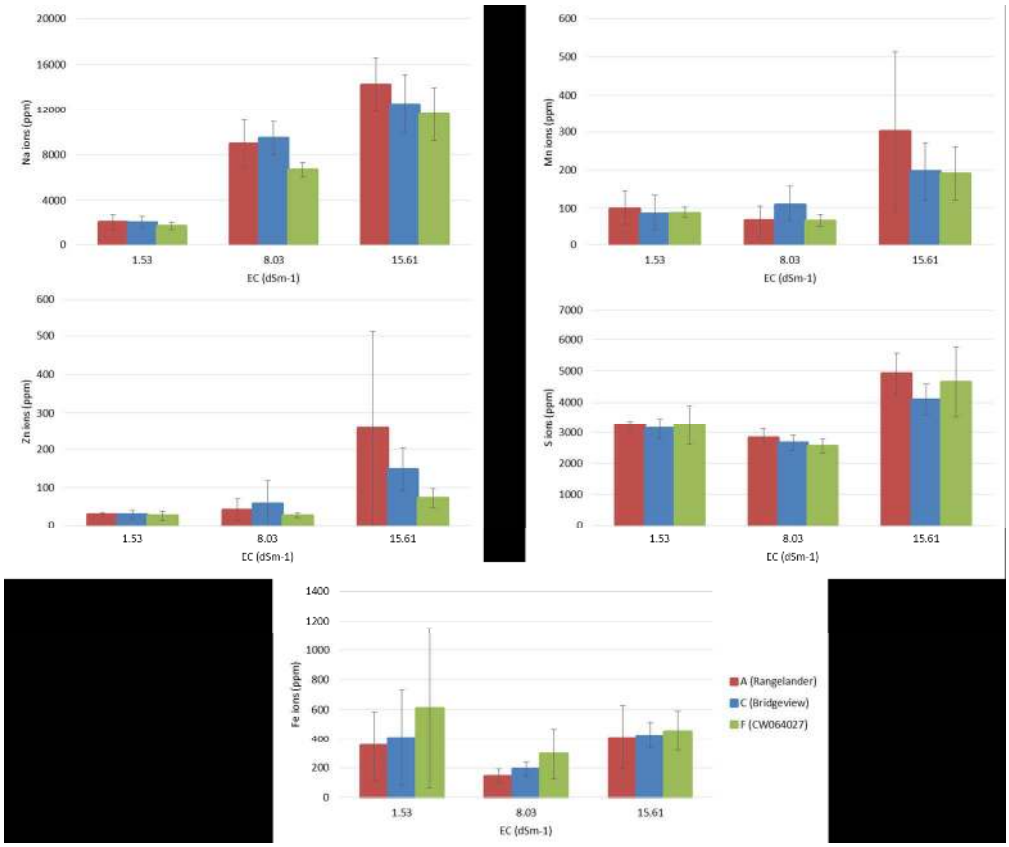


Figure 3. Root minerals (Na, Mn, Zn, S, Fe, Mg) as a function of increased electrical conductivity for saline-selected alfalfa populations and one non-selected population. Statistical tests are found on Supplementary Table S1. Error bars represent significant differences of the means at $p<0.05$.

302x253mm (150 x 150 DPI)

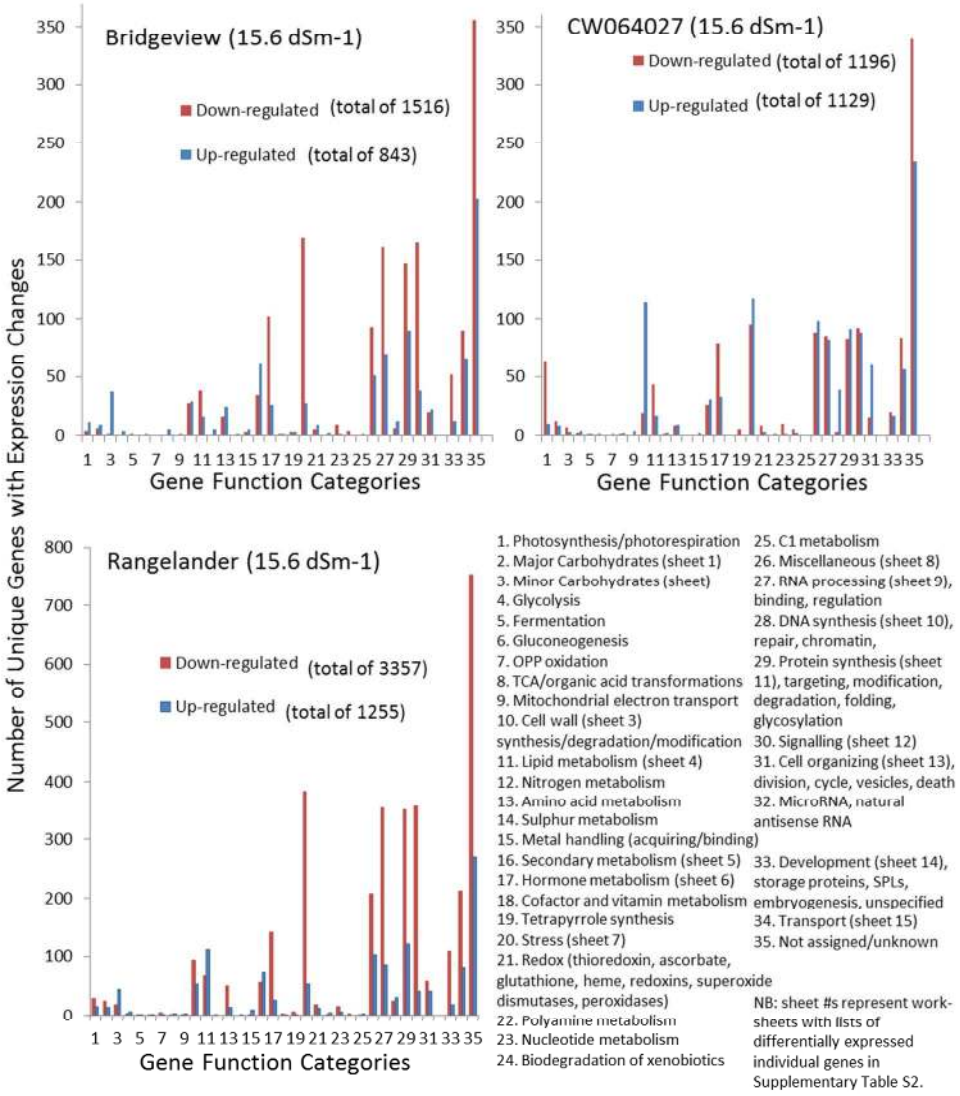


Figure 4. Forage transcriptome diversity (numbers of genes) for 34 Mapman general metabolism categories in salt-tolerant Bridgeview, salt-tolerant CW064027, and salt-sensitive Rangelander at 15.6 dSm-1 relative to Rangelander at 1.53 dSm-1 (control).

186x227mm (150 x 150 DPI)

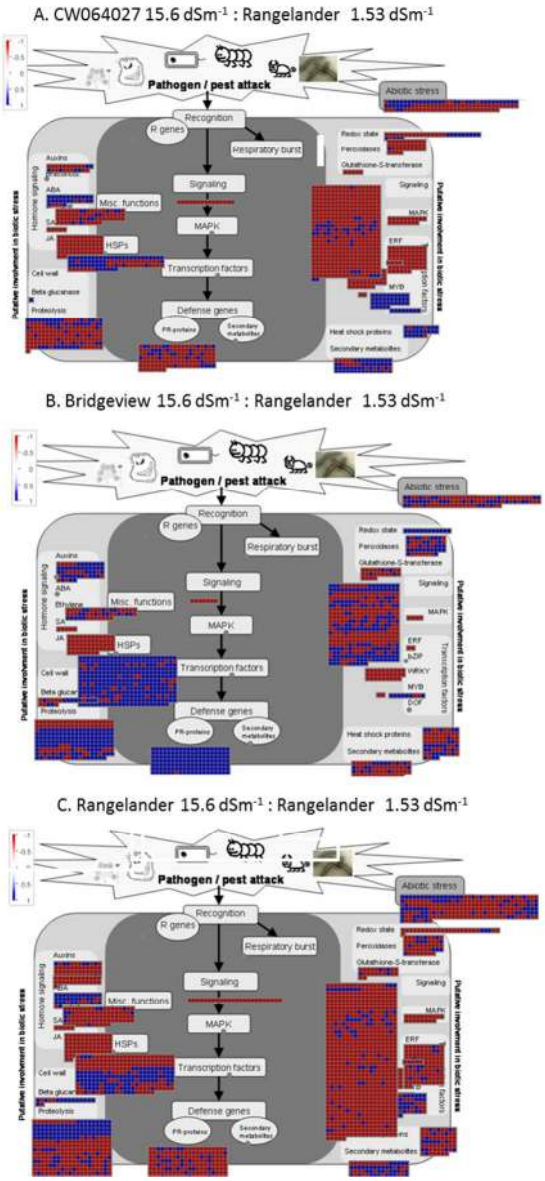


Figure 5. Mapman heatmap cartoon of forage stress transcriptome diversity (numbers of genes) in salt-tolerant Bridgeview, salt-tolerant CW064027, and salt-sensitive Rangelander at 15.6 dSm-1 relative to Rangelander at 1.53 dSm-1 (control). Red/blue colour intensity in heat maps equals negative/positive log2 change in significantly different transcript levels ($p<0.05$) relative to Rangelander at 1.53 dSm-1 (control). Maximum colour represents all transcript relative levels above that number.

111x241mm (150 x 150 DPI)

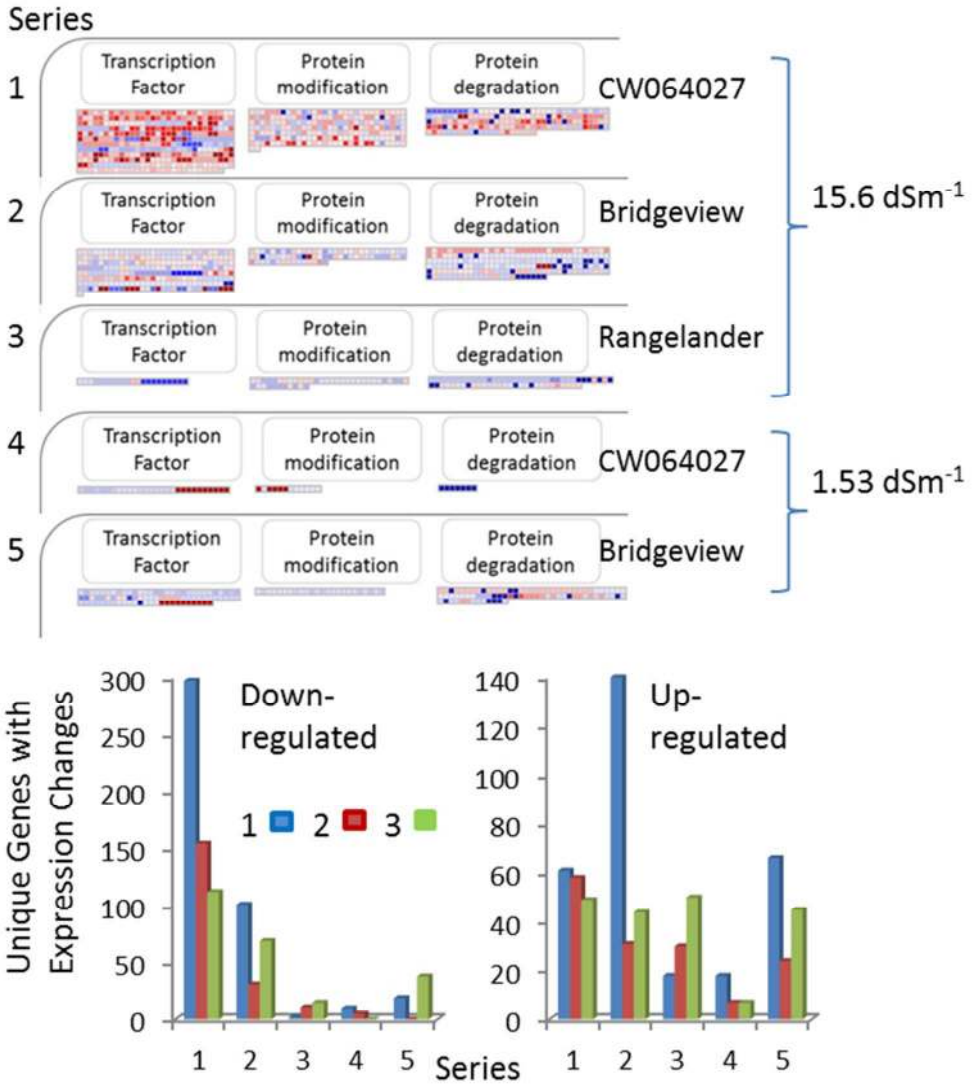


Figure 6. Forage transcriptome diversity for transcription factors and protein factors (based on Mapman heat map analysis) for salt-tolerant CW064027, salt-tolerant Bridgeview, and salt-sensitive Rangelander at 15.6 dSm-1 relative to the Rangelander shoot transcriptome at 1.53 dSm-1 (control). Red/blue colour intensity in heat maps equals negative/ log2 change in significantly different transcript levels ($p<0.05$) relative to Rangelander at 1.53 dSm-1 (control). Maximum colour represents all transcript relative levels above that number. Blue box, transcription factors. Red box, protein modification. Green box, protein degradation.

171x192mm (104 x 104 DPI)

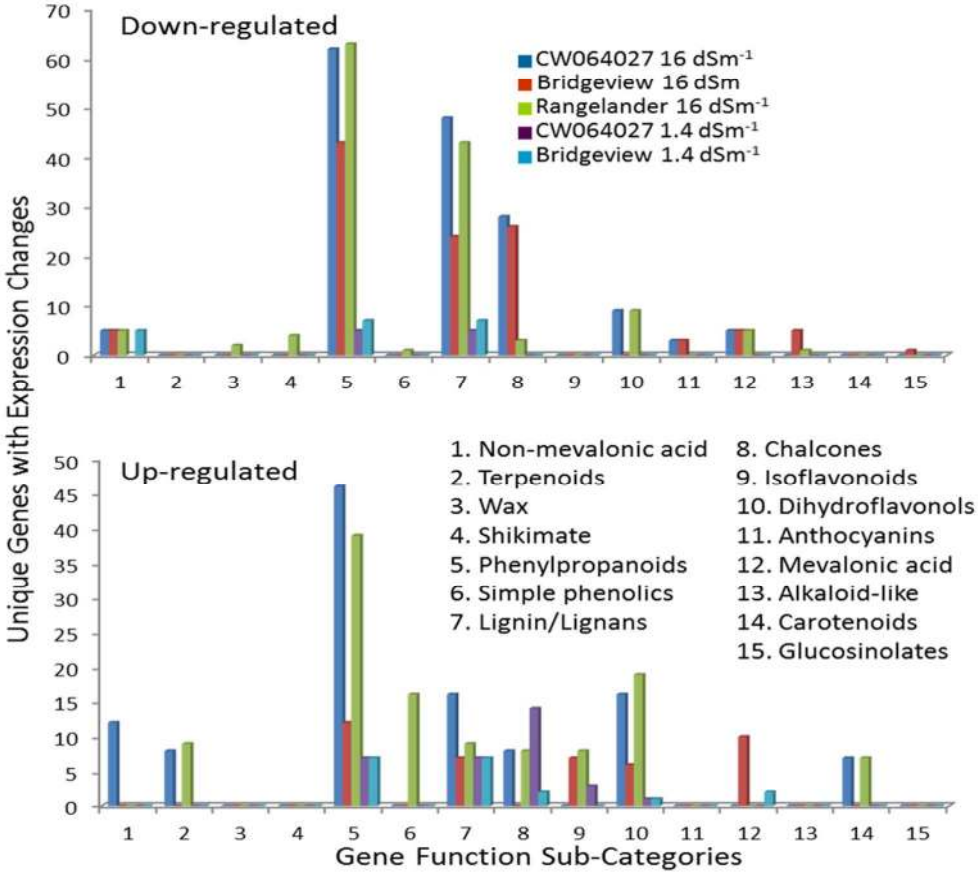
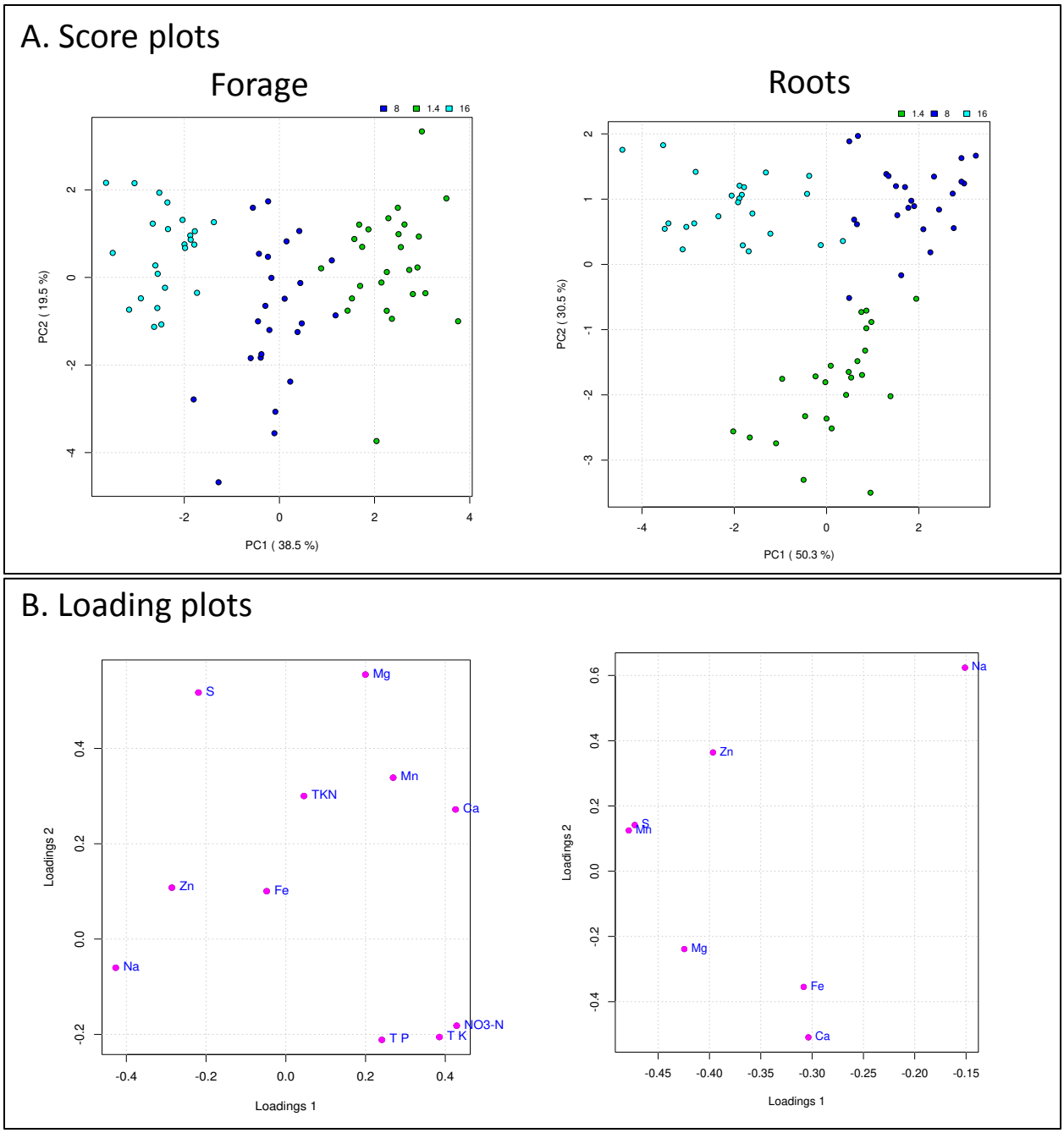
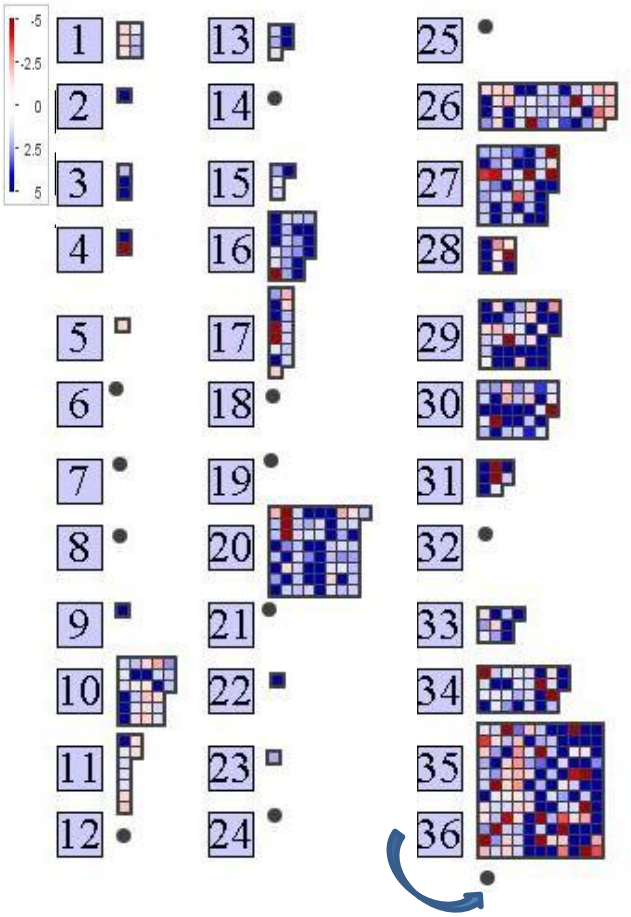


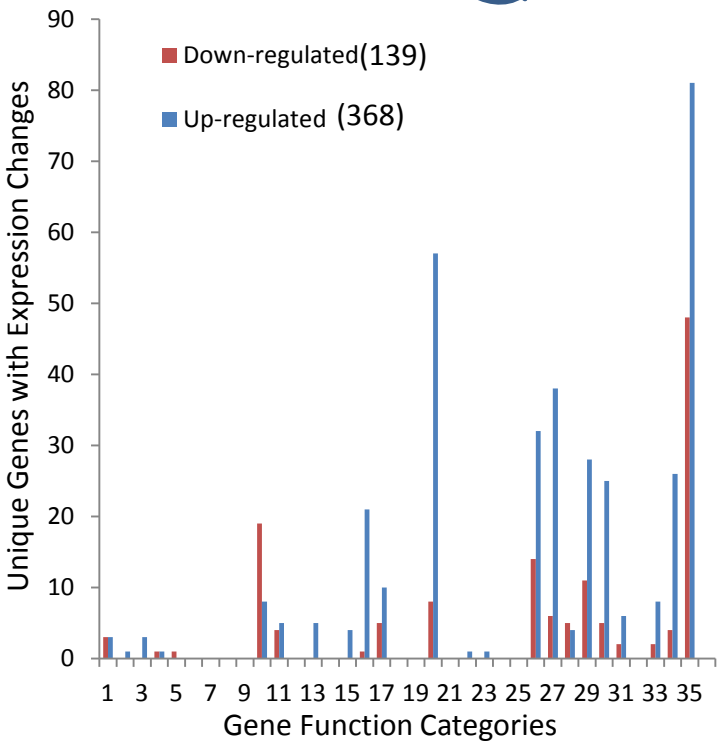
Figure 7. Mapman heatmap cartoon of forage transcriptome diversity for secondary metabolite factors (after Mapman analysis) for salt-tolerant CW064027, salt-tolerant Bridgeview, and salt-sensitive Rangelander at 15.6 dSm-1 relative to the Rangelander shoot transcriptome at 1.53 dSm-1 (control).

181x196mm (146 x 123 DPI)



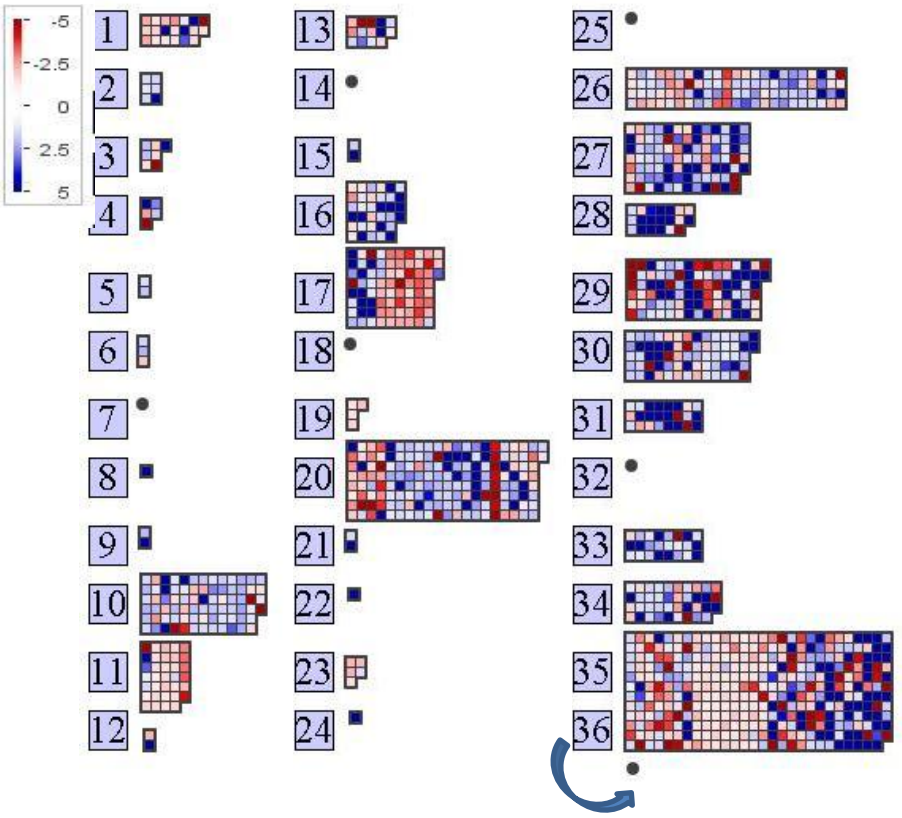
Supplementary Figure S1. Principle component score plots and loading plots for shoot and root ions (and other shoot minerals).

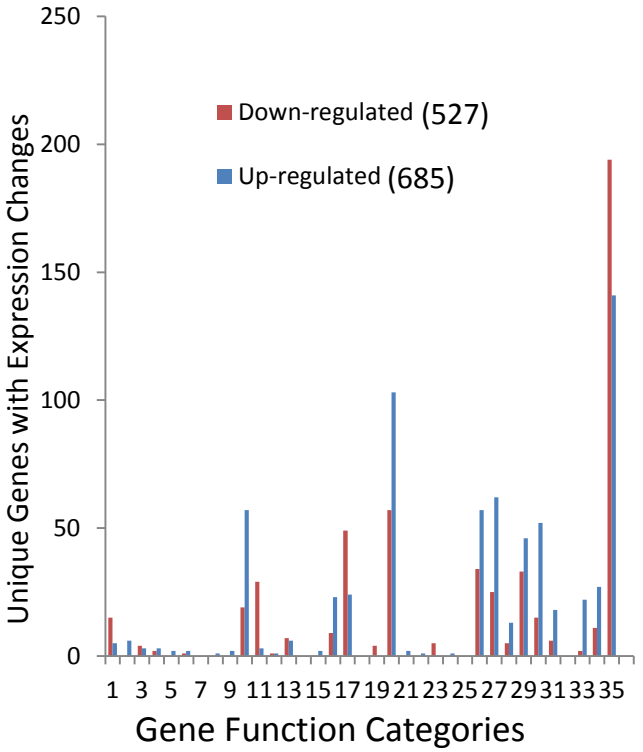




1. Photosynthesis/photorespiration
2. Major Carbohydrates
3. Minor Carbohydrates
4. Glycolysis
5. Fermentation
6. Gluconeogenesis
7. OPP oxidation
8. TCA/organic acid transformations
9. Mitochondrial electron transport
10. Cell wall synthesis/degradation/modification
11. Lipid metabolism
12. Nitrogen metabolism
13. Amino acid metabolism
14. Sulphur metabolism
15. Metal handling (acquiring/binding)
16. Secondary metabolism
17. Hormone metabolism
18. Cofactor and vitamin metabolism
19. Tetrapyrrole synthesis
20. Stress
21. Redox (thioredoxin, ascorbate, glutathione, heme, redoxins, superoxide dismutases, peroxidases)
22. Polyamine metabolism
23. Nucleotide metabolism
24. Biodegradation of xenobiotics
25. C1 metabolism
26. Miscellaneous
27. RNA processing, binding, regulation
28. DNA synthesis, repair, chromatin,
29. Protein synthesis, targeting, modification, degradation, folding, glycosylation
30. Signalling
31. Cell organization, division, cycle, vesicles, death
32. MicroRNA, natural antisense RNA
33. Development, storage proteins, SPLs, embryogenesis, unspecified
34. Transport
35. Not assigned/unknown
36. trichomes

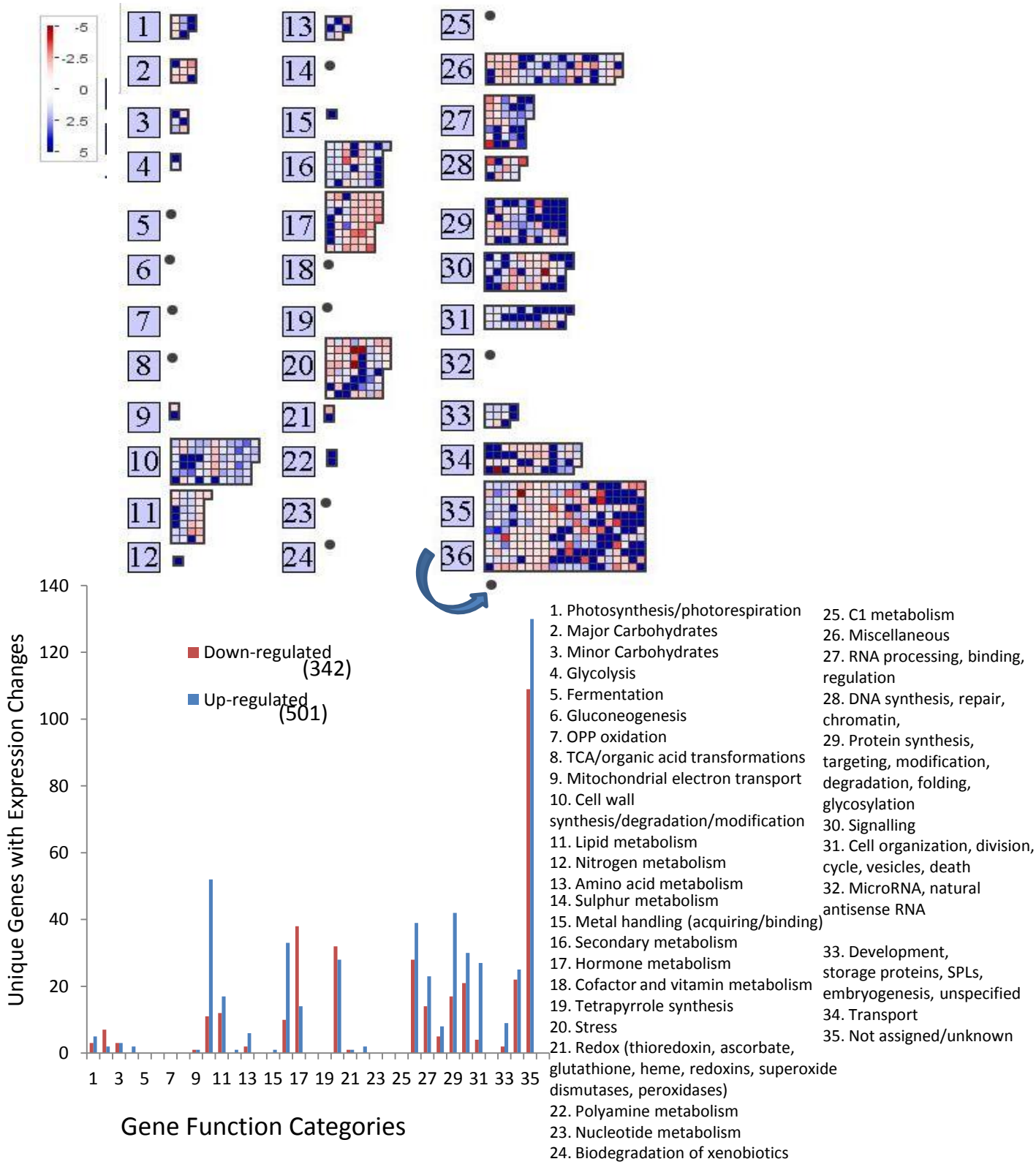
Supplementary Figure S2. Heat map for general metabolism EST shoot transcriptome changes for Bridgeview (1.53 dSm^{-1}) compared with Rangelander (1.53 dSm^{-1}).



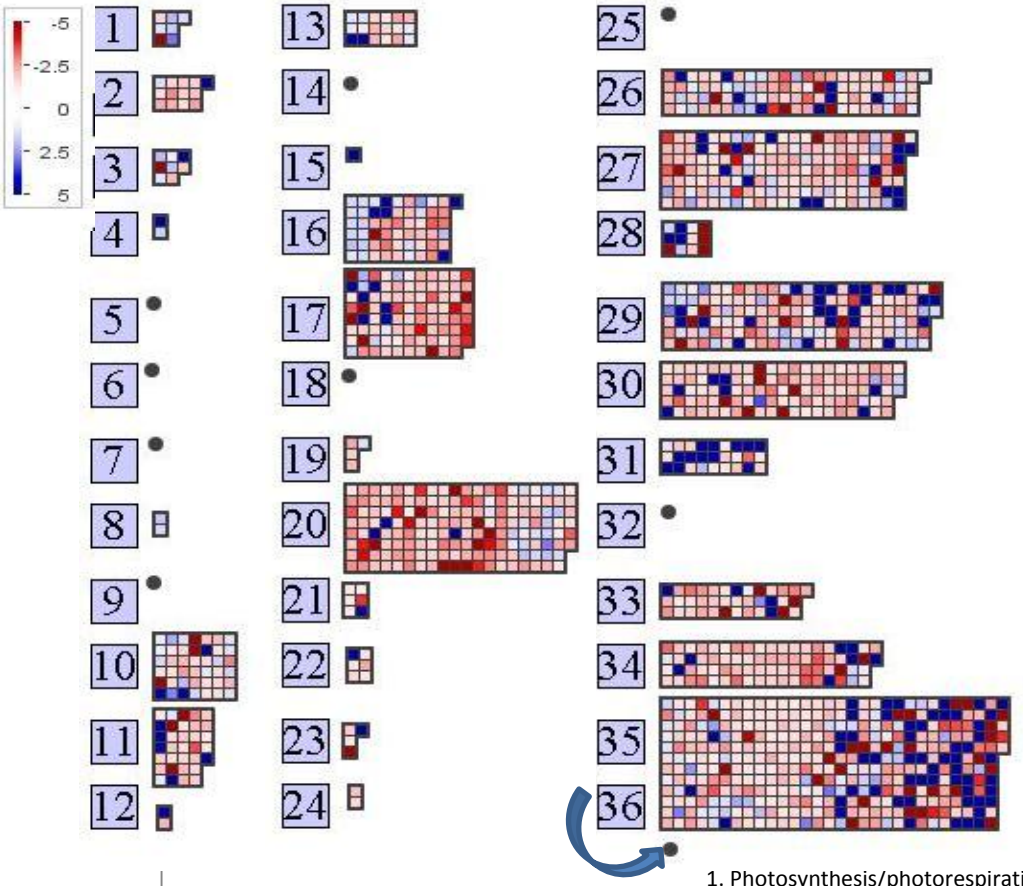


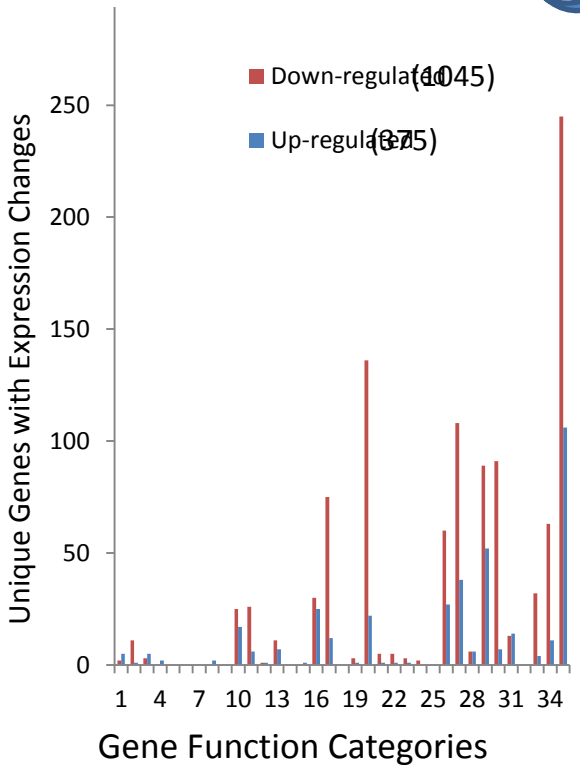
1. Photosynthesis/photorespiration
2. Major Carbohydrates
3. Minor Carbohydrates
4. Glycolysis
5. Fermentation
6. Gluconeogenesis
7. OPP oxidation
8. TCA/organic acid transformations
9. Mitochondrial electron transport
10. Cell wall synthesis/degradation/modification
11. Lipid metabolism
12. Nitrogen metabolism
13. Amino acid metabolism
14. Sulphur metabolism
15. Metal handling (acquiring/binding)
16. Secondary metabolism
17. Hormone metabolism
18. Cofactor and vitamin metabolism
19. Tetrapyrrole synthesis
20. Stress
21. Redox (thioredoxin, ascorbate, glutathione, heme, redoxins, superoxide dismutases, peroxidases)
22. Polyamine metabolism
23. Nucleotide metabolism
24. Biodegradation of xenobiotics
25. C1 metabolism
26. Miscellaneous
27. RNA processing, binding, regulation
28. DNA synthesis, repair, chromatin,
29. Protein synthesis, targeting, modification, degradation, folding, glycosylation
30. Signalling
31. Cell organization, division, cycle, vesicles, death
32. MicroRNA, natural antisense RNA
33. Development, storage proteins, SPLs, embryogenesis, unspecified
34. Transport
35. Not assigned/unknown
36. Trichomes

Supplementary Figure S3. Heat map for general metabolism EST changes for CW064027 (1.53 dSm^{-1}) compared with Rangelander (1.53 dSm^{-1}).



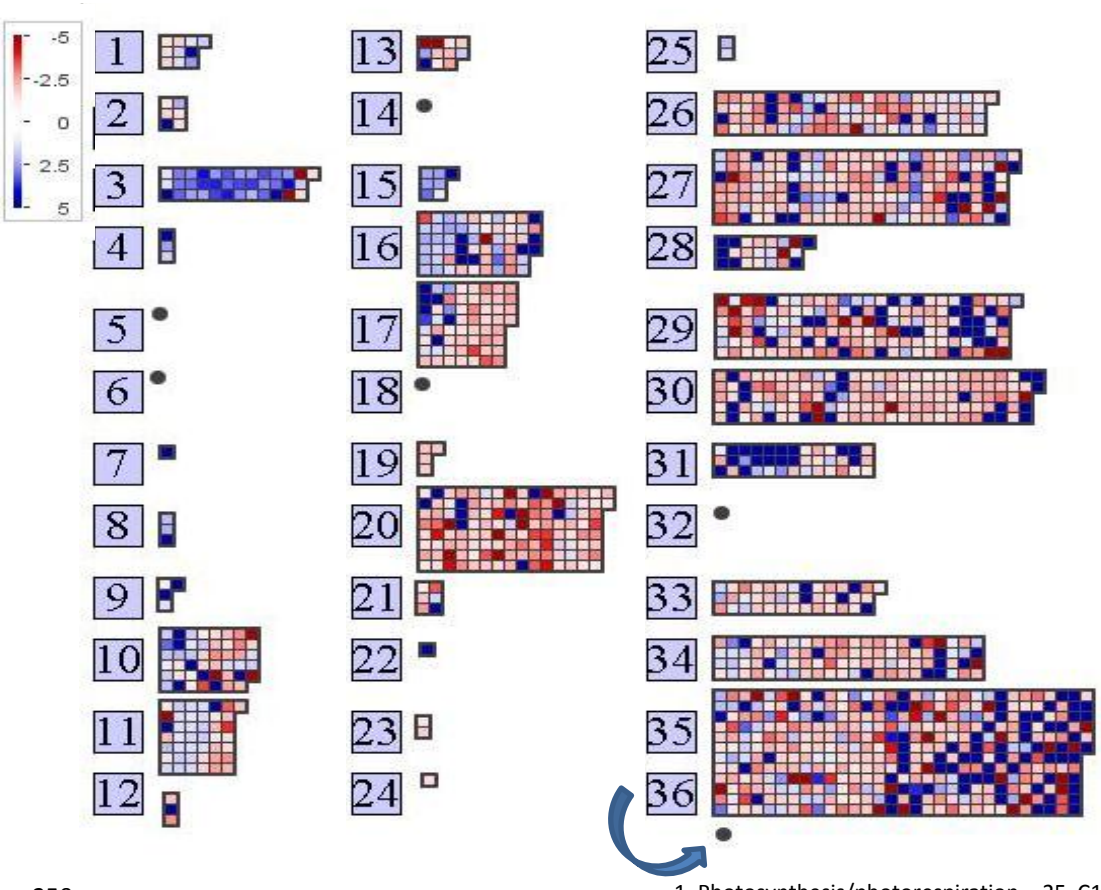
Supplementary Figure S4. Heat map for general metabolism EST changes for Rangelander (8 dSm^{-1}) compared with Rangelander (1.53 dSm^{-1}).

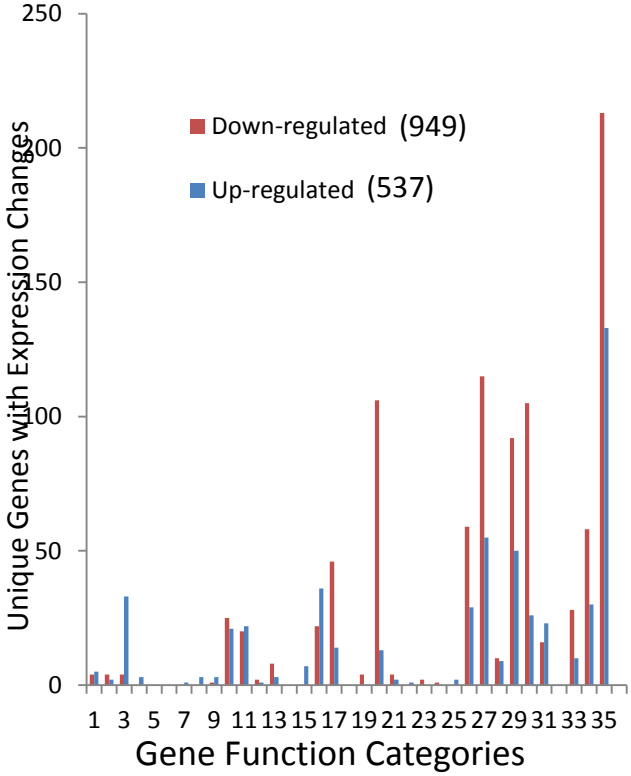




1. Photosynthesis/photorespiration
2. Major Carbohydrates
3. Minor Carbohydrates
4. Glycolysis
5. Fermentation
6. Gluconeogenesis
7. OPP oxidation
8. TCA/organic acid transformations
9. Mitochondrial electron transport
10. Cell wall synthesis/degradation/modification
11. Lipid metabolism
12. Nitrogen metabolism
13. Amino acid metabolism
14. Sulphur metabolism
15. Metal handling (acquiring/binding)
16. Secondary metabolism
17. Hormone metabolism
18. Cofactor and vitamin metabolism
19. Tetrapyrrole synthesis
20. Stress
21. Redox (thioredoxin, ascorbate, glutathione, heme, redoxins, superoxide dismutases, peroxidases)
22. Polyamine metabolism
23. Nucleotide metabolism
24. Biodegradation of xenobiotics
25. C1 metabolism
26. Miscellaneous
27. RNA processing, binding, regulation
28. DNA synthesis, repair, chromatin,
29. Protein synthesis, targeting, modification, degradation, folding, glycosylation
30. Signalling
31. Cell organization, division, cycle, vesicles, death
32. MicroRNA, natural antisense RNA
33. Development, storage proteins, SPLs, embryogenesis, unspecified
34. Transport
35. Not assigned/unknown
36. Trichomes

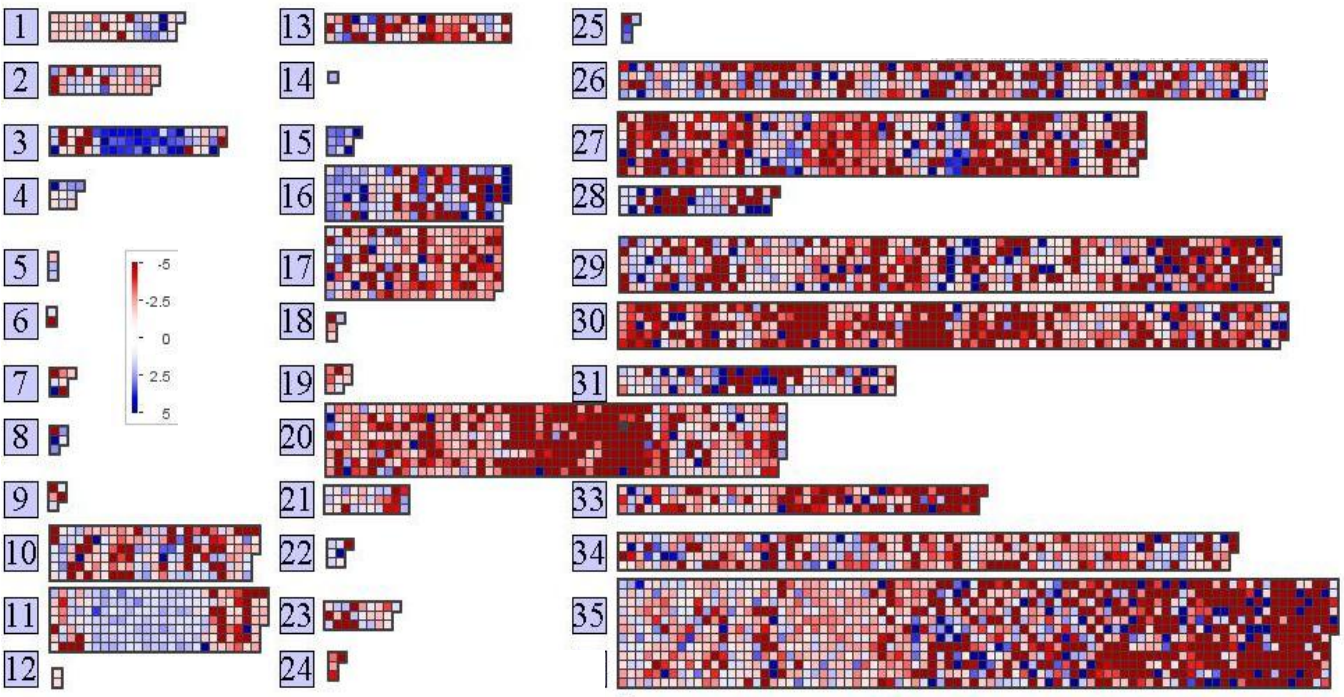
Supplementary Figure S5. Heat map for general metabolism EST changes for Bridgeview (8 dSm^{-1}) compared with Rangelander (1.53 dSm^{-1}).

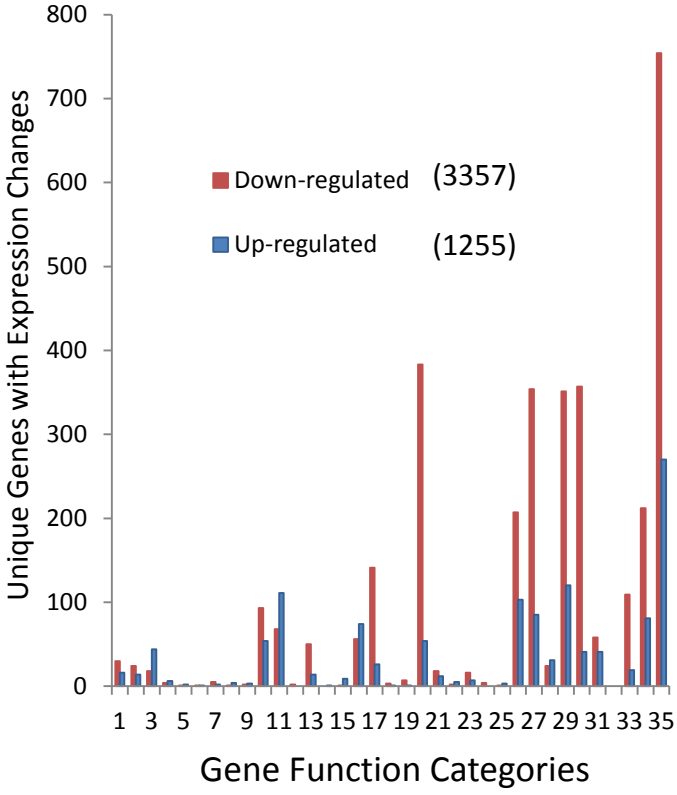




1. Photosynthesis/photorespiration
2. Major Carbohydrates
3. Minor Carbohydrates
4. Glycolysis
5. Fermentation
6. Gluconeogenesis
7. OPP oxidation
8. TCA/organic acid transformations
9. Mitochondrial electron transport
10. Cell wall synthesis/degradation/modification
11. Lipid metabolism
12. Nitrogen metabolism
13. Amino acid metabolism
14. Sulphur metabolism
15. Metal handling (acquiring/binding)
16. Secondary metabolism
17. Hormone metabolism
18. Cofactor and vitamin metabolism
19. Tetrapyrrole synthesis
20. Stress
21. Redox (thioredoxin, ascorbate, glutathione, heme, redoxins, superoxide dismutases, peroxidases)
22. Polyamine metabolism
23. Nucleotide metabolism
24. Biodegradation of xenobiotics
25. C1 metabolism
26. Miscellaneous
27. RNA processing, binding, regulation
28. DNA synthesis, repair, chromatin,
29. Protein synthesis, targeting, modification, degradation, folding, glycosylation
30. Signalling
31. Cell organization, division, cycle, vesicles, death
32. MicroRNA, natural antisense RNA
33. Development, storage proteins, SPLs, embryogenesis, unspecified
34. Transport
35. Not assigned/unknown
36. Trichomes

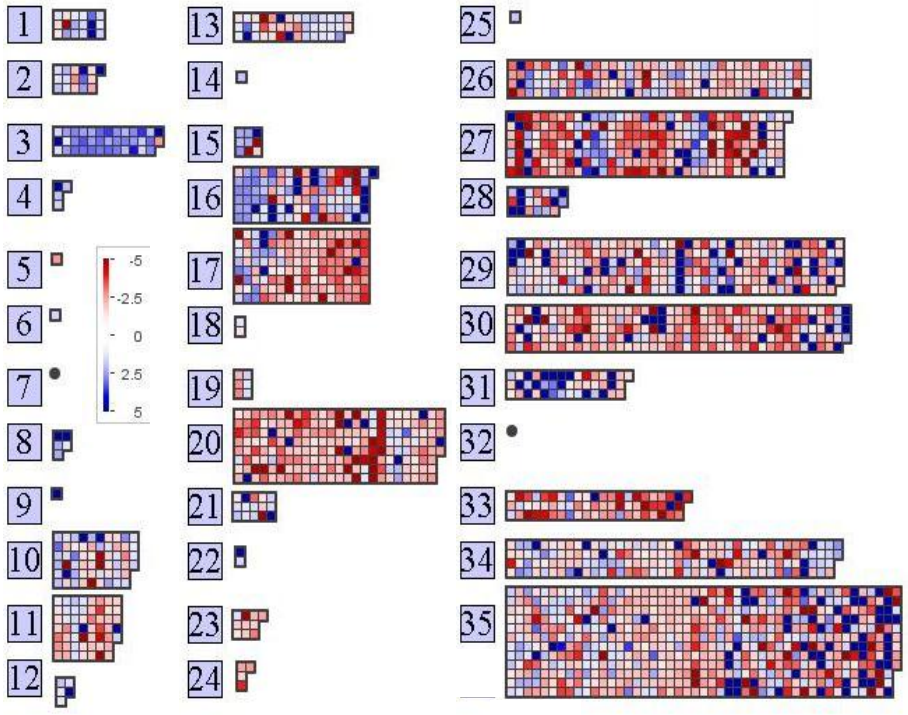
Supplementary Figure S6. Heat map for general metabolism EST changes for CW064027 (8 dSm^{-1}) compared with Rangelander (1.53 dSm^{-1}).



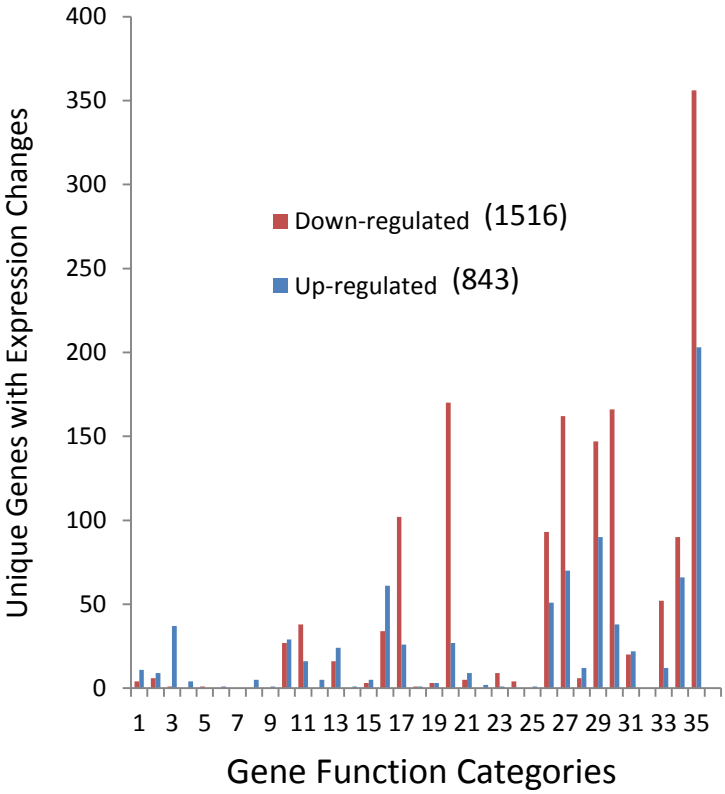


1. Photosynthesis/photorespiration
2. Major Carbohydrates
3. Minor Carbohydrates
4. Glycolysis
5. Fermentation
6. Gluconeogenesis
7. OPP oxidation
8. TCA/organic acid transformations
9. Mitochondrial electron transport
10. Cell wall synthesis/degradation/modification
11. Lipid metabolism
12. Nitrogen metabolism
13. Amino acid metabolism
14. Sulphur metabolism
15. Metal handling (acquiring/binding)
16. Secondary metabolism
17. Hormone metabolism
18. Cofactor and vitamin metabolism
19. Tetrapyrrole synthesis
20. Stress
21. Redox (thioredoxin, ascorbate, glutathione, heme, redoxins, superoxide dismutases, peroxidases)
22. Polyamine metabolism
23. Nucleotide metabolism
24. Biodegradation of xenobiotics
25. C1 metabolism
26. Miscellaneous
27. RNA processing, binding, regulation
28. DNA synthesis, repair, chromatin,
29. Protein synthesis, targeting, modification, degradation, folding, glycosylation
30. Signalling
31. Cell organization, division, cycle, vesicles, death
32. MicroRNA, natural antisense RNA
33. Development, storage proteins, SPLs, embryogenesis, unspecified
34. Transport
35. Not assigned/unknown

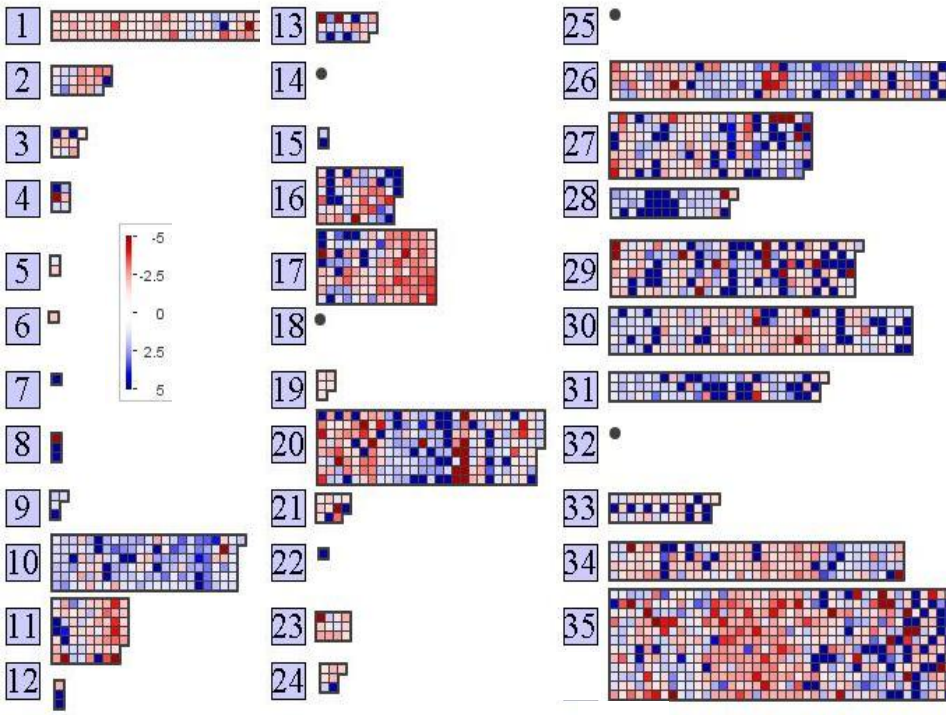
Supplementary Figure S7. Heat map for general metabolism EST changes for Rangelander (15.6 dSm^{-1}) relative to Rangelander (1.53 dSm^{-1}).



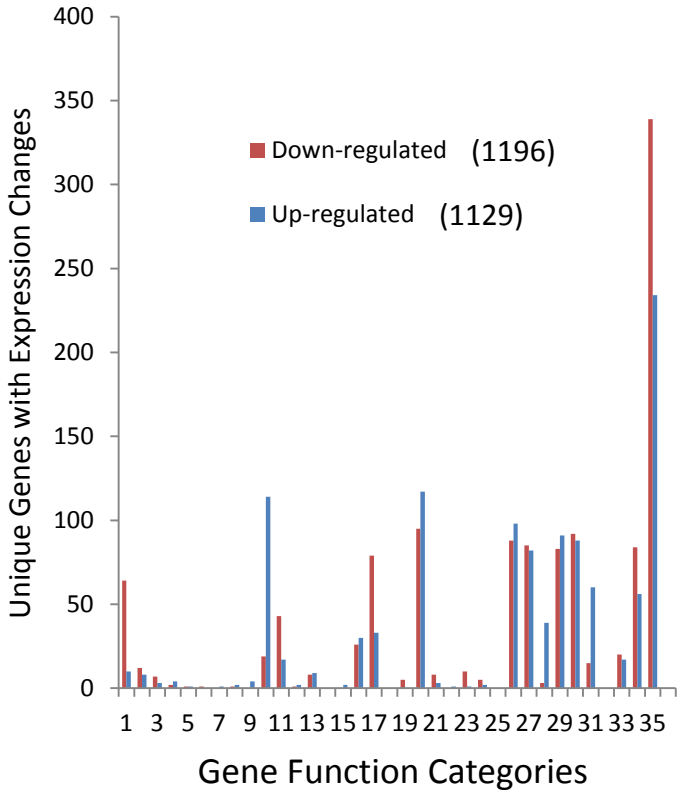
1. Photosynthesis/photorespiration
2. Major Carbohydrates
3. Minor Carbohydrates
4. Glycolysis
5. Fermentation
6. Gluconeogenesis
7. OPP oxidation
8. TCA/organic acid transformations
9. Mitochondrial electron transport
10. Cell wall synthesis/degradation/modification
11. Lipid metabolism
12. Nitrogen metabolism
13. Amino acid metabolism
14. Sulphur metabolism
15. Metal handling (acquiring/binding)
16. Secondary metabolism
17. Hormone metabolism
18. Cofactor and vitamin metabolism
19. Tetrapyrrole synthesis
20. Stress
21. Redox (thioredoxin, ascorbate, glutathione, heme, redoxins, superoxide dismutases, peroxidases)
22. Polyamine metabolism
23. Nucleotide metabolism
24. Biodegradation of xenobiotics
25. C1 metabolism
26. Miscellaneous
27. RNA processing, binding, regulation
28. DNA synthesis, repair, chromatin,
29. Protein synthesis, targeting, modification, degradation, folding, glycosylation
30. Signalling
31. Cell organization, division, cycle, vesicles, death
32. MicroRNA, natural antisense RNA
33. Development, storage proteins, SPLs, embryogenesis, unspecified
34. Transport
35. Not assigned/unknown



Supplementary Figure S8. Heat map for general metabolism EST changes for salt-tolerant Bridgeview (15.6 dSm^{-1}) relative to Rangelander (1.53 dSm^{-1}).



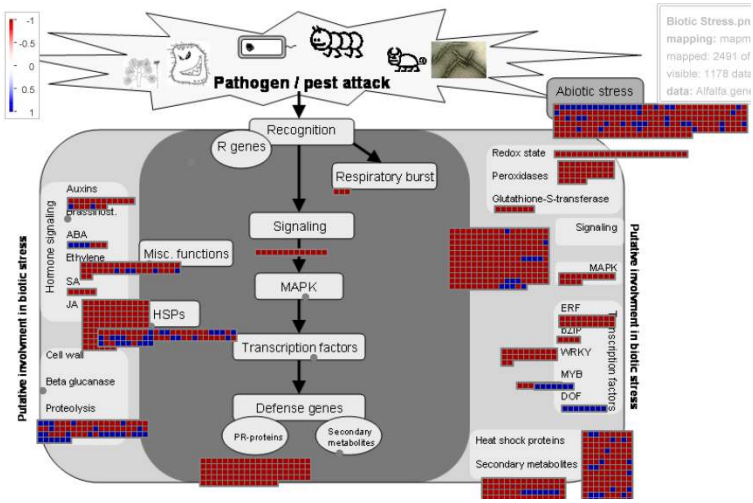
1. Photosynthesis/photorespiration
2. Major Carbohydrates
3. Minor Carbohydrates
4. Glycolysis
5. Fermentation
6. Gluconeogenesis
7. OPP oxidation
8. TCA/organic acid transformations
9. Mitochondrial electron transport
10. Cell wall synthesis/degradation/modification
11. Lipid metabolism
12. Nitrogen metabolism
13. Amino acid metabolism
14. Sulphur metabolism
15. Metal handling (acquiring/binding)



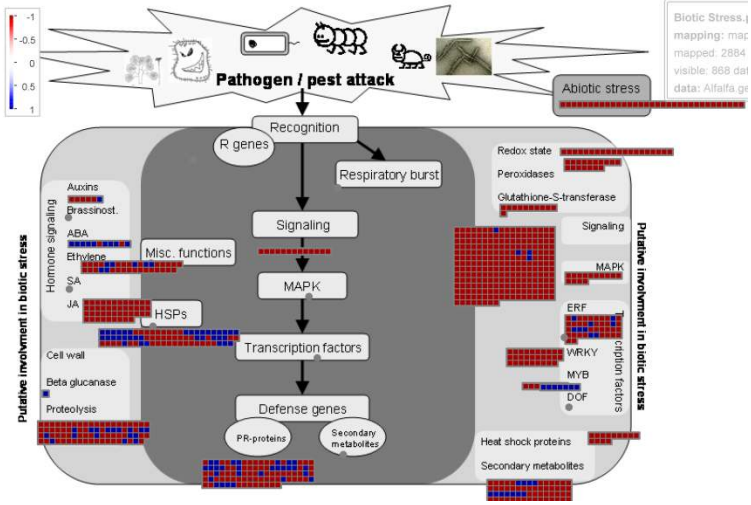
16. Secondary metabolism
17. Hormone metabolism
18. Cofactor and vitamin metabolism
19. Tetrapyrrole synthesis
20. Stress
21. Redox (thioredoxin, ascorbate, glutathione, heme, redoxins, superoxide dismutases, peroxidases)
22. Polyamine metabolism
23. Nucleotide metabolism
24. Biodegradation of xenobiotics
25. C1 metabolism
26. Miscellaneous
27. RNA processing, binding, regulation
28. DNA synthesis, repair, chromatin,
29. Protein synthesis, targeting, modification, degradation, folding, glycosylation
30. Signalling
31. Cell organization, division, cycle, vesicles, death
32. MicroRNA, natural antisense RNA
33. Development, storage proteins, SPLs, embryogenesis, unspecified
34. Transport
35. Not assigned/unknown

Supplementary Figure S9. Heat map for general metabolism EST changes for salt-tolerant CW06027 (15.6 dSm^{-1}) relative to Rangelander (1.53 dSm^{-1}).

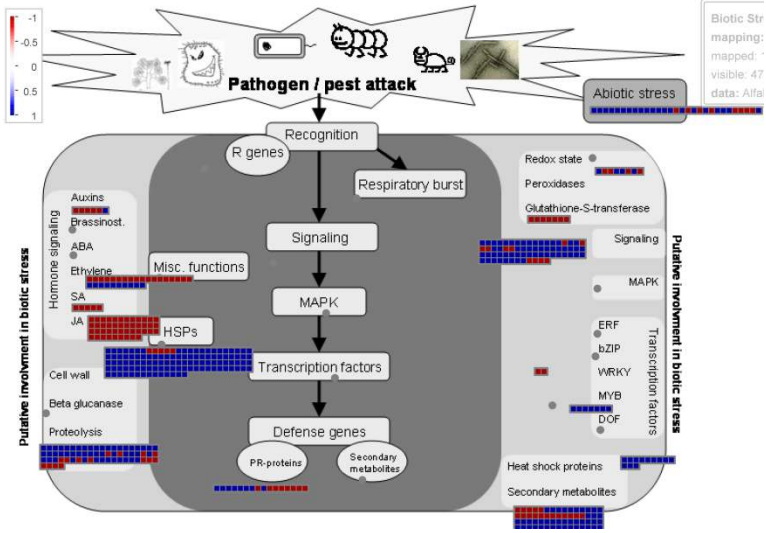
A. CW064027 8 dSm^{-1} : Rangelander 1.53 dSm^{-1}



B. Bridgeview 8 dSm^{-1} : Rangelander 1.53 dSm^{-1}

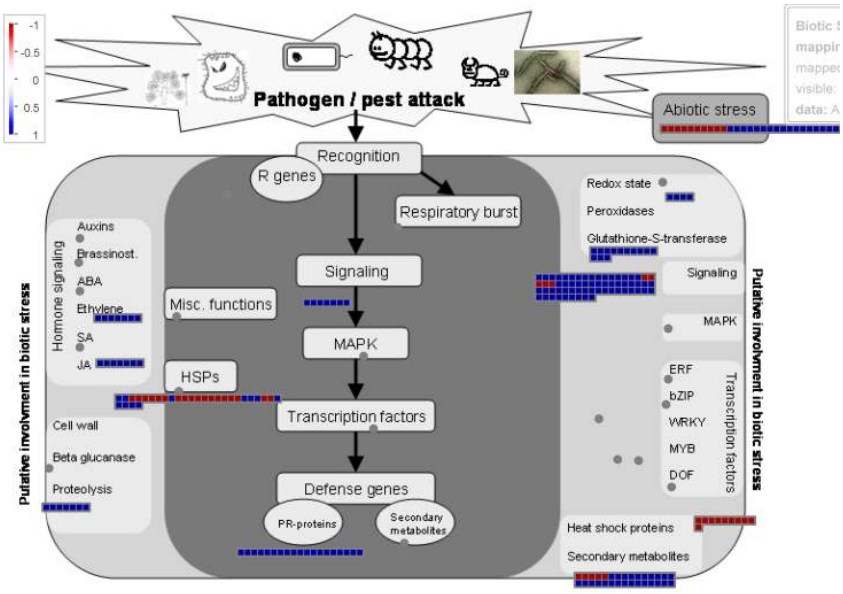


C. Rangelander 8 dSm^{-1} : Rangelander 1.53 dSm^{-1}

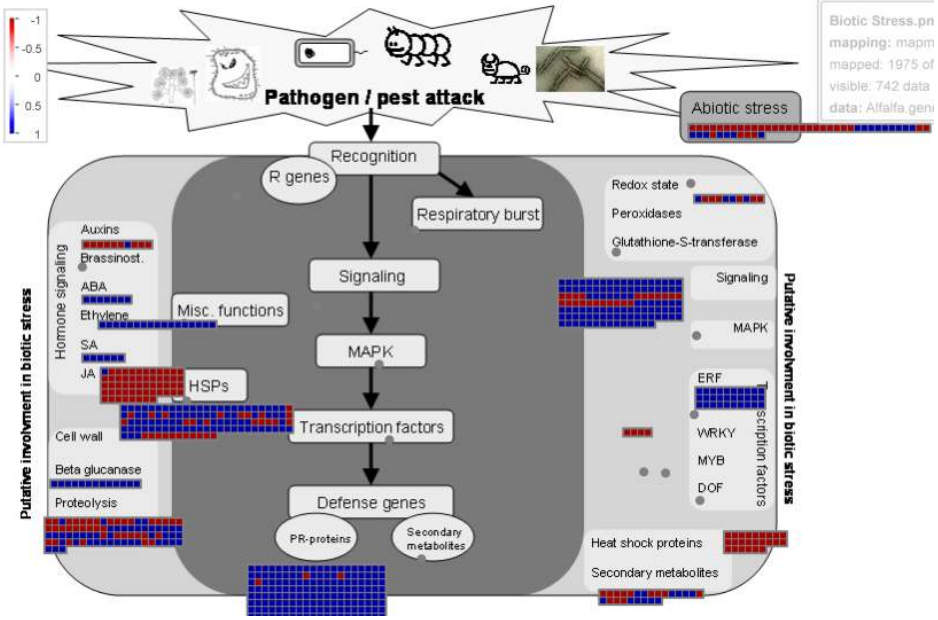


Supplementary Figure S10. Forage stress transcriptome at 8 dSm^{-1} for CW064027, Bridgeview, and Rangelander compared with Rangelander (at 1.53 dSm^{-1}).

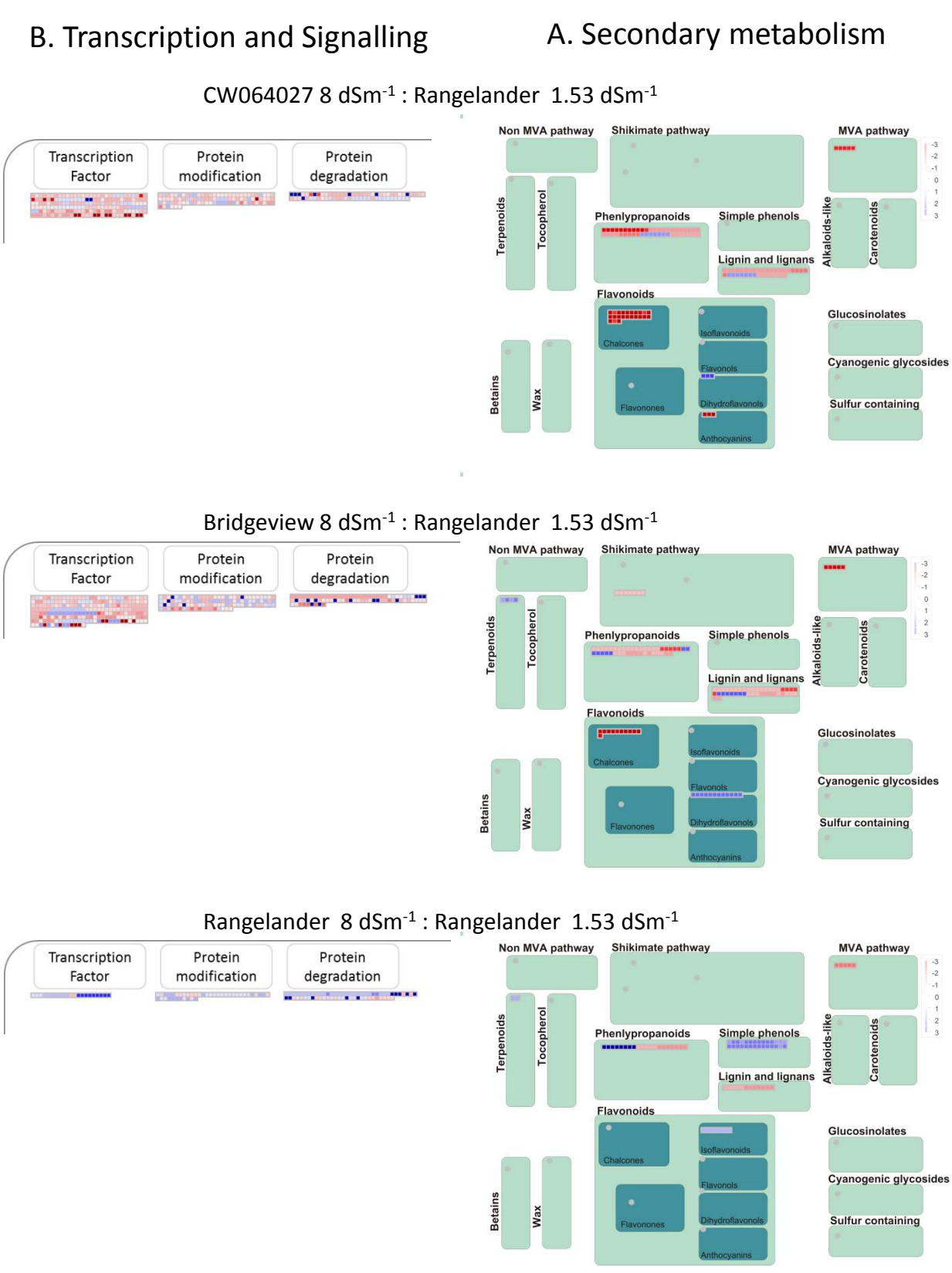
A. CW064027 1.53 dSm^{-1} : Rangelander 1.53 dSm^{-1}



B. AC Saltlander 1.53 dSm^{-1} : Rangelander 1.53 dSm^{-1}



Supplementary Figure S11. Shoot stress transcriptome at 1.53 dSm^{-1} for CW064027 and Bridgeview compared with Rangelander (at 1.53 dSm^{-1}).



Supplementary Figure S12. Shoot transcriptome at 8 dSm^{-1} for secondary metabolites, transcription factors, and signalling factors for CW064027, Bridgeview, and Rangelander compared with Rangelander (at 1.53 dSm^{-1}).

Supplementary Table S1. **Accumulated Ions and Minerals** in forage of two saline-selected alfalfa populations and one non-selected population. ND, not determined. NA, not applicable.

EC (dSm^{-1})	Cultivar Code	Value	Ions									Nutritional Components		
			Na (ppm)	Ca (ppm)	Mg (ppm)	Fe (ppm)	Mn (ppm)	Zn (ppm)	NO_3-N (ppm)	TK (10^4) (ppm)	K/Na selectivity	TKN (%)	TP (%)	S (%)
1.53 Shoot	A (Rangelander)	mean	1072.4	18945.5	2844.5	103.1	80.6	28.0	11641.5	5.2	48.3	4.6	0.4	2780.3
		SD	374.9	1528.1	749.0	85.5	12.0	4.0	826.8	0.4	11.1	0.5	0.0	270.6
	C (Bridgeview)	mean	1000.8	16155.6	2705.7	91.5	68.3	24.3	8660.2	4.7	46.8	4.9	0.4	2936.8
		SD	364.7	2699.4	136.2	67.9	17.1	4.9	425.5	0.4	10.1	0.3	0.0	313.8
	F (CW064027)	mean	966.9	17031.5	2422.6	63.6	59.0	18.8	9585.9	5.2	53.6	4.4	0.3	2768.7
		SD	334.5	4248.2	207.3	6.8	10.8	2.7	579.5	0.2	6.8	0.4	0.1	185.0
8.03 Shoot	A (Rangelander)	mean	3528.5	13739.7	2183.7	125.3	69.7	31.0	7768.6	4.8	13.6	4.3	0.4	3256.8
		SD	1065.4	1118.9	278.6	67.2	17.5	6.5	1186.2	0.3	2.4	0.3	0.0	233.1
	C (Bridgeview)	mean	4414.0	10947.4	1971.3	55.8	67.4	24.7	7315.1	4.8	10.8	4.2	0.4	2554.8
		SD	690.3	1933.8	339.1	18.8	37.2	2.8	2132.9	0.4	5.6	0.3	0.0	466.5
	F (CW064027)	mean	4008.8	12233.9	1978.6	56.4	66.2	23.8	7350.8	4.6	11.6	4.8	0.4	2915.5
		SD	322.1	515.7	150.8	2.1	11.4	2.8	1649.2	0.3	9.4	0.4	0.0	170.4
15.61 Shoot	A (Rangelander)	mean	6677.3	10784.2	2465.8	74.3	54.4	34.5	5080.8	4.3	6.4	4.5	0.3	4407.9
		SD	1522.4	698.5	182.2	3.9	6.4	4.6	884.2	0.2	1.6	0.2	0.0	482.5
	C (Bridgeview)	mean	6137.5	9213.1	2136.5	200.9	41.5	38.5	5282.4	4.3	7.0	4.7	0.3	3475.6
		SD	304.3	1782.3	258.7	116.4	8.9	4.8	1127.1	0.2	8.1	0.3	0.0	704.1
	F (CW064027)	mean	5643.9	9691.2	2133.0	58.8	52.1	28.8	5096.8	4.2	7.4	4.5	0.3	3309.3
		SD	956.0	849.3	166.7	5.1	8.0	3.1	330.7	0.3	2.8	0.6	0.0	315.3
1.53 Root	A (Rangelander)	mean	1952.1	6354.2	2147.6	350.1	99.6	30.0	ND	ND	ND	ND	ND	3266.4
		SD	699.2	1727.3	381.5	234.6	46.7	5.6						118.4
	C (Bridgeview)	mean	1935.6	6367.6	2304.8	410.8	86.2	29.6	ND	ND	ND	ND	ND	3127.6
		SD	497.1	1677.5	281.0	324.6	47.7	10.1						343.4
	F (CW064027)	mean	1614.6	6673.8	2441.8	608.4	87.0	26.3	ND	ND	ND	ND	ND	3251.0
		SD	328.7	2260.9	342.9	544.8	15.6	11.6						637.6
8.03 Root	A (Rangelander)	mean	8962.2	3246.3	1425.1	143.0	63.3	41.7	ND	ND	ND	ND	ND	2828.6
		SD	2102.3	500.9	257.4	48.1	42.7	28.9						284.3
	C (Bridgeview)	mean	9454.4	3479.0	1678.2	191.3	110.1	58.7	ND	ND	ND	ND	ND	2660.0
		SD	1484.1	260.0	158.8	48.3	46.9	59.9						257.5
	F (CW064027)	mean	6684.1	3131.1	1585.4	295.4	62.3	27.2						2550.6
		SD	655.1	125.8	148.5	173.8	16.3	5.6	ND	ND	ND	ND	ND	222.5
15.61 Root	A (Rangelander)	mean	14233.8	5209.3	2454.4	409.2	301.9	256.0	ND	ND	ND	ND	ND	4926.0
		SD	2355.7	1176.4	191.7	218.9	210.6	255.7						640.2
	C (Bridgeview)	mean	12546.7	4907.8	2129.7	424.8	196.8	147.7	ND	ND	ND	ND	ND	4088.9

	SD	2570.1	662.3	294.9	92.4	74.1	56.6						498.2
F (CW064027)	mean	11607.6	4541.3	2500.2	454.6	190.9	72.8	ND	ND	ND	ND	ND	4658.0
	SD	2376.4	728.8	493.5	135.5	70.5	24.3						1115.1
Statistical Tests:													
EC adjusted (shoots)		7.5E-30	2.6E-16	9.2E-08	8.7E-01	2.1E-03	9.5E-08	4.6E-15	3.8E-11	NA	4.6E-02	2.9E-04	5.6E-08
Cultivar adjusted (shoots)		1.0E+00	6.5E-01	3.9E-01	1.0E+00	1.0E+00	3.9E-01	1.0E+00	1.0E+00	NA	1.0E+00	7.9E-02	4.8E-01
Interaction adjusted (shoots)		1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	NA	1.0E+00	1.0E+00	1.0E+00
EC adjusted (roots)		5.8E-33	5.0E-13	4.3E-16	3.1E-06	1.3E-11	2.1E-13	NA	ND	ND	ND	ND	2.0E-15
Cultivar adjusted (roots)		8.8E-02	7.0E-01	5.1E-03	1.0E+00	1.0E+00	1.4E-01	NA	ND	ND	ND	ND	3.0E-01
Interaction adjusted (roots)		1.0E+00	1.0E+00	3.5E-01	1.0E+00	1.0E+00	1.0E+00	NA	ND	ND	ND	ND	1.0E+00

Supplementary Table S2.1. Major carbohydrate transcripts. ±1000 in relative intesity shows the f

Rangelander 15.6 dSm^{-1} relative to Rangelander 1.53 dSm^{-1}						Bridgeview
Bin Code	Bin Name	ID	Type	Description	Relative Intensity	Bin Code
Sugars						
2.1.1.2	major CHO	m.s.atnode_79570_	Transcript	highly similar tc	-1000	2.2.1.3.1
2.1.2.4	major CHO	m.s.atnode_83123_	Transcript	nearly identical	-1000	2.2.2.2
2.2.1.3.1	major CHO	m.s.atnode_17501_	Transcript	highly similar tc	-1000	2.2.1.3.1
2.2.1.5	major CHO	m.s.atnode_67862_	Transcript	nearly identical	-1000	2.2.1.3.1
2.2.2.1	major CHO	m.s.atnode_66484_	Transcript	highly similar tc	-1000	2.2.2.2
2.2.2.1.2	major CHO	m.s.at146148881	Transcript	highly similar tc	-2.97736	2.2.2.1.2
2.2.2.2	major CHO	m.s.atnode_83916_	Transcript	nearly identical	-2.89876	2.1.2.1
2.2.2.1.2	major CHO	m.s.at146148890	Transcript	highly similar tc	-2.62283	2.1.1.1
2.2.1.3.1	major CHO	m.s.atnode_12977_	Transcript	highly similar tc	-2.37474	2.1.1.1
2.2.2.2	major CHO	m.s.atnode_86692_	Transcript	nearly identical	-2.34164	2.1.1.1
2.2.1.3.1	major CHO	m.s.attc11627	Transcript	highly similar tc	-2.30101	2.1.1.1
2.2.2.8	major CHO	m.s.attc12267	Transcript	highly similar tc	-2.28469	2.1.1.1
2.2.1.3.1	major CHO	m.s.atnode_1428_le	Transcript	highly similar tc	-2.27816	2.2.2.1
2.2.2.1.2	major CHO	m.s.atnode_85141_	Transcript	highly similar tc	-2.22448	2.2.2.1
2.2.2.1	major CHO	m.s.attc3493	Transcript	highly similar tc	-2.09338	2.2.1.3.1
2.2.2.1.2	major CHO	m.s.at146148811	Transcript	highly similar tc	-1.98536	
2.2.2.1.2	major CHO	m.s.attc11565	Transcript	highly similar tc	-1.90739	
2.2.2.1.2	major CHO	m.s.attc19171	Transcript	highly similar tc	-1.85795	
2.2.2.2	major CHO	m.s.atnode_25589_	Transcript	nearly identical	-1.78408	
2.2.2.1.2	major CHO	m.s.at146148988	Transcript	highly similar tc	-1.78203	
2.2.2.1.2	major CHO	m.s.at124567571	Transcript	highly similar tc	-1.71101	
2.2.2.8	major CHO	m.s.atnode_37715_	Transcript	highly similar tc	-1.63464	
2.2.2.1.2	major CHO	m.s.at136141795	Transcript	highly similar tc	-1.54442	
2.2.2.1	major CHO	m.s.at136141617	Transcript	moderately sim	-1.11826	
2.2.2.1	major CHO	m.s.at50320524	Transcript	highly similar tc	1.39152	
2.2.2.1	major CHO	m.s.attc8190	Transcript	highly similar tc	1.39829	
2.2.2.1	major CHO	m.s.atf7snd4003f6c	Transcript	highly similar tc	1.55441	
2.2.1.5	major CHO	m.s.atf7snd4003g7p	Transcript	nearly identical	1.55962	
2.2.1.5	major CHO	m.s.atnode_148_ler	Transcript	highly similar tc	1.5728	
2.2.1.4	major CHO	m.s.atnode_20594_	Transcript	highly similar tc	1.79045	
2.2.1.5	major CHO	m.s.atf7snd4004jrq4	Transcript	nearly identical	1.86374	
2.2.2.1	major CHO	m.s.atf7snd4004h9l	Transcript	highly similar tc	1.88976	
2.2.2.1.2	major CHO	m.s.atnode_17692_	Transcript	highly similar tc	1.90285	
2.2.1.3.3	major CHO	m.s.attc9096	Transcript	highly similar tc	1.90545	
2.2.2.1.2	major CHO	m.s.atf7snd4004i8a	Transcript	highly similar tc	2.22114	
2.2.2.1	major CHO	m.s.atnode_69228_	Transcript	highly similar tc	2.46514	
2.1.1.1	major CHO	m.s.atnode_25891_	Transcript	nearly identical	2.50055	

old change for cases where either numinator or denominator was equal to zero for that target gene.

15.6 dSm^{-1} relative to Rangelander 1.53 dSm^{-1}					CW064027 15.6 dSm^{-1} relative to I		
Bin Name	ID	Type	Description	Relative Intensity	Bin Code	Bin Name	ID
major CHO metabolism.de	m.s.atnode	Transcript	highly simil	-2.69347	2.2.2.1.2	major CHO	m.s.at1461
major CHO metabolism.de	m.s.atnode	Transcript	nearly iden	-2.32473	2.2.2.1.2	major CHO	m.s.atnode
major CHO metabolism.de	m.s.atnode	Transcript	highly simil	-1.97411	2.2.2.1.2	major CHO	m.s.at1361
major CHO metabolism.de	m.s.attc11(	Transcript	highly simil	-1.90344	2.2.2.1.2	major CHO	m.s.at1245
major CHO metabolism.de	m.s.atnode	Transcript	nearly iden	-1.76593	2.2.2.1.2	major CHO	m.s.atf7snc
major CHO metabolism.de	m.s.at1361	Transcript	highly simil	-1.57677	2.2.2.1.2	major CHO	m.s.at1461
major CHO metabolism.sy	m.s.attc17(	Transcript	highly simil	1.37336	2.2.2.1.2	major CHO	m.s.at1461
major CHO metabolism.sy	m.s.at1461	Transcript	nearly iden	1.39589	2.2.2.1.2	major CHO	m.s.at1461
major CHO metabolism.sy	m.s.atnode	Transcript	nearly iden	1.52282	2.2.2.1.2	major CHO	m.s.attc11(
major CHO metabolism.sy	m.s.at1245	Transcript	nearly iden	1.52731	2.2.2.1.2	major CHO	m.s.at1461
major CHO metabolism.sy	m.s.atnode	Transcript	nearly iden	2.03676	2.2.2.1	major CHO	m.s.atnode
major CHO metabolism.sy	m.s.atnode	Transcript	nearly iden	2.18003	2.2.1.3.1	major CHO	m.s.atnode
major CHO metabolism.de	m.s.atnode	Transcript	highly simil	2.50011	2.2.1.5	major CHO	m.s.at1579
major CHO metabolism.de	m.s.atnode	Transcript	highly simil	3.10743	2.2.1.5	major CHO	m.s.at5032
major CHO metabolism.de	m.s.atnode	Transcript	highly simil	1000	2.2.2.1	major CHO	m.s.attc81(
					2.2.2.1	major CHO	m.s.atf7snc
					2.2.2.1	major CHO	m.s.at5032
					2.2.2.1.2	major CHO	m.s.atf7snc
					2.2.2.1	major CHO	m.s.atf7snc
					2.2.2.4	major CHO	m.s.atnode

Rangelander 1.53 dSm^{-1}		
Type	Description	Relative Intensity
Transcript	highly simil	-3.05246
Transcript	highly simil	-2.74092
Transcript	highly simil	-2.71535
Transcript	highly simil	-2.60091
Transcript	highly simil	-2.42324
Transcript	highly simil	-2.3218
Transcript	highly simil	-2.20061
Transcript	highly simil	-2.18719
Transcript	highly simil	-2.13473
Transcript	highly simil	-2.0115
Transcript	moderately	-1.51955
Transcript	highly simil	-1.32827
Transcript	nearly iden	1.41419
Transcript	highly simil	1.52208
Transcript	highly simil	1.54625
Transcript	highly simil	1.6808
Transcript	highly simil	1.71357
Transcript	highly simil	1.73294
Transcript	highly simil	2.79935
Transcript	weakly sim	1000