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Cell type-specific responses to salinity – the epidermal bladder cell transcriptome of *Mesembryanthemum crystallinum*

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Summary

- *Mesembryanthemum crystallinum* (ice plant) exhibits extreme tolerance to salt. Epidermal bladder cells (EBCs), developing on the surface of aerial tissues and specialized in sodium sequestration and other protective functions, are critical for the plant's stress adaptation. We present the first transcriptome analysis of EBCs isolated from intact plants, to investigate cell type-specific responses during plant salt adaptation.
- We developed a *de novo* assembled, nonredundant EBC reference transcriptome. Using RNAseq, we compared the expression patterns of the EBC-specific transcriptome between control and salt-treated plants.
- The EBC reference transcriptome consists of 37 341 transcript-contigs, of which 7% showed significantly different expression between salt-treated and control samples. We identified significant changes in ion transport, metabolism related to energy generation and osmolyte accumulation, stress signalling, and organelle functions, as well as a number of lineage-specific genes of unknown function, in response to salt treatment.
- The salinity-induced EBC transcriptome includes active transcript clusters, refuting the view of EBCs as passive storage compartments in the whole-plant stress response. EBC transcriptomes, differing from those of whole plants or leaf tissue, exemplify the importance of cell type-specific resolution in understanding stress adaptive mechanisms.

Introduction

The annual succulent *Mesembryanthemum crystallinum* (ice plant) is a model halophyte which has been used to search for physiological, biochemical, and molecular mechanisms fundamental to plant salt and water stress responses (Lüttge *et al.*, 1978; Adams *et al.*, 1998; Bohnert & Cushman, 2000; Barkla *et al.*, 2009). The adult ice plant reveals a series of adjustments to cope with stress, including dramatic metabolic changes from C₃ to Crassulacean acid metabolism (CAM), in response to a variety of conditions, such as high photon flux density, drought, and salinity (Adams *et al.*, 1998; Winter & Holtum, 2007). CAM permits the net uptake of CO₂ at night, and thus significantly increases water use efficiency (Cushman & Bohnert, 1999).

One outstanding trait of *M. crystallinum* is the presence of epidermal bladder cells (EBCs) on the aerial parts of the plant. These nonglandular, nonsecreting, single-cell trichomes line the plant's leaves, stems, and flower buds. EBCs remain compressed to the epidermal surface in unstressed plants, but expand to comprise up to 25% of the total aerial volume once the plants have responded to stresses (Steudle *et al.*, 1975). Fully developed

EBCs accumulate Na⁺, with concentrations up to > 1 M measured in EBC extracts (Adams *et al.*, 1992), and also compatible solutes, predominantly ononitol and pinitol (Paul & Cockburn, 1989; Vernon & Bohnert, 1992a). A mutational study implicated the presence of EBCs in *M. crystallinum* as a substantial contributing factor for salt tolerance (Agarie *et al.*, 2007). Enlarged EBCs may function in UV protection and defense by accumulating flavonoids and betacyanins, and act as reflectors protecting the underlying mesophyll cells from heat damage (Vogt *et al.*, 1999; Ibdah *et al.*, 2002).

Mesembryanthemum crystallinum expressed sequence tags (ESTs) have been used to compare gene expression in different developmental states, stress conditions, and diurnal cycles (Kore-eda *et al.*, 2004; Cushman *et al.*, 2008). Recently, proteomic approaches identified salt-responsive proteins from *M. crystallinum* leaves (Cosentino *et al.*, 2013). These studies measured molecular signatures in the context of the entire leaf, ignoring any cell type-specific processes and stress responses. The salt stress response is strikingly illuminated by the EBCs more than by any other cell type in *M. crystallinum*. Yet biochemical or molecular functions underlying the physiological and morphological changes in this specialized cell type have to date not been studied separately from the rest of the leaf tissue.

Despite the many biochemical and molecular data sets that have been produced (Barkla *et al.*, 2013; Borland *et al.*, 2014), few attempts have been made to dissect the role of the cell type-specific response in salt stress adaptation. A cell type-specific transcriptome atlas in rice (*Oryza sativa*) (Jiao *et al.*, 2009) confirmed that many cell-specific mRNAs are not detectable in whole-tissue or whole-organ samples. Obtaining cell type-specific transcriptomes remains a challenge despite the recognized need for microgenomics to access cell type-specific gene expression (Bailey-Serres, 2013).

A high-throughput method was developed to extract sap from EBCs, enabling the resolution of functional changes of this unique cell type (Barkla *et al.*, 2012). In a first attempt to explore the proteome of EBC sap from salt-treated plants, we identified proteins involved in ion and water homeostasis and found evidence for active participation in CAM by EBCs (Barkla *et al.*, 2012, 2013). These studies suggested that EBCs are metabolically highly active, refuting previous views of EBCs as mere metabolically passive reservoirs of carbon (as inositols and malate, for example), salt, and water (Lüttge *et al.*, 1978).

We report here the transcriptome of *M. crystallinum* EBCs from mature plants. We have assembled an EBC-specific reference transcriptome *de novo*, and have quantified transcript levels with and without plant reorganization in response to salt stress. Our results indicate the salinity-dependent activation of metabolic pathways in EBCs that may lead to compatible solute and pigment accumulation, as well as maintenance of a large arsenal of transporter systems. Our results also reveal EBC-specific pronounced transcriptome responses to salt in a number of precisely defined pathways. These responses differ from canonical plant 'salt stress' responses, and mirror changes in signal transduction and organelle functions, probably as a consequence of the salt-induced accumulation of osmolytes and the drastic anatomical modification of this particular cell type.

Materials and Methods

Plant material

Mesembryanthemum crystallinum L. plants derived from material originally collected by Winter *et al.* (1978) were grown in Metro-Mix510 soil (SunGro Horticulture, Bellevue, WA, USA), in a glasshouse under natural irradiation and photoperiod, where photosynthetic photon flux density reached a midday maximum of $1300 \mu\text{mol m}^{-2} \text{s}^{-1}$. Temperature was maintained at $25 \pm 3^\circ\text{C}$. Plants were watered daily, and half-strength Hoagland's medium (Hoagland & Arnon, 1950) was supplied weekly. NaCl treatment (200 mM) was initiated 6 wk after germination for a period of 14 d.

Extraction of EBC sap

EBC sap was obtained from cells in stems and on the leaf abaxial epidermal surface by vacuum aspiration using a fine-gauge insulin needle (27G; 13 mm) attached to a collection reservoir maintained on ice. The needle was oriented horizontally to the leaf or

stem axis to avoid removing sap from underlying tissue. The procedure was visualized using a Nikon SMZ645 stereo-microscope (Nikon, Mexico City, Mexico). The sap from *c.* 3000 EBCs from a single mock- or salt-treated plant was pooled to obtain *c.* 1 ml of sample. Two biological replicates were harvested for each condition. Sap was collected at the end of the dark phase, and tissue was kept in the dark until the collection.

Salt responses of EBCs

The osmolality of the EBC sap was measured in 50- μl samples with a cryoscopic osmometer (Osmomat030; Genotec, Gangelt, Germany). Na^+ and K^+ concentrations were determined using Flame photometry (Model 943; Instrumentation Laboratory, Lexington, MA, USA). Anthocyanin content in EBC sap was measured as described by Neff & Chory (1998), by measuring absorbance of the solution at 530 and 657 nm using a diode array spectrophotometer (Hewlett Packard, Palo Alto, CA, USA), after extraction with an equal volume of acidified methanol (1% HCl), incubation at 4°C for 1 h, and centrifugation. Betacyanins and betaxanthins were measured according to Stintzing *et al.* (2003). EBC sap samples were mixed with an equal volume of 100% ethanol, and absorbance of supernatants was determined at 480 and 538 nm, after centrifugation.

Sequencing, assembly, and annotation of the reference transcriptome

Total RNA was extracted from the EBC sap using the RNeasy Plant Mini kit (Qiagen, Mexico City, Mexico). RNA-sequencing (RNA-seq) libraries were prepared using the TruSeq Stranded RNA Sample Prep Kit (Illumina, San Diego, CA, USA). Libraries from control and salt-treated samples, with two biological replicates, were bar-coded and sequenced in a single Illumina HiSeq2000 flowcell, generating >41 million high-quality 100-nucleotide (nt) single-end RNA-seq reads per sample.

For the *de novo* reference transcriptome assembly, all RNA-seq reads were pooled and used as input for the Trinity pipeline (version 'trinityrnaseq_r2012-01-25', Kcov = 2 and -S option for strand specificity; Haas *et al.*, 2013). After removing contaminants, artefacts, and contigs with low read support (Table 1), all assembled transcript contigs showing $>95\%$ sequence identity over $>70\%$ of the total length were clustered using all-to-all BLASTN. We selected a representative transcript containing the longest continuous open reading frames (ORFs) for each cluster to generate a nonredundant *Mesembryanthemum crystallinum* epidermal bladder cell (McEBC) transcriptome.

Annotation was based on best BLASTN hits found in the National Center for Biotechnology Information (NCBI) database 'refseq_rna' (April 2014), as well as the TAIR10 Arabidopsis gene model sequence, with 10^{-5} as the e-value cut-off. Sequence comparison with *M. crystallinum* full-length cDNAs (NCBI, November 2013) and ESTs (<http://compbio.dfci.harvard.edu/tgi/>, July 2008) was performed using NUCMER (–minmatch 11 –mincluster 25; Kurtz *et al.*, 2004). ORFs in each contig were identified with TRANSDCODER (<http://transdecoder.sourceforge.net/>). The

Table 1 *De novo* assembly of a *Mesembryanthemum crystallinum* epidermal bladder cell (McEBC) transcriptome

Assemblies	Transcriptome stats			RNA-seq read mapping stats ^a		
	Number of transcript contigs	Median contig length ^b	Total base assembled (Mbase)	Total mapped reads (%)	Uniquely mapped reads (%)	Reads mapped to multiple contigs (%)
Trinity assembly ^c	101 077	769	119.0	86.96 ± 5.67	64.18 ± 3.88	22.78 ± 1.81
After removing nonplant contigs ^d	100 044	778	118.4	86.52 ± 5.79	63.78 ± 3.99	22.74 ± 1.82
After removing missense contigs ^e	95 423	825	115.7	86.32 ± 5.77	63.63 ± 3.97	22.68 ± 1.82
After removing low-abundance contigs ^f	85 492	996	113.3	86.27 ± 5.76	63.59 ± 3.97	22.68 ± 1.82
After removing redundant contigs ^g	37 341	665	39.4	82.48 ± 5.48	82.40 ± 5.44	0.08 ± 0.04

^aMean ± SE from four RNA-seq samples (control and salt-treated; two biological replicates) are shown. All RNA-seq samples include at least 41.99 million high-quality reads. RNA-seq reads were mapped to the reverse strand of the contigs from each assembly.

^bFor all assemblies, the minimum and maximum contig lengths were 201 nt and 12 252 nt, respectively.

^cStrand-specific assembly with minimum Kmer coverage 2.

^dContigs showing BLASTN hit score with e-value < 10⁻⁵ only to a nonplant RNA sequence were removed.

^eRNA-seq was designed to generate short reads specifically from the complementary sequences of mRNAs. Contigs showing a higher number of reads mapped to the forward strand than the complementary strand were considered as artifacts and removed.

^fContigs supported with no more than 10 RNA-seq reads were removed.

^gSee the Materials and Methods section and Supporting Information Fig. S1 for details.

transcriptome assembly and annotation were deposited at the NCBI (BioProject ID: PRJNA260050).

RNA-seq analysis

RNA-seq reads from each sample were aligned to the McEBC transcriptome using BOWTIE (Langmead *et al.*, 2009), with the seed length set to 50 nt. The number of reads uniquely aligned to each contig was counted for each sample. Contigs with significantly different uniquely mapped RNA-seq reads between control and salt-treated samples were identified as differently expressed transcript contigs (DETs), based on false discovery rate (FDR) < 0.01 (Benjamini–Hochberg correction), using the DESeq pipeline (Anders & Huber, 2010). We also calculated reads mapped per kilobase of transcript contig per million reads (RPKM) for each contig, following Mortazavi *et al.* (2008). Gene ontology (GO) terms enriched in DETs were analysed via BINGO (Maere *et al.*, 2005), using the GO annotation assigned to the most similar Arabidopsis homolog of each DET. RNA-seq results for selected transcript contigs were visualized using INTEGRATIVE GENOME VIEWER (Thorvaldsdóttir *et al.*, 2012).

Results

Salt responses in McEBC

EBCs were present at all stages of development on the aerial tissue of the *M. crystallinum* plant. In unstressed adult plants the EBCs were small and appressed to leaf, stem, or flower bud surfaces (Fig. 1a,b; control). In salt-treated plants of the same age, the cells expanded and appeared as liquid-filled balloons (Fig. 1a–c; salt). When plants were exposed to continuous long-term stress, EBCs accumulated visible amounts of pigments (Fig. 1d).

Treatment of plants with 200 mM NaCl resulted in a more than three-fold increase in the osmolarity of EBC sap isolated from leaves and stems, compared with the mock-treated control

(Fig. 2a). [Na⁺] in the EBC sap was 87.7 and 820 mM in the control and salt-treated samples, respectively, while [K⁺] showed no significant differences (Fig. 2a). To determine the nature of pigment accumulation (Fig. 1d), we measured the amounts of anthocyanins, betacyanins, and betaxanthins (Fig. 2b, Supporting Information Fig. S1). While anthocyanins were unchanged by salt treatment, betacyanins and betaxanthins were present in larger amounts in untreated samples and further increased by 2.4- and 7.1-fold, respectively, following salt treatment (Fig. 2b).

De novo assembly and annotation of the McEBC transcriptome

We sequenced RNA samples derived from extracts of EBCs isolated from 8-wk-old *M. crystallinum* plants treated for 2 wk with mock solution for control or with 200 mM NaCl. Two biological repeats were prepared for each condition. RNA-seq reads pooled from all biological replicates were used for *de novo* Trinity assembly, to produce a reference transcriptome. The initial assembly contained 101 077 contigs. Mapping of RNA-seq reads to the assembled transcript contigs using BOWTIE revealed *c.* 22% of total reads were mapped to multiple contigs, indicating the presence of redundant assembled sequences within the transcriptome (Table 1). After filtering out nonplant, missense, and low-abundance contigs, the remaining contigs were subjected to clustering and cleaning of redundant sequences, as described in the Materials and Methods section and Fig. S2. This generated a reference McEBC transcriptome of 37 341 transcript contigs with the median contig length of 665 nt. Compared with the initial transcriptome assembly, RNA-seq reads mapped to multiple contigs were reduced to < 1.2%, indicating that the curated reference transcriptome is nonredundant (Table 1).

The nonredundant McEBC transcriptome was annotated based on sequence similarity as described in the Materials and Methods section (Tables 2, S1). When compared with the NCBI reference RNA sequence database (as of April 2014), 16 984

Fig. 1 Epidermal bladder cells (EBCs) from *Mesembryanthemum crystallinum*. (a) EBCs on the aerial parts of a 8-wk-old plant, including the leaves, stems and flower buds, from control and salt-treated (200 mM NaCl for 2 wk) plants. (b) Cells are appressed to the stem surface under control conditions (control) but enlarge when plants are salt-treated (salt). (c) Magnification of a single EBC after salt treatment. (d) EBCs accumulate pigments when exposed to high concentrations of salt for prolonged periods (upper and lower right), while unstressed plants only accumulate minimal amounts of the pigments, primarily at leaf tips and on flower buds (upper and lower left). Plants shown are 13 wk old and have been salt-treated for 7 wk.

contigs showed a BLASTN hit with a plant sequence (e -value $< 10^{-5}$), with $c.$ 70% of contigs showing the highest similarity to sequences derived from *Vitis vinifera* (8352 contigs), *Populus trichocarpa* (1849 contigs), or *Ricinus communis* (1534 contigs). Comparison with the TAIR10 Arabidopsis annotation identified 14 521 contigs, or 38.9% of the total, showing a BLASTN hit ($e < 10^{-5}$) with an Arabidopsis gene model (Table 2). Among all contigs, 25 670 (68.7%) contained an ORF encoding a polypeptide longer than 50 aa (Table 2). The McEBC transcriptome was also compared with the 145 *M. crystallinum* full-length cDNAs and 10399 unique ESTs, retrieved from NCBI and The Institute for Genomic Research databases, respectively. In total, 100 full-length cDNAs and 5650 ESTs showed $> 70\%$ overlap with a contig in the McEBC transcriptome, with at least 98% sequence identity. Among them, 63 cDNAs and 4042 ESTs showed a perfect or near-perfect match ($> 98\%$ overlap and $> 98\%$ identity; Table 2).

Salt-regulated DET contigs in the McEBC transcriptome

We used the DESeq package to compare the two biological replicates of control and salt-treated samples to identify salt-regulated DETs, based on the number of RNA-seq reads uniquely mapped to each transcript contig in the McEBC transcriptome. We identified 2490 up-regulated and 2849 down-regulated DETs with

expression significantly ($FDR < 0.01$) altered in response to salt treatment (Fig. 3a). More than 86% (4595) of all DETs contained an ORF. Among them, 1028 did not show significant similarity (BLASTN $e < 10^{-5}$) with a known RNA sequence, probably encoding lineage-specific peptides (Fig. 3b).

To access the abundance of transcript contigs in the McEBC transcriptome, we quantified RPKM of each transcript contig. We found lineage-specific transcripts enriched among rarely expressed transcripts. When ranked from the most abundant to the least abundant transcripts, the top 25% most abundant transcripts include $< 5\%$ of the total lineage-specific transcripts. By contrast, the remaining 21%, 36% and 38% of the lineage-specific transcripts were found in the second, third and fourth quartiles, respectively (Fig. 3c). It is possible that the majority of these lineage-specific transcript contigs with low expression represent fragments of transposable elements, which show greater sequence variation compared with nontransposon genes.

The top 60 most abundant transcript contigs, ranked based on their average RPKMs in the control and salt-treated samples, are listed in Table 3. Among them, a number of transcripts encoding abscisic acid (ABA)- and dehydration-induced proteins, nonspecific lipid transport proteins, defense-related proteins, and chlorophyll *a/b* (*Chla/b*)-binding proteins were down-regulated by salt treatment (e.g. ranks 1, 2, 3, 7, 11, 13, 16 and 17, in Table 3). By contrast, those encoding enzymes related to stress

Fig. 2 Salt stress responses of epidermal bladder cells (EBCs). (a) Osmolarity, Na⁺ and K⁺ measurements and (b) anthocyanin, betacyanin and betaxanthin contents of EBC sap isolated from *Mesembryanthemum crystallinum* plants grown in the absence (control) or presence (salt) of 200 mM NaCl for 2 wk. Shown are the mean $\pm$ SD of 10 replicates. Unpaired two-tailed Student's *t*-test: *n* = 10; ***, *P* < 0.01.

response and carbohydrate catabolism were up-regulated by salt treatment. These included a cysteine protease, a *myo*-inositol-1-phosphate synthase (INPS), a pyruvate phosphate dikinase (PPDK), an aldolase, and a beta-amylase homolog (ranks 8, 23, 29, 35 and 41, respectively, in Table 3). A plasma membrane (PM) aquaporin homolog was ranked at 52, indicating that it was the most abundant among all transcripts encoding transporter/channel proteins, while its expression was not significantly affected by salt (FDR = 0.14; Table 3). A number of putative lineage-specific transcripts, showing similarity to *M. crystallinum* ESTs, but no significant similarity (BLASTN *e* < 10⁻⁵) to a known RNA sequence from any other species, were also found among the top 60 most abundant transcripts (ranks 21, 31, 42, 49, 53 and 57, in Table 3).

Networks of GO terms enriched among DETs

Approximately 60% of all salt-regulated DETs showed significant sequence similarity (BLASTN *e* < 10⁻⁵) with an Arabidopsis gene, enabling GO enrichment analysis on DETs, based on GO annotations of their Arabidopsis homologs. We selected 1234 up-regulated and 1669 down-regulated DETs showing sequence similarity in their ORF regions with Arabidopsis ORF sequences, and used BINGO to identify and visualize GO terms

Table 2 Annotation of the reference *Mesembryanthemum crystallinum* McEBC transcriptome

Category	Cutoff	Numbers in total 37 341 contigs
Shows BLASTN hit with a plant refseq_rna sequence	BLASTN e-value < 10 ⁻⁵	16 984
Shows BLASTN hit with an Arabidopsis gene model	BLASTN e-value < 10 ⁻⁵	14 521
Contains an ORF ^a	Deduced protein $\geq$ 100 aa Deduced protein $\geq$ 50 aa	16 652 25 670
Shows similarity with a known <i>M. crystallinum</i> full-length cDNA	> 98% identity, > 70% coverage > 98% identity, > 98% coverage	100 ^b 63 ^b
Shows similarity with a known <i>M. crystallinum</i> EST	> 98% identity, > 70% coverage > 98% identity, > 98% coverage	5650 ^c 4042 ^c

^aIncluding partial open reading frames (ORFs), identified using TRANSCODER.

^bOut of 145 full-length *M. crystallinum* cDNA sequences retrieved from NCBI.

^cOut of 10399 nonredundant expressed sequence tags (ESTs) retrieved from TIGR (<http://compbio.dfci.harvard.edu/tgi/>).

enriched among them (Figs 4, S3, S4; Tables 4, S2, S3). Fig. 4 illustrates networks of GO terms enriched among DETs, constructed based on their parent-child relationships. A network consisting of 'transport' (GO:0006810), 'generation of precursor metabolites and energy' (GO:0006091), and their children was identified among GO terms enriched in up-regulated DETs. This network (Fig. 4a) included salt-induced DETs encoding components of the vacuolar proton transporting ATPase (vacuolar H⁺-ATPase (VHA)) and cation transporters, as well as enzymes in the glycolytic pathway (Table 4, BP-U3; Table S3). Similarly, there was enrichment of 'response to stimulus' (GO:0050896) and its child GO terms in DETs down-regulated by salt. This network (Fig. 4b) consisted of genes annotated as responsive to various biotic and abiotic stresses, especially salt and osmotic stresses (Table 4, BP-D1; Table S3).

Comparison between all networks for up- and down-regulated DETs identified distinct salt-regulated branches of GO networks (summarized in Table 4). For example, while the GO term 'response to stimulus' (GO:0050896) was overrepresented in both salt up- and down-regulated DETs, the overrepresentation was caused specifically by enrichment of child GO terms 'response to radiation' (GO:0009314) and 'response to hormone stimulus' (GO:0032870) for DETs up-regulated by salt. By contrast, 'response to salt stress' (GO:0009651), 'response to fungus' (GO:0009620), and 'response to water deprivation' (GO:0009414) were enriched in DETs down-regulated by salt (Table 4; BP-U1 and BP-D1). Similarly, distinct child GO terms of 'small molecule metabolic process' (GO:0044281) and 'transport' (GO:0006810) were enriched in DETs up- (Table 4; BP-U2 and BP-U3) as well as down-regulated (Table 4; BP-D2 and

Fig. 3 Identification of differentially expressed transcript contigs (DETs) in the *Mesembryanthemum crystallinum* epidermal bladder cell (EBC) transcriptome. (a) Scatter plot showing mean normalized uniquely mapped RNA-seq read counts for control (x-axis) and salt-treated (y-axis) samples. DETs were identified based on a false discovery rate (FDR) < 0.01 (Benjamini–Hochberg multiple testing adjustment) using DESeq (Anders & Huber, 2010). Red and blue spots signify DETs up- and down-regulated by salt treatment, respectively. The complete DESeq result is given in Supporting Information Table S1. (b) Venn diagram representing overlaps among DETs, transcript contigs containing an open reading frame (ORF) encoding a protein larger than 50 aa and transcript contigs showing a BLASTN hit with a known plant reference RNA or an Arabidopsis gene model sequence with an e-value cutoff of 10^{-5} (BLASTN). (c) The percentage of lineage-specific transcripts is shown for transcript contigs within indicated expression abundance quartiles (based on reads mapped per kilobase of transcript contig per million reads (RPKM): 1st quartile = the most abundant 25%; 4th quartile = the least abundant 25%). Lineage-specific transcripts are those without a BLASTN hit ($e\text{-value} < 10^{-5}$) with any sequence in the National Center for Biotechnology Information (NCBI) reference RNA ('refseq_rna') database, as described in the Materials and Methods section.

BP-D3) by salt. For the GO terms in the category Cellular Component, different child terms of 'plastid' (GO:0009536) were overrepresented in DETs both up- and down-regulated by salt (Table 4; CC-U1 and CC-D1). In addition, 'vacuole' (GO:0005773) and its child GO terms were highly represented in salt-induced DETs, while 'endoplasmic reticulum' (GO:0005783), 'Golgi apparatus' (GO:0005794), 'plant-type cell wall' (GO:0009505) and 'tubulin complex' (GO:0045298) were overrepresented in DETs down-regulated by salt (Table 4; CC-U2 and CC-D2). Analysis of GO terms in the Molecular Function category revealed a similar result comparable to that for the Biological Process category (data not shown). In their entirety, the networks are presented in Figs S3 and S4, and transcript contigs included in each GO term are listed in Table S3.

Salt response of the McEBC transcriptome

Based on GO enrichment analysis, we focused on gene families and pathways that highlighted different aspects of the collective salt

response of the McEBC transcriptome. These included genes involved in ion transport, cellular energy metabolism, canonical stress signal transduction and responses, and intracellular organelle functions. Transcript contigs encoding proteins involved in each aspect are curated (Tables S4–S7), and RNA-seq results for representative transcripts are plotted as read abundance across the transcript model under control and salt-stressed conditions (Figs 5, 6).

Ion transport The salt response of ion transporters known to be involved in Na^+ transport and sequestration, including vacuolar proton pumps, is shown in Fig. 5(a) and Table S4. A homolog of the chloroplast-localized Na^+ -HYDROGEN ANTIPORTER 1 (NHDI) was induced 2.5-fold by salt. While transcripts encoding full-length homologs of Na^+ - H^+ EXCHANGER 2 (NHX2) and SALT OVERLY SENSITIVE 1 (SOS1) were ranked in the top 10% most abundant transcripts (Table S1), their expression levels were not significantly affected by salt. Similarly, cation exchangers in the CATION H^+ EXCHANGER (CHX) family were unaffected by salt, with the exception of a CHX19 homolog

Table 3 Top 60 most abundant transcript contigs in the *Mesembryanthemum crystallinum* epidermal bladder cell (McEBC)^a

Rank overall	Transcript contig ID	Control RPKM (rank)	Salt RPKM (rank)	Log ₂ (salt: control) ^b	FDR ^b	Contig length	Longest ORF coordinates	Similar to ^c	Annotation ^d
1	comp24427_c0_seq1	14566 (1)	4898 (6)	-1.6	5.E-10	927	155–499	TC9327	Weakly similar to nonspecific lipid-transfer protein (<i>Atriplex nummularia</i>)
2	comp15895_c0_seq1	11608 (3)	6178 (3)	-0.9	8.E-04	870	165–629	TC8964	Similar to abscisic acid (ABA)- and environmental stress-inducible protein (<i>Medicago sativa</i>)
3	comp5206_c0_seq1	12095 (2)	2980 (9)	-2.0	4.E-11	520	3–299	TC10307	Antimicrobial peptide 1 precursor
4	comp19657_c2_seq1	5968 (4)	8715 (1)	0.6	5.E-02	2006	269–1612	TC9865/At2g39730	Rubisco activase (RCA)
5	comp24428_c0_seq1	5550 (5)	5112 (5)	-0.1	8.E-01	1131	403–735	TC7834	Similar to abscisic stress ripening protein (<i>Musa acuminata</i>)
6	comp23899_c2_seq1	4733 (8)	5639 (4)	0.3	4.E-01	1109	245–787	M38316/At5g38430	Ribulose-1,5- biphosphate carboxylase small subunit (rbcS-2)
7	comp13365_c0_seq1	5502 (6)	2925 (10)	-0.9	9.E-03	223	2–223	AI823120	Weakly similar to nonspecific lipid-transfer protein (<i>Spinacia oleracea</i>)
8	comp13359_c0_seq1	293 (302)	8058 (2)	4.8	2.E-70	1597	198–1256	U30322/At5g50260	Cysteine proteinase
9	comp24432_c0_seq1	4162 (10)	3672 (7)	-0.2	7.E-01	405	1–405	TC7894/At4g05320	Ubiquitin family protein
10	comp13349_c0_seq1	3524 (11)	3324 (8)	-0.1	9.E-01	559	2–559	TC9545/At4g05320	Ubiquitin family protein
11	comp24439_c0_seq1	4475 (9)	1958 (18)	-1.2	4.E-06	892	101–892	TC9016	Similar to fruit-ripening protein (<i>Lycopersicon esculentum</i>)
12	comp24434_c0_seq1	3230 (14)	2807 (12)	-0.2	6.E-01	271	2–271	BE035072/At5g20620	Ubiquitin
13	comp13351_c1_seq1	5195 (7)	833 (63)	-2.6	5.E-26	546	3–335	TC10534	Weakly similar to Arabidopsis ABA-inducible protein
14	comp24429_c0_seq1	3120 (16)	2839 (11)	-0.1	8.E-01	228	3–227	TC7894/At4g05050	Ubiquitin family protein
15	comp13355_c0_seq2	3391 (13)	2541 (14)	-0.4	6.E-01	938	67–594	TC9951	Weakly similar to glycine-rich RNA-binding protein (<i>Zea mays</i>)
16	comp13357_c0_seq2	3496 (12)	1684 (20)	-1.0	2.E-04	718	153–494	TC8837	Weakly similar to nonspecific lipid-transfer protein (<i>Solanum tuberosum</i>)
17	comp19813_c0_seq2	3218 (15)	1187 (40)	-1.4	2.E-08	1093	2–865	AF003127/At2g34420	Chlorophyll <i>a/b</i> -binding protein (CAB)
18	comp24435_c0_seq1	2925 (18)	1439 (26)	-1.0	1.E-04	868	265–522	TC11100	Weakly similar to upstream regulatory element binding protein (<i>Homo sapiens</i>)
19	comp24436_c0_seq1	2981 (17)	1242 (38)	-1.3	8.E-07	1271	93–887	TC7871/At2g05100	Chl <i>a/b</i> -binding protein (CAB)
20	comp23899_c0_seq1	2371 (21)	1600 (22)	-0.6	1.E-01	1307	2–1024	TC9623/At4g24570	Mitochondrial dicarboxylate carrier
21	comp24444_c0_seq1	2606 (20)	1248 (37)	-1.0	6.E-05	856	3–572	TC9441	No homology
22	comp5278_c0_seq1	2679 (19)	1133 (41)	-1.2	2.E-06	1746	177–1400	TC10368/At5g25610	Similar to Responsive to Dehydration 22 (RD22)-like protein (<i>Knorreria sibirica</i>)
23	comp24437_c0_seq1	1122 (44)	2654 (13)	1.3	9.E-07	2814	200–1738	U32511/At2g22240	Myo-inositol-1-phosphate synthase
24	comp24440_c0_seq1	1700 (27)	1931 (19)	0.2	6.E-01	1526	1–1131	M29956/At1g13440	NAD-glyceraldehyde-3-phosphate dehydrogenase
25	comp24442_c0_seq1	2161 (23)	1352 (34)	-0.7	2.E-02	661	106–348	AF078912/At3g09390	Metallothionein
26	comp24446_c0_seq1	1868 (25)	1464 (24)	-0.3	3.E-01	2099	543–1649	TC8118/At5g15948	Similar to S-adenosylmethionine decarboxylase proenzyme 1 (<i>Dianthus caryophyllus</i>)
27	comp24438_c0_seq1	1644 (28)	1620 (21)	0.0	1.E + 00	205	2–205	BE034631/At4g02890	Ubiquitin family protein
28	comp15996_c0_seq2	2184 (22)	992 (49)	-1.1	1.E-05	1416	92–805	TC9526/At1g10370	Similar to glutathione S-transferase (<i>Salicornia brachiata</i>)
29	comp5195_c0_seq1	707 (88)	2354 (15)	1.8	2.E-12	4449	257–3106	TC8935/At4g15530	Pyruvate phosphate dikinase (PPDK)
30	comp22312_c0_seq2	1603 (30)	1420 (29)	-0.2	7.E-01	2152	128–1261	TC9344/At5g09810	Actin family protein
31	comp5207_c0_seq1	2146 (24)	852 (62)	-1.3	2.E-07	299	185–298	BE034064	Similar to genome sequence (<i>Vitis vinifera</i>)
32	comp21936_c0_seq4	1306 (40)	1517 (23)	0.2	5.E-01	1966	360–1553	TC10432/At4g38970	Fructose-bisphosphate aldolase 2
33	comp24417_c1_seq4	1219 (41)	1394 (30)	0.2	6.E-01	1930	105–1448	TC8050/At1g07920	Elongation factor 1- α
34	comp24449_c0_seq1	1633 (29)	899 (55)	-0.8	2.E-03	1453	2–1000	TC7853/At4g10340	Chlorophyll <i>a/b</i> -binding protein (CAB)

Table 3 (Continued)

Rank overall	Transcript contig ID	Control RPKM (rank)	Salt RPKM (rank)	Log ₂ (salt: control) ^b	FDR ^b	Contig length	Longest ORF coordinates	Similar to ^c	Annotation ^d
35	comp21936_c0_seq2	366 (237)	2157 (16)	2.6	2.E-08	1616	360–1553	AT2g21330	Fructose-bisphosphate aldolase 1
36	comp5204_c0_seq1	1847 (26)	642 (85)	-1.5	1.E-09	953	124–555	TC8662	Similar to predicted protein (<i>Chlamydomonas reinhardtii</i>)
37	comp24448_c0_seq1	1370 (37)	1099 (46)	-0.3	4.E-01	1358	240–1070	TC8166/At1g61520	PSI type III chlorophyll <i>a/b</i> -binding protein
38	comp5205_c0_seq1	1328 (39)	1104 (44)	-0.3	5.E-01	1618	191–1180	TC8028/At5g66570	Chloroplast oxygen-evolving enhancer protein 1
39	comp13395_c0_seq1	964 (51)	1458 (25)	0.6	3.E-02	2114	390–1502	U80071/At3g14420	Glycolate oxidase (GOX)
40	comp24457_c0_seq1	946 (52)	1374 (33)	0.6	1.E-01	830	1–528	AF003125/At1g60950	Ferredoxin I precursor
41	comp13353_c1_seq1	291 (305)	2027 (17)	2.8	9.E-06	2046	268–1899	TC8500/At5g18670	Similar to beta-amylase 3
42	comp24447_c1_seq1	1433 (36)	803 (67)	-0.8	3.E-03	558	3–218	TC10857	No homology
43	comp5196_c0_seq1	1011 (48)	1200 (39)	0.3	5.E-01	2854	799–2361	TC9507/At5g43760	Similar to photosystem II 10-kDa polypeptide (<i>Spinacia oleracea</i>)
44	comp24452_c0_seq1	779 (74)	1423 (28)	0.9	3.E-02	1878	175–1389	TC9000/At3g26650	Glyceraldehyde-3-phosphate dehydrogenase A subunit
45	comp24445_c0_seq1	828 (65)	1304 (36)	0.7	2.E-02	774	172–408	TC10036	Weakly similar to defensin (<i>Nelumbo nucifera</i>)
46	comp15932_c0_seq1	1586 (31)	537 (116)	-1.6	5.E-05	1439	3–779	TC10614/At5g20190	Similar to tetratricopeptide repeat (TPR)-like superfamily protein
47	comp24455_c0_seq1	1478 (35)	618 (96)	-1.2	1.E-06	674	2–403	TC8867	Weakly similar to nonspecific lipid-transfer protein (<i>Hordeum vulgare</i>)
48	comp13357_c0_seq1	1559 (32)	505 (124)	-1.6	7.E-06	463	298–432	TC8055	Cysteine proteinase (partial)
49	comp19862_c0_seq1	688 (94)	1375 (32)	1.0	1.E-04	737	138–362	TC9463	No homology
50	comp24454_c0_seq1	1107 (45)	940 (52)	-0.2	6.E-01	1529	240–1106	TC9464/At5g01530	Similar to light harvesting complex photosystem II (LHC4.1)
51	comp13376_c0_seq1	1505 (33)	484 (135)	-1.6	6.E-07	1168	82–939	TC9131/At1g80130	Similar to tetratricopeptide repeat (TPR)-like superfamily protein
52	comp5273_c0_seq1	1129 (43)	810 (64)	-0.5	1.E-01	1392	2–1021	AF133530/At3g53420	Water channel protein (MipH)
53	comp19764_c0_seq1	942 (53)	955 (51)	0.0	1.E+00	1189	450–689	CA837931	No homology
54	comp24453_c0_seq1	510 (151)	1385 (31)	1.4	4.E-03	700	209–448	TC10158	Weakly similar to unknown protein (<i>Oryza sativa</i> subsp. <i>japonica</i>)
55	comp5192_c0_seq1	1483 (34)	354 (220)	-2.1	4.E-04	311	3–311	AI148374	Weakly similar to neurofilament heavy polypeptide-like (<i>Cicer arietinum</i>)
56	comp24451_c0_seq1	917 (57)	913 (54)	0.0	1.E+00	956	90–476	TC9013/At3g62410	Similar to Calvin cycle protein CP12 precursor (<i>Nicotiana tabacum</i>)
57	comp13387_c0_seq1	1101 (46)	706 (75)	-0.6	4.E-01	1158	136–408	TC9925	No homology
58	comp24461_c0_seq1	935 (56)	861 (60)	-0.1	8.E-01	1747	143–1321	U79767/At1g02500	Methionine adenosyltransferase
59	comp24459_c0_seq1	658 (103)	1116 (42)	0.8	4.E-03	1198	135–815	BE034185/At4g03280	Similar to cytochrome b6-f complex iron-sulfur subunit (<i>Spinacia oleracea</i>)
60	comp5200_c0_seq1	273 (330)	1427 (27)	2.4	2.E-04	1300	213–620	TC10081	30S ribosomal protein S5, chloroplast precursor

^aTranscript contigs are ranked based on their abundance quantified as their average reads mapped per kilobase of transcript contig per million reads (RPKM) values over all samples. Ranks in control and salt-treated samples are also given in separate columns.

^bFold changes and *P*-values showing significantly different expression are indicated in bold. Fold change values are color-coded with red for up-regulation and blue for down-regulation by salt treatment.

^cShown are *M. crystallinum* full-length cDNAs or expressed sequence tags (ESTs) with > 95% nucleotide identities and TAIR10 gene models with the best BLASTN hit (*e*-value > 10⁻⁵), compared with the transcript contig.

^dBased on Cushman *et al.* (2008) and Arabidopsis gene model annotation (TAIR10).

Fig. 4 Examples of networks representing gene ontology (GO) terms enriched among differently expressed transcript contigs (DETs) that are (a) up- or (b) down-regulated by salt treatment in the *Mesembryanthemum crystallinum* epidermal bladder cell (EBC) transcriptome. Enriched GO terms were identified using BINGO and visualized with Cytoscape, as described in the Materials and Methods section. GO terms are connected based on their ancestor–child relationships. Colors of circles indicate the *P*-value of enrichment. Sizes of circles represent the size of GO terms in the background TAIR10 annotation. GO terms selected for the summary in Table 4 are marked with (a) red and (b) blue dots. The complete networks and list of enriched GO terms are presented in Supporting Information Figs S3 and S4, and Table S3, respectively.

which showed a 5.8-fold induction (Fig. 5a; Table S4, cation- H^+ transporter). The two most abundant copies among the three *HIGH-AFFINITY K^+ TRANSPORTER 1* (*HKT1*) homologs, as well as a homolog of *K^+ TRANSPORTER 1* (*KT1*), were down-regulated by salt, with 2.8-, 17-, and 1.9-fold decreases, respectively (Fig. 5a; Table S4, K^+ / Na^+ transporter). The entire subunit components of the VHA complex were transcriptionally salt-induced by 2- to 4-fold (Table S4, V-ATPase V_0 complex and V_1 complex). Contrastingly, homologs of the vacuolar pyrophosphatases were unchanged or down-regulated by the salt treatment (Table S4, vacuolar pyrophosphatase).

Metabolism Transcript contigs encoding enzymes in hexose catabolism including glycolysis, *myo*-inositol metabolism, CAM, and proline biosynthesis pathways were all up-regulated by salt (Fig. 5b; Table S4). At least two copies of Na^+ /*myo*-inositol transporters, assembled as full-length transcripts, were also salt-induced, by 1.9- and 4-fold, respectively (Table S4). Genes encoding CAM-related enzymes, for example, pyruvate phosphate dikinase (PPDK), phosphoenolpyruvate carboxylase (PEPC), malic enzyme (ME) and malate dehydrogenase (MDH), showed up-regulation by salt (Table S4). In addition, genes involved in the regulation or activation of the enzymes

Table 4 Summary of gene ontology (GO) biological process (BP) and cellular components (CC) terms enriched in differently expressed transcript contigs (DETs) in the *Mesembryanthemum crystallinum* epidermal bladder cell (McEBC) transcriptome

Salt response	Network ^a	GO terms ^b	Number (%) in DETs	Number (%) in background ^c	Log (P-value) ^d	Representative genes or gene families ^e
Up	BP-U1	Response to stimulus	225 (19.9)	3207 (11.6)	−13.6	
		–Response to abiotic stimulus	101 (8.9)	1168 (4.2)	−9.9	<i>CA1, CBL10, CIPK3, OZS1</i>
		–Response to radiation	44 (3.9)	471 (1.7)	−4.8	<i>ELIP, IM, INPS, STO</i>
		–Response to hormone stimulus	56 (5.0)	767 (2.8)	−3.3	<i>CNI1, GH3, 1, GH3.3</i> , Gibberellin-regulated family, <i>MPK11</i> , SAUR-like family
	BP-U2	Small molecule metabolic process	103 (9.1)	1248 (4.5)	−9.0	
		–Carboxylic acid metabolic process	50 (4.4)	620 (2.2)	−3.9	<i>ACO, P5CS2, VTC2</i>
		–Fatty acid biosynthetic process	17 (1.5)	105 (0.4)	−4.4	<i>CER1, FAD8</i>
		–Hexose catabolic process	10 (0.9)	63 (0.2)	−2.4	<i>FBA1, HXK1</i> , enzymes of glycolysis
		–Flavonoid biosynthetic process	9 (0.8)	46 (0.2)	−2.8	<i>McIMT1</i>
		–Starch biosynthetic process	5 (0.4)	15 (0.1)	−2.4	<i>SBPase</i>
	BP-U3	Transport	94 (8.3)	1502 (5.4)	−3.2	Amino acid transporter family, <i>BAC2, CAT2, CLC-B, NRT1.1/1.5, SAD2, SULTR4;1</i>
		–Cation transport	28 (2.5)	271 (1.0)	−3.7	<i>NHD1, CHX19</i> , VHA subunits
		Generation of precursor metabolites and energy	26 (2.3)	199 (0.7)	−5.1	<i>PETC, PGRL1B</i>
		–ATP synthesis coupled proton transport	10 (0.9)	33 (0.1)	−4.8	VHA subunits
		–Glycolysis	8 (0.7)	40 (0.1)	−2.5	Enzymes of glycolysis
Down	BP-D1	Response to stimulus	265 (17.1)	3207 (11.6)	−8.3	
		–Response to abiotic stimulus	113 (7.3)	1168 (4.2)	−5.9	
		–Response to salt stress	48 (3.1)	360 (1.3)	−5.6	<i>AOC3, BGLU22, CIPK6, HKT1, HOS2, NCED3, RD19, RD22, SNRK2.7</i>
		–Response to water deprivation	25 (1.6)	188 (0.7)	−2.7	<i>ABA2, HIS1-3, NAC3, SIP1</i>
	BP-D2	–Response to biotic stimulus	60 (3.9)	550 (2.0)	−4.3	<i>CRK4/5/20, EDS1, WRKY17/23/40/53</i>
		–Response to fungus	23 (1.5)	156 (0.6)	−3.0	<i>ANAC081, BAG6, GSTF7, PR5K, TRX5</i>
		Small molecule metabolic process	125 (8.1)	1248 (4.5)	−7.5	
		–Carboxylic acid metabolic process	75 (4.8)	620 (2.2)	−7.4	<i>ABA4, JAR1, KCS1/9, NAS3, PLD</i>
		–Cellular amino acid biosynthetic process	24 (1.5)	142 (0.5)	−4.1	<i>CAD4, DHS2, IMS1, MTO3, PGDH, trpB</i> -containing family
	BP-D3	–Vitamin metabolic process	14 (0.9)	63 (0.2)	−3.4	<i>QS, THI1</i>
		Transport	122 (7.9)	1502 (5.4)	−3.0	<i>AKT1, BAT2, ENT3, GONST4/5, HMTD, HKT1, NIP4;1, PILS7, PTR1, Sec14p</i> -like
		–Anion transport	13 (0.8)	67 (0.2)	−2.6	<i>AAP6, BOR2, NAP3, PHT1;7, NRT1;6, SULTR3;5</i>
Up	CC-U1	Plastid	196 (17.3)	2139 (7.8)	−23.8	<i>AIM1, DPE1, GLDP1, LDA, STR16</i>
		–Plastid stroma	48 (4.2)	354 (1.3)	−10.4	<i>AGT1, CA1, CP33, HCF136, PGM, PRXQ, RH3, ROC4</i>
	CC-U2	–Plastid thylakoid	32 (2.8)	254 (0.9)	−6.0	<i>FBKF13/16-2, NDH18, PSB27, PPL1/2</i>
		Vacuole	53 (4.7)	455 (1.6)	−9.0	<i>CAS1</i> , Major facilitator family, <i>PAP3/26</i>
		–Vacuolar membrane	13 (1.1)	77 (0.3)	−3.5	<i>CAT2, McITR3</i> , VHA subunits
Down	CC-D1	Plastid	159 (10.3)	2139 (7.8)	−2.4	<i>ERD9, FD3, NIR1, QS, TPR</i> -like, <i>SIP1</i>
		–Plastoglobule	12 (0.8)	55 (0.2)	−2.8	<i>CCD4</i> , LHC components
	CC-D2	Endoplasmic reticulum	43 (2.8)	329 (1.2)	−4.7	Chaperones, PDI-like
		Golgi apparatus	35 (2.3)	194 (0.7)	−7.0	<i>ERD2B, GAUT1/3/9, GONST4/5, SAM</i> family
		Plant-type cell wall	29 (1.9)	180 (0.7)	−4.6	<i>EXL2/3, PAP10/12, PDL1, SCPL46</i>
		Tubulin complex	6 (0.4)	13 (0.0)	−2.8	<i>TUA2, TUB4/7/9</i>

^aThe entire networks described here are presented as Figs S2 and S3 in the Supporting Information.^bGO terms best describing all branches of the network are selected.^cGO annotation of *Arabidopsis thaliana* (TAIR10, *n* = 28 352).^dHypergeometric test for enrichment, after Benjamini & Hochberg False Discovery Rate (FDR) correction.^eUnless otherwise specified, the name of the closest *Arabidopsis* homolog (TAIR10) is shown. The full list of gene abbreviations is given in Table S7 in the Supporting Information.

Fig. 5 Salt response of *Mesembryanthemum crystallinum* transcripts involved in (a) Na⁺ ion transport and (b) metabolic pathways for osmolyte accumulation. Histograms indicate the counts of uniquely mapped RNA-seq reads per million total reads (RPM), of control (blue) and salt-treated (red) samples. The range of RPM, indicated on the y-axis of the uppermost histogram, is for all four histograms. Gray bars below the histograms signify the transcript with the deduced open reading frame (ORF) shown as thicker boxes. 'Log₂FC' is the log value of fold change (FC) produced by salt treatment, with statistically significant differences (false discovery rate (FDR) < 0.01) indicated in bold.

in the CAM cycle, such as PPDK kinase and PEPC kinase, were induced by 3.5- and 17-fold, respectively, upon salt treatment. Transcripts encoding two key enzymes in proline biosynthesis, a delta 1-pyrroline-5-carboxylate synthase (McP5CS; AF067967) and a homolog of pyrroline-5-carboxylate reductase (P5CR), also showed 2.6- and 1.8-fold higher expression, respectively, in salt-treated samples (Fig. 5b; Table S4).

Stress signalling Transcripts homologous to genes canonically identified as stress-responsive in *Arabidopsis* and other

nonhalophyte species were either unchanged or down-regulated by salt in EBCs. Homologs of genes involved in ABA synthesis and stress signalling, such as *ABA DEFICIENT 1 (ABA1)*, *ABA3*, *ABA INSENSITIVE 1 (ABI1)*, *ABI5*, *ABA RESPONSIVE ELEMENT-BINDING FACTOR 3 (ABF3)* and *ABI5 BINDING PROTEIN 1 (ABP1)*, were not significantly affected (i.e. FDR > 0.01; Table S1). Other genes in this category were down-regulated by salt, for example: *ABA2*, *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3)* and transcription factors listed in the 'ABA signalling' section of Table

Fig. 6 Salt response of *Mesembryanthemum crystallinum* transcripts involved in (a) stress signaling and responses, and (b) endoplasmic reticulum (ER) and Golgi functions. Histograms indicate the counts of uniquely mapped RNA-seq reads per million total reads (RPM), of control (blue) and salt-treated (red) samples. The range of RPM, indicated on the y-axis of the uppermost histogram, is for all four histograms. Gray bars below the histograms signify the transcript with the deduced open reading frame (ORF) shown as thicker boxes. 'Log₂FC' is the log value of fold change (FC) produced by salt treatment, with statistically significant differences (false discovery rate (FDR) < 0.01) indicated in bold.

S6. Likewise, all homologs of the *RESPONSIVE TO DESICCATION* and *RESPONSIVE TO DEHYDRATION* (RD) family genes, including an *RD2*, an *RD19*, two *RD28* and two *RD22* homologs, were either unchanged or down-regulated in EBCs by salt (Fig. 6a; Tables S1, S6). Exceptions to this trend include two homologs of *CALCINEURIN B-LIKE PROTEIN 10* (*CBL10*) and a *CBL-INTERACTING PROTEIN KINASE 3* (*CIPK3*) homolog, which showed 2.0- to 5.9-fold increases in response to salt treatment (Table S6). Among the stress-responsive genes, those involved in high light or radiation responses were highly salt-induced in EBCs. For example, expression of an *IMMUTANS* (*IM*) homolog and three *EARLY LIGHT-INDUCIBLE PROTEIN 2* (*ELIP2*) homologs were induced by 8.6- to 42.2-fold in salt-treated EBC samples (Fig. 6a; Table S1).

Organelle functions Among genes involved in organelle functions, one of the most conspicuous changes upon salt treatment in EBCs was the down-regulation of almost all transcript contigs involved in protein folding and modification, and localized to the endoplasmic reticulum (ER) and Golgi apparatus, as shown in Table S7. Among the 442 EBC transcript contigs annotated with

the GO terms 'endoplasmic reticulum' or 'Golgi apparatus', 90 contigs (c. 20%) showed down-regulation, while only 20 (4.5%) were up-regulated by salt (FDR < 0.01). Homologs of ER chaperones and protein disulphide isomerase (PDI) family genes showed 1.7- to 3.0-fold lower expression in the salt-treated samples (Fig. 6b; Table S7, 'ER chaperones'). Similarly, transcripts encoding enzymes and transporters targeted to the Golgi apparatus were down-regulated by 1.6- to 3.0-fold by salt treatment (Fig. 6b; Table S7, 'Golgi apparatus'). Salt-up-regulated transcripts that encode ER- or Golgi apparatus-targeted proteins included homologs of vesicle transport-related genes (Table S7, 'Vesicle transport in ER and Golgi'), as well as two homologs of Arabidopsis *ECERIFERUM 1* (*CER1*) (Table S1).

Discussion

We constructed a cell-type-specific transcriptome for EBCs by *de novo* assembly, and characterized the transcriptome response of this single cell type to salt stress. To facilitate quantification of the transcript abundance based on nonambiguously mapped RNA-seq reads, we aimed to assemble a reference transcriptome

with minimum possible sequence redundancy and misassembled chimeras. Based on comparisons of multiple assemblers (data not shown; Yang & Smith, 2013), we chose the Trinity assembler and a custom filtering pipeline to create the nonredundant EBC transcriptome.

The quantification of expression highlighted biological processes that are enriched in EBCs, as well as their cell type-specific salt regulation. For example, a homolog of *GLABRA 2* (*GL2*), a transcription factor required for trichome development in *Arabidopsis* (Rerie *et al.*, 1994), was up-regulated by salt (5.8-fold; $\text{FDR} < 10^{-21}$) in EBCs (Table S1), mirroring the development process of EBCs from trichome initiating cells (Adams *et al.*, 1998). This approach also captured transcripts encoding proteins that had been previously found to be highly abundant in the EBC-specific proteome (Jou *et al.*, 2007; Barkla *et al.*, 2012). Examples include homologs for all VHA subunits, which were among the top 2% most abundant EBC transcripts (Tables S1, S4), as well as a RUBISCO small subunit, and cysteine protease genes (ranks 6 and 8 in Table 3). We also compared the composition of McEBC transcripts with published leaf/shoot transcriptomes, to highlight the cell specificity (Tables S8, S9).

Sodium and water transport captured in the McEBC transcriptome

The Na^+ sequestration in EBC vacuoles appears to be a critical component of salt stress adaptation in *M. crystallinum*, given that EBCs represent up to 25% of the aboveground volume of the plants (Lüttge *et al.*, 1978; Barkla *et al.*, 2002). Vacuolar Na^+ deposition requires energy, probably provided by vacuolar proton pumps, which is supported by the increase in activity and abundance of the VHA proteins in salt-treated *M. crystallinum* epidermal leaf tissue peels (Barkla *et al.*, 1995, 2009). Our data also support this view by showing higher expression of transcripts encoding all of the VHA subunits in EBCs from salt-treated plants (Fig. 7; Table S4).

The EBC PM has been reported to have a high conductivity to both water and salt (Steudle *et al.*, 1975). However, salt-induced transcriptional activation of known PM Na^+ transporters was not significant in our study. While the EBC transcriptome captured homologs of transporters suggested to function in low-affinity Na^+ entry into plant cells, such as HKT1 (Benito *et al.*, 2014; Maathuis, 2014), including near full-length copies of the previously known *McHKT1* and *McHKT2* (Su *et al.*, 2003) and a third novel *HKT1* homolog, these all showed decreased expression in EBCs from salt-treated plants (Fig. 7; Tables S1, S4). One explanation is the presence of still unknown components of the cellular Na^+ uptake pathways, such as the nonselective cation channels that have been identified at the PM of root cells (Demidchik & Tester, 2002). Another explanation would be to assume posttranslational mechanisms rather than transcription or transcript stabilization are collectively responsible for the regulation of flux required to deposit Na^+ into the cell.

The sequestration of Na^+ into vacuoles is thought to be mediated by Na^+ - H^+ exchangers. EBC tonoplast Na^+ - H^+ exchange activity is induced by salt and significantly higher than that

measured in leaf mesophyll cells (Barkla *et al.*, 2002). Members of the monovalent Cation: Proton Antiporter 1 (CPA1)-like transporters include tonoplast Na^+ - H^+ or K^+ - H^+ exchangers (Bassil *et al.*, 2011; Barragán *et al.*, 2012). Multiple transcripts homologous to *AtNHX2*, *AtNHX4* and *AtNHX6* were captured as full-length transcripts in the McEBC transcriptome. Among them, the *NHX2* homolog showed the highest basal expression level (Table S4), ranked within the top 2% most abundant (Table S1). However, none of the NHX family homologs showed significant induction in response to salt treatment (Tables S1, S4). Other possible candidates for vacuole Na^+ uptake are members of the CPA2-like transporter family, which have been implicated in salt tolerance (Qi *et al.*, 2014). One member of this family, a homolog of *AtCHX19*, localized to the prevacuolar compartment and late endosomes (Chanroj *et al.*, 2011), showed transcript levels increased by 5.7-fold in salt-treated samples (Fig. 7; Table S4).

Prevention of passive leakage of Na^+ back into the cytosol is another critical aspect of vacuolar Na^+ sequestration, minimizing futile Na^+ cycling between the cytosol and vacuole. The fast vacuolar (FV) and slow vacuolar (SV) tonoplast channels show nonselective monovalent cation permeability and thus mediate Na^+ transport between the cytosol and vacuole in both halophytes and glycophytes (Pantoja *et al.*, 1989; Bonales-Alatorre *et al.*, 2013a; Shabala, 2013). A recent study illustrated that the negative regulation of SV and FV tonoplast channels reduced Na^+ leak, to establish successful Na^+ sequestration in vacuoles and confer salt tolerance in the halophytic crop quinoa (*Chenopodium quinoa*) (Bonales-Alatorre *et al.*, 2013b). While the transcript identity of FV channels remains unknown, the *TWO-PORE CHANNEL 1* (*TPC1*) gene is known to encode an SV channel (Peiter *et al.*, 2005; Hedrich & Marten, 2011). Our results show a 3.3-fold repression of a *TPC1* homolog by salt in EBCs (Fig. 7; Table S10).

McNHD1, an Na^+ - H^+ exchanger localized to the chloroplast envelope, was transcriptionally up-regulated in EBCs by salt (Fig. 5a). NHD1 protects the photosynthetic machinery from toxic concentrations of Na^+ , by maintaining a low Na^+ concentration in the stroma (Müller *et al.*, 2014). In addition to ion transporters, there has been speculation that Na^+ transport could be mediated through vesicular trafficking between the PM, prevacuolar compartments, and the vacuole (MacRobbie, 1999; Garcia de la Garma *et al.*, 2014). In the EBCs, a relatively large number of transcript contigs showing altered expression in response to salt were observed among genes associated with vesicle trafficking (Fig. 7, Vesicle/Endosome).

The transmembrane movement of water is facilitated by channels of the major intrinsic protein (MIP) family in plants (Kirch *et al.*, 2000; Quigley *et al.*, 2002). Among 28 MIP transcripts in the McEBC transcriptome, the PM intrinsic protein (PIP) subfamily was the most abundant (e.g. rank 52 in Table 3). Five out of a total number of 11 PIP subfamily members ranked within the top 2% most abundant transcripts. None of the PIP homologs showed significantly altered expression after salt stress, except for a PIP1;5 homolog, which was significantly down-regulated by 2.7-fold ($\text{FDR} < 10^{-7}$; Fig. 7; Table S1). Expression differences by salt

Collectively, a large number of transcripts for diverse membrane transport functions were identified in the McEBC transcriptome and regulated by salt (Fig. 7). The presence of ion channels and transporters, as well as transporters of amino acids, sugars, glutathione, xanthine, xenobiotics, lipids, inositols, water, hormones such as auxin, and other compounds, is fundamental to the McEBC physiology, and their regulation by salinity is key to the understanding of how McEBCs sense and respond to salinity.

Inorganic ions, especially Na^+ , accumulating in the vacuoles of salt-treated EBCs are balanced by metabolites such as pinitol and proline in the cytosol. Pinitol has been shown to comprise 71% of the soluble carbohydrate fraction in salt-treated leaves of *M. crystallinum*, compared with only 5% in controls (Paul & Cockburn, 1989). Concentrations of 1 mM proline and 2.2 mM pinitol have been measured in salt-treated EBCs (Adams *et al.*, 1998). Our RNA-seq results suggest salt induction of metabolic pathways that lead to synthesis, accumulation, transport, and conversion to other metabolically active groups contributing to stress management, in EBCs (Figs 5b, 8; Table S5). For example, beta-amylase, invertase and hexokinase participate in starch degradation to provide glucose-6-phosphate that can be converted to *myo*-inositol, and then to D-pinitol and D-ononitol. These and a variety of other sugar alcohols have been shown to accumulate

Fig. 8 Metabolic pathways activated in *Mesembryanthemum crystallinum* epidermal bladder cells (EBCs) by salt, resulting in accumulation of osmoticums. BAM, beta-amylase; INV, invertase; HXK, hexokinase; INPS, *myo*-inositol-1-phosphate synthase; IMT, inositol methyl transferase; P5CS, delta 1-pyrroline-5-carboxylate synthase; P5CR, pyrroline-5-carboxylate reductase; PFK, phosphofructokinase; FBA, fructose-bisphosphate aldolase; PGK, phosphoglycerate kinase; PK, pyruvate kinase; PPK, pyruvate phosphate dikinase; PPC, phosphoenolpyruvate carboxylase; MDH, malate dehydrogenase; ME, malic enzyme; PPCK, phosphoenolpyruvate carboxylase kinase.

under salt and water stress in many halophytes including *M. crystallinum* (Flowers & Colmer, 2008).

The synthesis of *myo*-inositol, the precursor for the sugar alcohols ononitol and pinitol that accumulate under salt stress (Vernon & Bohnert, 1992a), is also enhanced by salinity (Ishitani *et al.*, 1996). In EBCs, transcripts encoding *myo*-inositol-1-phosphate synthase (INPS; Ishitani *et al.*, 1996) and *myo*-inositol O-methyltransferase 1 (IMT1; Vernon & Bohnert, 1992a,b), two key enzymes in the pathway leading to D-pinitol *via* D-ononitol, were highly abundant (ranks 23 and 78, respectively, in Tables 3 and S1), and up-regulated by salt, with the *IMT1* transcript exhibiting one of the highest fold changes observed in our study (> 170-fold; Table S5). *IMT* became one of the most abundant transcripts in the salt-treated transcriptome (rank 35), while showing highly suppressed expression in the control samples (rank 10232), suggesting a strict regulatory mechanism by salt in EBCs (Table S1, comp5305_c0_seq1).

Homologs of PM and tonoplast H⁺ or Na⁺/*myo*-inositol transporter (ITR) genes, *INT1* and *INT4*, were up-regulated by salt (Fig. 7; Table S10). PM-localized ITRs have been associated with the long-distance Na⁺ transport and vacuolar Na⁺ unloading that lead to the transport of *myo*-inositol into the cytoplasm, where it serves as a precursor for D-ononitol and D-pinitol (Vernon & Bohnert, 1992a; Nelson *et al.*, 1999; Chauhan *et al.*, 2000). These polyols may function in scavenging hydroxyl radicals that are not targeted by reactive oxygen species (ROS) scavenging enzymes (Smirnov & Cumbes, 1989; Peshev *et al.*, 2013). Interestingly,

the most abundant ITR in EBCs was *Mitr3* (AY233386; F. Quigley *et al.*, unpublished), while *Mitr2*, previously known as the leaf-specific paralogue (Chauhan *et al.*, 2000), was not detected in the McEBC transcriptome, suggesting cell type-specific transcript compartmentalization (Table S1).

Salt stress initiates a shift from C₃ to CAM carbon fixation mode in *M. crystallinum* (Adams *et al.*, 1998). Proteomic profiling of EBC extracts identified several CAM-specific isoforms of enzymes in these cells, suggesting that EBCs may contribute to the carbon fixation process in the leaf (Barkla *et al.*, 2012). Our RNA-seq identified transcripts for multiple essential CAM genes, with many showing significant salt induction (Table S5). These included PEPC kinase, induced by the largest fold difference among CAM-related transcripts at > 16-fold. PEPC kinase regulates PEPC activity, thus controlling the primary assimilation of CO₂ in CAM plants (Taybi *et al.*, 2000). CAM-related transcripts show diurnal fluctuation in their expression, while generally found to have higher basal-level expression in salt-stressed leaf tissues compared with unstressed samples (Cushman *et al.*, 2008). The accumulation of malic acid and other organic acids, and their diurnal fluctuations and physiological role in EBCs, possibly as osmoticums, as observed in some CAM plant species (Herrera, 2009), remain to be elucidated in future studies.

EBC salt stress-responsive gene expression

EBCs illustrate a salt response centered on the energy-intensive process of Na⁺ and osmolyte accumulation, including the proline synthesis pathway (Table S5), while deprioritizing canonical stress responses. RNA-seq results for EBC samples from salt-treated plants indicate that cells are in a stress-adapted state and therefore do not show hallmarks of transcript induction under salt stress as observed for glycophytes (Gong *et al.*, 2005).

Indeed, salt treatment appeared to relieve EBCs of osmotic stress when compared with the control samples, arguably using the accumulated Na⁺ and other compatible solutes as osmoticums (Adams *et al.*, 1998). GO terms enriched in DETs with decreased expression in response to salt treatment include 'response to salt stress', 'response to water deprivation' and 'response to biotic stress' (Fig. 4b; Table 4, BP-D1). These include homologs induced by ABA, salt, and osmotic stresses in *Arabidopsis* and other glycophytes (Table S5), as well as defense-related genes. Notably, transcripts encoding homologs of RD or early response to dehydration (ERD) family proteins showed down-regulation by salt in EBCs (Fig. 6a; Tables S1, S5).

In contrast, we observed salt induction of transcripts that provide protection from certain abiotic stresses, for example, transcripts in the GO term 'response to radiation' (Table 4, BP-U1; Fig. S3a). Furthermore, transcripts encoding homologs of CBL and CIPK family proteins showed increased expression upon salt treatment. Two copies of *CBL10* homologs were identified in the EBC transcriptome. Both showed up-regulation in response to salt. *CBL10*, often found duplicated in salt-adapted species (Oh *et al.*, 2014; Tang *et al.*, 2014), functions as a calcium sensor together with CIPK24 to regulate Na⁺ transport at the tonoplast (Kim *et al.*, 2007), and as a regulator of PM K⁺ transport by

modulating AKT1 activity via a direct interaction (Ren *et al.*, 2013), in Arabidopsis. These dual roles of CBL10 may facilitate both K⁺ uptake and Na⁺ sequestration in the cell, coordinating K⁺ : Na⁺ ratios in the cytoplasm. CBL10 is also involved in anthocyanin accumulation in Arabidopsis, as a response to UV irradiation (Quan *et al.*, 2007; Heyndrickx & Vandepoele, 2012). When plants are exposed to prolonged stress, McEBCs accumulate betacyanins and betaxanthins (Figs 1d, 2b, S1), which provide protection from strong irradiation (Vogt *et al.*, 1999). Supporting this finding, a transcript contig encoding a 4,5-DOPA-extradial dioxygenase, a key enzyme in the biosynthesis of the precursor of betacyanins and betaxanthin (Harris *et al.*, 2012), showed a three-fold increase in expression in response to salt (Table S1, comp28325_c0_seq1).

Finally, our RNA-seq study revealed the gene regulation associated with the massive intracellular functional reorganization within EBCs upon salt treatment. This included concerted down-regulation of genes involved in ER and Golgi functions, as well as cell wall components (Table 4, CC-D2; Table S7). However, transcripts linked to the GO term 'vacuole' remained up-regulated in the salt-treated samples, suggesting the continued importance of vacuoles in stress-regulated EBCs (Table 4, CC-U2; Table S7).

Conclusions

The McEBC transcriptome carries a unique profile that represents cell type-specific roles in the whole-plant integrated stress adaptation mechanisms. Transcripts associated with most of the signature pathways for salinity or water stress were down-regulated in the salt-adapted EBCs compared with EBCs from control plants. However, transcripts in pathways critical for metabolically active cellular functions under prolonged salt stress and pathways involved in protection against radiation were up-regulated. Our results support the notion of EBCs as metabolically active cells with unique stress responses.

About 32% of transcripts showing significant salt regulation in EBCs contained no sequence similarity (based on BLASTN $e < 10^{-5}$) to reference RNA sequences from any other plant species and were therefore functionally uncharacterized. These potentially lineage-specific transcripts may make important contributions to the development of the unique cell types and, in turn, the whole-plant salt-adaptation capacity of *M. crystallinum*. Our research demonstrates the utility of cell type-specific approaches, and provides resources for future investigations of the genetic mechanism of EBC functions.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Anthocyanin, betacyanin and betaxanthin content of bladder cell sap from *Mesembryanthemum crystallinum*.

Fig. S2 Flow chart depicting the processes in the development of a nonredundant reference transcriptome based on *de novo* assembly of RNA-seq reads.

Fig. S3 Networks representing gene ontology (GO) terms in the biological process (BP) category enriched in differently expressed transcription contigs (DETs).

Fig. S4 Networks representing gene ontology (GO) terms in the cellular component (CC) category enriched in differently expressed transcription contigs (DETs).

Table S1 List of contigs, annotation and RNA-seq results for the McEBC transcriptome

Table S2 List of gene and protein abbreviations used in Table 4

Table S3 Full list of gene ontology (GO) terms enriched in differently expressed transcription contigs (DETs) and transcript contigs included in each GO term

Table S4 Salt regulation of transcript contigs related to Na⁺ transport and H⁺ gradient generation

Table S5 Salt regulation of transcripts in metabolic pathways related to osmolyte accumulation

Table S6 Salt regulation of transcript contigs in gene ontology (GO) terms related to salt and osmotic stress responses

Table S7 Salt regulation of transcript contigs involved in endoplasmic reticulum (ER) and Golgi apparatus functions

Table S8 Comparison of the McEBC transcriptome with published RNA-seq data for leaf tissues in diverse plant species

Table S9 Data used for Table S8

Table S10 List of transcript contigs shown in Fig. 7, with the intracellular localization of the encoded protein and salt response of expression in McEBCs

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