

**New Phytologist**  
**Supporting Information**

STALK CELL POLAR ION TRANSPORT PROVIDE FOR BLADDER-BASED SALINITY TOLERANCE IN  
CHENOPODIUM QUINOA

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## supporting Methods

### Staining

For fluorescein diacetate (FDA) fluorescence detection, SCs from epidermal peels or petioles were isolated as described above. Prior to tissue staining, small sections of the petioles were taken. All drying steps were done at room temperature and in darkness. Tissues were stained using BSM containing  $5 \mu\text{g ml}^{-1}$  FDA and incubated for 15 min. They were then washed in BSM for 3 min to remove residual dyes before measuring the fluorescence intensity. The stained tissues were visualised using a fluorescence microscope (Leica MZ12, Leica Microsystems, Wetzlar, Germany) with UV illumination and a 13-wavelength filter. Photographs were taken with a megapixel digital colour camera (Leica DFC295, Leica Microsystems) and images were acquired using LAS V3.8 software (Leica Microsystems).

SCs were stained using 1 mM Lucifer yellow (LY) fed for 36 hours with via the petiole or the dye was iontophoretically loaded into the EC that is adjacent to a SC using a single barrel microelectrode. Staining of an EBC was performed using  $5 \mu\text{M}$  CFDA ((5-(and-6)-carboxyfluorescein diacetate)) diffused through a glass-capillary. Dyes were purchased at (Sigma-Aldrich). Pictures were taken using a Zeiss LSM 510 Meta laser scanning confocal microscope. The fluorescence of LY and CFDA was imaged using a 400-488 nm laser for excitation and emission collected between 520 and 570 nm (LY) and 520 and 530 nm (CFDA).

For staining of SCs with voltage sensitive dye ANNINE-6plus (Fromherz et al. 2008), quinoa leaves were submerged for 20 min with a dye concentration of  $5 \mu\text{g/ml}$  and 0.01% Triton X-100 (Thermo Scientific) in deionized water. The leaves were subsequently washed in deionised water and fluorescence images were taken with a Zeiss LSM 510 Meta laser scanning confocal microscope. Excitation was performed at 480 nm with a bandwidth of 10 nm and detection was between 515 and 560 nm. After images were taken under stable conditions, the samples were treated with 1% saponin to break down the potential gradient. After 5 min saponin treatment the image was taken again. Fluorescence intensities at the epidermal- and the bladder facing side of the SCs were calculated using ImageJ (v 1.51) before ( $F$ ) and after ( $F_{\text{end}}$ ) saponin treatment.

All images were processed with using ImageJ (v 1.51).

### RNAseq library preparation and sequencing

Library preparation and RNAseq were performed at the service facility “KFB - Center of Excellence for Fluorescent Bioanalytics” (Regensburg, Germany; [www.kfb-regensburg.de](http://www.kfb-regensburg.de)).

Library preparation and RNAseq were carried out as described in the Illumina TruSeq Stranded mRNA Sample Preparation Guide, the Illumina NextSeq 500 System Guide (Illumina, Inc., San Diego, CA, USA), and the KAPA Library Quantification Kit - Illumina/ABI Prism User Guide (Kapa Biosystems, Inc., Woburn, MA, USA). In brief, 200 ng of total RNA was used for purifying the poly-A containing mRNA molecules using poly-T oligo-attached magnetic beads. Following purification, the mRNA was fragmented to an average insert size of 200-400 bases using divalent cations under an elevated temperature (94 °C for 4 minutes). Next, the cleaved RNA fragments were reverse transcribed into first strand cDNA using reverse transcriptase and random hexamer primers. Actinomycin D was added to improve strand specificity by preventing spurious DNA-dependent synthesis. Blunt-ended second strand cDNA was synthesised using DNA Polymerase I, RNase H and dUTP nucleotides. The incorporation of dUTP, in place of dTTP, quenched the second strand during the later PCR amplification because the polymerase does not incorporate past this nucleotide. The resulting cDNA fragments were adenylated at the 3' ends, the indexing adapters were ligated, and subsequently specific cDNA libraries were created by PCR enrichment. The libraries were quantified using the KAPA SYBR FAST ABI Prism Library Quantification Kit. Equimolar amounts of each library were sequenced on a NextSeq 500 instrument using a 150 Cycles High Output Kit with the single index, paired-end (PE) run parameters. Image analysis and base calling resulted in .bcl files, which were converted into .fastq files with the bcl2fastq v2.18 software.

### Processing of raw RNAseq data, mapping and DGE

The raw RNAseq reads were analysed for quality with FastQC (FASTQC: A Quality Control tool for High Throughput Sequence Data. <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and afterwards trimmed with trimmomatic (Bolger et al. 2014) with parameters “ILLUMINACLIP:Illumina\_PE\_adapters.fasta:2:30:10:8:true LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:40”. The processed data was again analysed with FastQC

resulting in high quality trimmed reads. We constructed a genome-guided *de novo* reference transcriptome with trinity (Haas et al. 2013). For this, we used the quinoa reference genome (Jarvis et al. 2017) and the RNAseq data from this publication (Array Express ID: E-MTAB-10363) and from our ABA studies (Array Express ID: E-MTAB-10419) as input for trinity. The trinity reference transcriptome, which was built for this study, contained 113451 transcripts with BUSCO (Benchmarking Universal Single-Copy Orthologs) (Simão et al. 2015) values C:98.1%[S:12.2%,D:85.9%],F:1.4%,M:0.5%,n:1375 (1349 Complete BUSCOs (C), 168 Complete and single-copy BUSCOs (S), 1181 Complete and duplicated BUSCOs (D), 19 Fragmented BUSCOs (F), 7 Missing BUSCOs (M), 1375 Total BUSCO groups searched (n)) which shows a nearly complete transcriptome reference and reflects the tetraploidy of quinoa (D:85.9%). BUSCO was used with the lineage dataset *embryophyta\_odb10* ([https://busco.ezlab.org/list\\_of\\_lineages.html](https://busco.ezlab.org/list_of_lineages.html), creation date: 2017-12-01, number of species: 60, number of BUSCOs: 1375). In order to gain raw counts and TPM (Transcripts Per Million) values, we mapped the trimmed RNAseq libraries to the new reference transcriptome with kallisto (Bray et al. 2016) using standard parameters. We functionally annotated the transcriptome with AHRD (<https://github.com/groupschoof/AHRD>) to gain *Arabidopsis thaliana* description lines and gene identifiers as well as Gene Ontology (GO) terms. MapMan (Thimm et al. 2004) BINs and descriptions were constructed with Mercator (Schwacke et al. 2019). The R package EdgeR (Robinson et al. 2010) was used for differential gene expression (DGE). A gene was called differentially expressed when its false discovery rate (FDR) was below 0.05. Two major contrasts were analysed, called SCe (SC-enriched, “lightly brushed” vs “hard brushed”) and EBCe (EBC-enriched, “non brushed” vs “lightly brushed”).

The AHRD tool classified the transcripts into high confidence (HC) and low confidence (LC). The AHRD quality-code consists of a three-character string, where each character is either ‘\*’ if the respective criteria is met or ‘-’ otherwise.

Position 1: Bit score of the blast result is > 50 and e-value is < e-10,

Position 2: Overlap of the blast result is > 60%,

Position 3: Top token score of assigned HRD is > 0.5 (<https://github.com/groupschoof/AHRD#241-tab-delimited-table>). The strings “--\*” and “---” were treated as LC. In order to define the stalk cell transcriptome, we used both HC and LC transcripts, but for further analysis we focused only on HC transcripts.

### Enrichment of MapMan, GO and KEGG terms

We received the MapMan BINs from Mercator (Thimm et al. 2004) , the GO terms from AHRD (<https://github.com/groupschoof/AHRD>) and the KEGG (Kyoto encyclopaedia of genes and genomes) identifiers of *Arabidopsis thaliana* (Ath) from EdgeR (Robinson et al. 2010). In order to convert the trinity quinoa transcript IDs to Ath gene IDs, we used the AHRD Ath best BLAST (Basic Local Alignment Search Tool) hits and the R package tximport (Soneson et al. 2016).

To enrich the MapMan BINs, the gene ontology (GO) terms and the KEGG pathways, we used the tool camera coming with the R package limma (Ritchie et al. 2015). The heatmaps were designed using the R package pheatmap (Kolde 2012). Pheatmap: pretty heatmaps (1.2). R package. <https://cran.r-project.org/package=pheatmap> and ggplot2 (Wickham 2016). Significant enrichments were used for the plots.

### Cell purity of samples

To test purity via cell type enrichment or its absence in our approach, we first undertook a principal component analysis (PCA) (Figure S1A). This showed that the different samples (non-brushed, lightly-brushed and hard-brushed) (1) clustered impressively discrete, (2) were found very well separated, demonstrating a very fine-grained and clear split of the different experiments and RNA fractions from the different treatments. It is noteworthy mention that the dimensions of PC axes 1 and 2 (77% and 7% respectively; sum 84%) explain the different expression characteristics found in the three treatments and are remarkably high and pronounced. This already indicates a strong bias in expression characteristics among samples that can be very well separated and, of course, indicates different gene expression characteristics present in the different samples.

Next, we compared the current EBC-enriched genes (non-brushed vs. lightly-brushed) with the gene expression patterns of isolated EBCs (Bohm et al. 2018). The EBC enrichment comprises 1431 DEGs, thereof 231 being up-regulated and 1200 being down-regulated. We mapped the RNAseq data from isolated EBCs to our current reference and generated their mean TPM values. By doing so we could connect the fold changes obtained with both methods. The mean TPM values of expressed genes (TPM > 0.5) of isolated EBCs were 1529 in up-regulated DEGs and 21 in down-regulated DEGs, meaning a 72-times higher expression of EBC-enriched genes

compared to down-regulated genes. This suggests that the current approach using non-brushed and lightly-brushed samples (which cluster less distant because of similar tissue compartments than the hard brushed sample, see Figure S1A) provides EBC-enriched genes which are highly expressed in isolated EBCs. Comparing the clearly separated lightly and hard brushed samples should then result in an even more specific SC-enriched gene set.

As an additional test, we focused on trichome specific genes, based on the evolution of the SC-EBC complex from typical trichomes (Shabala et al. 2014). Thus in analogy trichome specific genes can be used as SC-EBC specific marker genes. For this purpose we used a published dataset of highly trichome specific genes (Jakoby et al. 2008) described in Arabidopsis. We identified the respective Arabidopsis orthologs in our data and compared the expression of the three samples non-, lightly- and hard-brushed. We could detect a significantly higher expression of the trichome specific genes in lightly- and non-brushed samples compared to hard-brushed sample (Figure S2A), also pointing to a high cell purity of the samples. The finding that an entire cluster of genes is specific to a cell type gives a clear advantage over a single marker gene.

In summary the observations underpin the ability to detect SC specific genes by contrasting different mechanically treated tissue fractions.

### *Xenopus oocyte preparation*

Investigations on quinoa channels and transporters were performed in oocytes of the African clawfrog *Xenopus laevis*. Permission for keeping *Xenopus* exists at the Julius-von-Sachs Institute and is registered at the government of Lower Franconia (Reference Number 55.2-2532-2-1035). Mature female *X. laevis* frogs were kept at 20 °C on a 12/12 h day/night cycle in dark grey 96 litre tanks (five frogs per tank). For oocyte isolation, mature female *X. laevis* frogs were anesthetised by immersion in water containing 0.1% 3-aminobenzoic acid ethyl ester. Following partial ovariectomy, stage V or VI oocytes were treated with 0.14 mg/ml collagenase I in Ca<sup>2+</sup>-free ND96 buffer (10 mM HEPES pH 7.4, 96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>,) for 1.5 h. Subsequently, oocytes were washed with Ca<sup>2+</sup>-free ND96 buffer and kept at 16 °C in ND96 solution (10 mM HEPES pH 7.4, 96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>) containing 50 mg/l gentamycin. For oocyte electrophysiological experiments, 10 ng of

each cRNA was injected into selected oocytes. Oocytes were incubated for 2 to 4 days at 16 °C in ND96 solution containing gentamycin.

#### RNA preparation for oocyte expression

cDNA was prepared using 2 µg of total RNA after DNA contamination was removed by RNase free DNase I (Thermo Scientific) digestion in the presence of RiboLock RNase Inhibitor (Thermo Scientific) following the manufacturers specifications. RNA was precipitated with 5 M NH<sub>4</sub>Cl in isopropanol and washed with 70% ethanol. The RNA pellet was resuspended in diethyl pyrocarbonate (DEPC)-treated H<sub>2</sub>O and first strand cDNA was synthesised using the M-MLV RT RNase (H-) point mutant (Promega) following the manufacturers specifications. This cDNA was used for the amplification of the transport proteins. Using gene-specific oligonucleotide primers (shown below), which comprised the CDS of the transport proteins and Advantage cDNA Polymerase Mix (Clontech), revealed for each gene, a sequence identical to that expected from the RNAseq data set.

Gene-specific oligonucleotide primers: CqSLAH3; fwd 5` ATGGAGAAGGCCGCGGAGC`3, rev 5` CTAGACTTCAACATTTTCCTTTTCAT`3; CqAMT1; fwd 5` ATGTCTTCTTCCACGGCACC`3, rev 5` TTATGAAGTTGACCCATATCCATC `3; CqSKOR; fwd 5` ATGACGCCGAAGAAAATGGTAC`3, rev 5` CTATTCTGTTTCACTTATCAGATACAAC`3

For localisation in *X. laevis* oocytes, the cDNA was cloned into the oocyte expression vectors (based on pGEM vectors) by an advanced uracil excision-based cloning technique as described by Nour-Eldin et al. 2006 (Nour-Eldin et al. 2006). CqAMT1 was fused N-terminally to the vector-based YFP. The measurements were performed on a Leica SP5 confocal laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) using the water immersion objective lens Leica HCX IRAPO L25×/0.95W for confocal imaging. YFP fusion proteins were excited with a 514-nm diode laser, and YFP fluorescence emission was detected between 530 and 560 nm.

In two-electrode voltage-clamp (TEVC) studies, oocytes were perfused with standard bath solution containing 10 mM MES/Tris (pH  $\geq$  5.5), 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 1 mM LaCl<sub>3</sub>. The osmolality of each solution was adjusted to 220 mOsm/kg using D-sorbitol. For the cation transport proteins, the ionic strength for measurements under varying substrate concentrations was balanced with lithium to give a total concentration of 100 mM. For CqSLAH3, we compensated for the chloride or nitrate variations with gluconate. For current-voltage relations of CqSKOR, single-voltage pulses were applied in 30 mV decrements from +40 to -140 mV starting from a holding potential of 0 mV. Any deviations of this standard pulse protocol are mentioned in the figure legends.

To estimate the relative open probability of CqSKOR, instantaneous currents were extracted immediately after the voltage jump from the test pulses to the holding potential. For calculation of the relative open probability, the Boltzmann function was used:

$$P_{\text{Open}} = \frac{I_{\text{off}} + I_{\text{on}}}{I_{\text{off}} + I_{\text{on}} + I_{\text{on}} \exp\left(\frac{V_{1/2} - V_m}{z}\right)} \quad (1)$$

where  $V_{1/2}$  is the half-maximal activation voltage,  $V_m$  is the membrane potential and  $z$  is the slope of the Boltzmann function. The currents were normalised to the saturation value of the calculated Boltzmann distribution. For recording representative current traces and steady-state currents of CqSLAH3-expressing oocytes, the standard voltage protocol was as follows: starting from a holding potential of 0 mV, single 20 s-voltage pulses were applied in 20 mV decrements from +40 to -180 mV. Steady-state currents were extracted after 19 s of the respective voltage pulse. Instantaneous currents were extracted 45 ms after the voltage jump from the holding potential of 0 mV to 50 ms test pulses ranging from +70 to -150 mV. The reversal potentials ( $V_{\text{rev}}$ ) used for the calculation of the relative permeability of CqSLAH3 were recorded in the current-clamp mode in the presence of 50 mM of the respective anion. For determination of  $V_{\text{rev}}$  for the respective anion, oocytes were preincubated in 50 mM NO<sub>3</sub><sup>-</sup> to gain full activity of SLAH3. The relative permeability was calculated as described previously (Chen et al. 2010). The expression of CqAMT1 N-terminally tagged with an YFP was observed as described in 'Localisation in *X. laevis* oocytes'. CqAMT1-expressing oocytes were clamped at -160 mV and the steady-state currents ( $I_{\text{ss}}$ ) were recorded and compared to control (water injected) cells.

For statistical analysis and graph preparations the software Igor Pro7 (waveMetrics, Inc., Lake Oswego, Oregon, USA) and Excel (Microsoft Corp. Redmond, Washington, USA) was used.

### Ion-flux and membrane potential measurements

Ion selective electrodes were ion-fluxes were measured from isolated SCs 1 h after brushing the petioles for 5 minutes using commercially available  $K^+$  and  $Cl^-$  liquid ion exchanger cocktails (both from Sigma-Aldrich, St Louis, MO, USA). The  $Na^+$  liquid ion exchanger was made by mixing the following chemicals (all purchased from Sigma-Aldrich): 95.9 mg of 2-Nitrophenyl octyl ether (cat#: 73732), 0.6 mg of Sodium tetrphenylborate (cat#: 72018) and 3.5 mg of Sodium ionophore X (cat#: 71747). pulled from borosilicate glass capillaries w/o filament ( $\emptyset$  1.0 mm, Science Products GmbH) with a vertical puller (Narishige Scientific Instrument Lab). They were baked over night at 220 °C and silanised with N, N-Dimethyltrimethylsilylamine (Sigma-Aldrich) for 1 h. For ion-flux determination two independent techniques were applied.

i) The Microelectrode Ion-Flux Estimation (MIFE, University of Tasmania, Hobart, Australia) technique was used to measure ion-fluxes in Table 1, Figure 3D/E, 4A-C, D-E and S9B-F.

ii) The SISE (Scanning Ion Selective Electrodes) technique was applied to measure fluxes in Table 1, in Figure 3D/E, and S9G.  $K^+$ -and  $NH_4^+$ -selective electrodes were backfilled with 100 mM of the respective ion and the tip was filled with Potassium ionophore I cocktail B or Ammonium ionophore I cocktail A (Sigma-Aldrich). Calibration of the selective electrodes was performed at 1, 10 and 100 mM KCl or  $NH_4Cl$ . Only electrodes that recorded a shift in voltage of around 59 mV per  $pK^+$ ,  $pNH_4^+$  unit were used. The ion-selective electrodes were positioned with a Micromanipulator (PatchStar, Scientifica) approximately 4  $\mu m$  distance from the stalk cells using an inverted microscope (Axiovert 135, Carl Zeiss AG). The electrode was connected via an Ag/AgCl half-cell to the head stage of the microelectrode amplifier (custom-built). The electrode scanned at 10 s intervals over 50  $\mu m$ . Raw data were acquired with a NI USB 6259 interface (National Instruments), using custom-built Labview-based software "Ion-Flux Monitor". Raw voltage data were converted offline into ion-flux data (Bohm et al. 2018). A detailed description of the statistical analysis performed is available here: [https://github.com/TeamMacLean/peak\\_analysis](https://github.com/TeamMacLean/peak_analysis)

In brief, the mean values of the voltages measured with ion selective electrodes at both positions were calculated and the voltage difference  $dV$  between both positions was computed and inserted in equation (2). The Flux  $J$  was calculated using equation:

$$J = cuF(58/\text{Nernst slope})(dV/dx), \quad (2)$$

With the following parameters: the ion concentration  $c$  ( $\text{mol m}^{-3}$ ), the mobility of the ion  $u$  ( $\text{m}^2 \text{s}^{-1} \text{ volt}^{-1}$ ), the Nernst-slope originating from the calibration, the voltage difference  $dV$  (volt) between both positions, and the distance between both positions  $dx$  (m). For the spherical SC the distance was corrected using:

$$dx = r^2 \left[ \frac{1}{r+x} - \frac{1}{r+x+dx} \right] \quad (3)$$

With  $r$  being the radius of the SC,  $x$  the minimum distance to the SC and  $dx_1$  as the distance between both positions ( $50 \mu\text{m}$ ).

For membrane potentials, conventional microelectrodes with a tip diameter of  $0.5 \mu\text{m}$  were filled with  $1 \text{ M KCl}$  and used to measure MP via an Ag/AgCl half-cell with the MIFE electrometer (Shabala et al. 2002, Cuin et al. 2005). Membrane potentials were measured by impaling the stalk cells with the KCl-filled Ag/AgCl microelectrodes using a manually operated hydraulic micromanipulator (MHW-4, Narishige, Tokyo, Japan). Membrane potentials recorded for 2 min after stabilisation.

### Capillary electrophoresis

Due to the bladder-based turgor and the capillary force, the EBC sap was simultaneously pushed and sucked into the glass capillary tip. For each sample, 20 bladder cells were impaled. To determine the sap volume, images were taken of each capillary tip (Leica DFC295, Leica Microsystems, LAS V3.8 software) and the volume calculated with the equation for the volume of a cone  $V = (1/3)\pi r^2 h$ , using the following parameters: volume ( $V$ ), radius ( $r$ ) and height ( $h$ ).  $10 \mu\text{l}$  water was added to each sample and the resulting dilution factor was taken into account in the final concentration determination of the analysed cations.

Reagents: Standard solutions of NaCl, KCl and  $\text{NH}_4\text{Cl}$  ( $10, 50, 100$  and  $500 \mu\text{M}$  each) were prepared using analytical grade reagents from Sigma-Aldrich. The background electrolyte (BGE) was freshly prepared and consisted of  $50 \text{ mM}$  tris(hydroxymethyl)aminomethane (Tris) and  $50 \text{ mM}$  N-Cyclohexyl-2-aminoethanesulfonic acid (CHES).

Instrumentation: Hewlett Packard 3D-CE instrument consisting of an on-column diode array UV/VIS detector was used. The C4D signal was collected using an Agilent 35900 E analogue-to-digital converter and the capillary detection was achieved using a TraceDec C4D cell, placed

inside the cassette. The integration and processing of signals was achieved using the 3D-CE Chem Station Software.

Separation process: Silica fused capillaries with a total length of 75 cm and 75  $\mu\text{m}$  i.d. were used and flushed with BGE for 15 min at 1,000 mbar prior to their first use and after overnight storage. Before each run, the capillary was pre-conditioned with BGE for 2 min at 1,000 mbar. The samples were hydrodynamical injected at 40 mbar for 4 s (corresponds to 18.58 nl) at 25  $^{\circ}\text{C}$  with a separation voltage of 25 kV and a separation time of 5 min. UV detection was not performed.

### Microscopy

Pictures of the EBCs from the abaxial leaf of plants grown under different NaCl concentrations were taken using an environmental scanning electron microscope (FEI MLA650 ESEM, ThermoFisher Scientific, Oregon, United States). and examined at 25 kV, 600 Pa,  $\sim 10$  mm working distance and at  $\sim 5.0$   $^{\circ}\text{C}$ . Pictures of the leaves and SCs were taken with a conventional digital camera. All images were processed with ImageJ (v 1.51).

For detailed analysis of the SCs, quinoa epidermal cells were cut with a razor blade, carefully separated from the rest of the leaf with forceps then further prepared for transmission electron microscopy analysis as previously described (Bohm et al. 2018). Electron micrographs were recorded with TEM Center software (JEOL) at a JEOL JEM-1400Flash transmission electron microscope (TEM) at 120 kV with a Matataki camera and at a JEOL JEM-2100 TEM at 200kV with EM-Menu acquisition software and a TemCam F416 (TVIPS) camera. 3D visualisation of the stalk cell ultrastructure was performed with the computer software package, IMOD (Kremer et al. 1996).

To locate quinoa epidermal cells for EM preparation, a micro CT scan was taken using the Fraunhofer IIS ntCT (Fella et al. 2018). NtCT is a NanoCT setup based on an Excillums Nanotube X-ray source and Dectris Eiger 2 photon counting detector. The measurement was taken at 60 kV tube voltage, with 350mW electron beam power at a  $\sim 450$  nm (FWHM) X-ray spot. The CT dataset with a voxel sampling of 1.2  $\mu\text{m}$  was acquired with 1890 projections each 10 s, resulting in a total scan time of 6 h.

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