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Supplemental information

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Temperature changes in the root ecosystem affect plant functionality

Mary Paz González-Garcia*[1,2], Carlos M. Conesa*[1], Alberto Lozano[1], Barbara Simancas[1], Sara Navarro-Neila[1], María Sánchez-Bermúdez[1], Victoria Baca-Gonzalez[1], Elena Caro[1,2], Isai Salas-González[3], Gabriel Castrillo[4] and Juan C. del Pozo[1ψ]

[1] Centro de Biotecnología y Genómica de Plantas. Universidad Politécnica de Madrid (UPM) - Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA/CSIC). Campus Montegancedo 28223 Pozuelo de Alarcón (Madrid), Spain.

[2] Departamento de Biotecnología-Biología Vegetal, Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de Biosistemas, Universidad Politécnica de Madrid (UPM), 28040 Madrid, Spain

[3] Undergraduate Program in Genomic Sciences. Center for Genomics Sciences. Universidad Nacional Autonóma de México, Av. Universidad s/n. Col. Chamilpa, 62210. Cuernavaca Morelos, México.

[4] Future Food Beacon of Excellence & School of Biosciences, University of Nottingham, Sutton Bonington, UK

Supplemental Methods

TGRooZ device

The TGRooZ device has been patented as utility model (U202230407) and was developed in collaboration with Ibercex company (https://www.inilab.es/marca/ibercex-camaras-ultracongeladores/). The TGRooZ consists of a metallic growth box that holds a cold-regulable bottom container (Supplementary Fig. 1b). The temperature differences between the bottom and the top surface of the device generates a gradient. The gradient can be regulated (extended or limited) by modulating the temperature in the cold-regulable bottom container and is measured with a digital thermometer with a metallic probe of 35 cm long. The temperature is regulated by water chiller machine (Supplementary Fig. 1c). In the top of the device, different lids can be positioned. Here we designed two model, one to hold 12x12 cm petri dishes containing agar-based medium and other to hold zip bags until 40 cm wide.

To cultivate pots containing soil, a modified TGRooZ device was engineered. Similarly, the base of the device was refrigerated by circulating cold water using a water-cooling machine at 10ºC and the rest of the pot was isolated with polyurethane foam to allow to cool the base of the pot and force a gradient from bottom to the top of the soil. Additionally, in the base of the potholder, a small hole is made to evacuate the excess of irrigation.

Soil Gradient calculation

To determine the soil gradient a 5 digital thermometer with a 30 cm metallic probe were used. The probes were penetrated into the soil every 2 cm and temperature was recorded in the natural soil (GPS´s coordinates 40.40535848787632, -3.831371201424853) in May and June 2019 at 2 pm. The humidity and pH was recorded using a soil PhMetter (sinbadlab).

To analyze the fluctuation of the soil gradient, we measured the maximum and minimum temperature during 4 consecutive days (27^{th}-30^{th} of June, 2022) in the soil indicated above. We used some maximum-minimum digital thermometers with an extensible probe that were introduced into the soil at 5 and 15 cm from the top. We recorded the max –min temperature daily

and represented in Supplemental Figure 1C as the average of three measures from 3 different thermometers.

Tomato cultivation in germination paper

Lycopersicon esculentum, variety money maker seeds, were pre-germinated in darkness in filter paper wetted with water for 4 days. Homogeneous seedlings were then transferred to the germination paper system. This system consists in a germination paper of 20 cm wide x 30 cm height (AHLSTROM MUNKSJÖ, BINZ2.383.200350) into a transparent plastic zip bag. Small apertures were made in to bag where the seedlings is settled to allow the growth of the hypocotyl and shoot, remaining the rest of the zip closed to avoid the evaporation of the medium. The germination paper is wetted with 45 ml of one fourth MS liquid medium without sucrose at pH=5.8. The germination paper-zip system is then moved to the chamber at 26ºC (26SR), 26TGRooZ using an adapted holder for the zip bag, 36ºC (36SR) or 36TGRooZ with the same adapted holder. After 7 days growing, the root system was analyzed with the GiaRoots to quantify different parameters.

Stomatal conductance and leaf temperature

Arabidopsis seedlings were germinated in half-strength MS medium (MS1/2) with vitamins plus 1% sucrose and 1% Difco Agar, 0,05% MES and pH=5.8 using the D-Root. After 7 days, they were transferred to pots containing 3 L of greenhouse substrate soil. These seedlings were grown for 3 weeks at 22ºC in shoot and root; (22SR), 32ºC in root and shoot, (32SR) or 32TGRooZ (32ºC in shoot and gradient from 32 to 24ºC in the root) and irrigated with 100 ml of sterile water twice a week. The water content was recorded using a Teros 10 probe (Meter group) and the Zentra Z6 datalogger every 8 hours for an entire period of 3 weeks to verify that the water content was not limited in the experiment.

Stomatal conductance (mmol m−2 s−1) was measured using a leaf porometer (model SC-1, Decagon Devices, Inc., Pullman, WA, USA). Instrument calibration was done prior each set of measurements according to manufacturer's guidelines. Two leaves from the same position in each

plants was measured in three different replicates and temperature conditions. Limiting the number of leaves on which to measure stomatal conductance was done to minimize the variability in stomatal conductance due to meteorological factors. The total number of leaves used in the experiment was always greater than eighteen. Soil water content was measured to uncouple with stomatal conductance.

Thermal pictures were taken with a FLIR-E96 camera and leaf temperature was quantified using the FLIR research studio (https://www.flir.es/products/flir-thermal-studio-suite). We quantify more than 30 leaves per temperature treatment from 3 different experiments.

Gene network generation

A gene regulatory network is a collection of molecular regulators that interact with each other and with other substances in the cell to govern the gene expression levels of mRNA and proteins, which, in turn, determine the function of the cell. Gene Network Inference with Ensemble of trees (GENIE3), a GRN inference method based on variable selection with ensembles of regression trees. It produces a directed graph of regulatory interactions and naturally allows for the presence of feedback loops in the network. During DESeq2 analysis, the normalized counts were obtained for both root and shoot samples, using the median of ratios method. These normalized counts show a new value of the count divided by the total number of observations. For each comparison, 32SR_vs_22SR, TGRZ_vs_22SR and TGRZ_vs_32SR, it is created a new *.csv file, containing the normalized counts for each gene within the samples belonging to the temperature conditions required for each comparison. These files will be the source to create the expression matrix using the by GENIE3 software, where each row belongs to a gene and every column belongs to a sample at a certain temperature condition. Each entry in the matrix represents the expression level of a particular gene in a given sample. All the genes in every condition must have unique reads assigned to that gene in that sample in order to be processed by GENIE3. In order to add more biological meaning to the GRNs, additional information like identifying transcription factors among the whole list of genes can be given. This additional information would provide more reliability to the network and robustness to the interaction between genes. With the expression

matrix and the extra information, GENIE3 can generate the weight matrix for each comparison. The algorithm outputs a matrix containing the weights of the putative regulatory links, with higher weights corresponding to more likely regulatory links. The next step is to generate the linklists for each comparison, containing the ranking of links. This link list gets the weight matrix as the input and creates all the interactions between genes existing among the data. These linklist contain rows corresponding to regulatory links, whose first column shows the regulator (i), the second shows the target gene (j) and the last columns shows the weight (im) of the connection between genes (i and j). Since the algoritm has some randomness within, the same values in the same linklist executed at different times, will vary slightly. This linklist is a large file with as many rows as interactions set earlier. Usually there is no need to get all the links between genes, and some of the interactions might have a value of 0, so it is possible to get only the most regulatory links with or to filter the matrix adding a minimum weight threshold. This threshold is recommended to be set to erase all the links whose value is equal to 0 or close to 0.

Microbiota analyses

For the analyses of microbiota abundances, tomato seeds were germinated at 22ºC during 1 week in sterile vermiculite. Afterwards, seedlings were transferred to pots (1 per pot) filled with 3 liter of natural soil obtained from coordinates 40°24'21.6"N 3°49'58.1"W that were mixed with 1 part of clean river sand. These plants were cultivated for 3 weeks at three different temperatures 22ºC (22ºC shoot and root; 22SR), 36ºC (36ºC in root and shoot, 36SR) and 36TGRZ (36ºC in shoot and gradient from 36 to 20ºC in root) and irrigated with 150 ml of sterile water twice a week. We took 6 samples of the initial soil and maintained them at -20ºC. After 3 weeks, we took 6 samples of soil corresponding to rhizosphere and 6 samples of roots that were extensively washed to eliminate soil/microorganism traces) and freeze to -20ºC. Soil and roots were taken from the same area in the pot, corresponding to an intermedium part of the root system.

After collection, samples were sent for molecular analysis to Biome Makers laboratory (https://biomemakers.com/) in Sacramento, US. DNA extraction was performed with the DNeasy PowerLyzer PowerSoil Kit from Qiagen. To characterize bacterial communities associated with

bulk soils, rhizosphere and endophytes, the 16S rRNA were selected. Libraries were prepared following the two-step PCR Illumina protocol using custom primers amplifying the 16S rRNA V4 region as described in[1]. Sequencing was conducted in an Illumina MiSeq instrument using pair-end sequencing (2x300bp).

16S rRNA amplicon sequence analysis

Briefly, raw amplicon sequences were quality filtered using fastp and then merged using pear with default parameters. The resulting high-quality filtered sequences were then denoised and collapsed into amplicon sequence variants (ASVs) using DADA2 v.1.10.1[2]. Representative ASVs sequences were taxonomically classified with the mothur naive bayes classifier trained on the SILVA 132 database[3]. We filtered ASVs that were assigned to chloroplast, mitochondria, oomycete, archaea or did not have a known kingdom assignment. After the filtering of low quality ASVs, we created rarefied and relative abundance tables using a threshold of depth of 10,000 reads per sample. The resulting abundance tables were processed and analyzed with functions from the ohchibi package (https://github.com/isaisg/ohchibi).

To compare alpha diversity across conditions and fractions, we calculated the Shannon diversity and Richness indexes using the diversity function from the vegan package v2.5-5[4]. We used ANOVA to test for differences in alpha diversity between conditions across fractions. Beta diversity analyses (Principal coordinate analysis) was based on Bray-Curtis dissimilarity matrixes calculated from the abundance table. To compute the variance explained by the Fraction effect and Temperature treatments, we performed PERMANOVA using the function adonis from the vegan package v2.5-5[4]. Bar graphs showing raw and average relative abundance of phylums across conditions was computed using the chibi.phylogram function from the ohchibi R package. We used the R package DESeq2 v.1.24.0[5] to compute the intra-fraction (Intra rhizosphere, intra root) specific enrichment profiles across the three temperature conditions. For each taxonomic unit of the following taxonomic levels, Phylum, Class, Order, Family, Genus, and ASV, we

estimated their abundance differences in each of the three temperature conditions, using a generalized linear model (GLM) with the following design:

Abundance ~ Temperature condition

Within each fraction (rhizoshphere and root), we performed the all vs all contrasts between temperature conditions (3 possible contrasts in total) and kept taxonomic units with a False Discovery Rate (FDR) p-value < 0.05. We visualized the results of the contrasts using a heatmap, in which we showed the enrichment patterns (log2 fold change) of all statistically significant taxonomic units across the three contrasts using ggplot2 v.3.2.1 R package [6]

Analysis of leaf mineral elemental profile (ionome)

The elemental profiles of leaves were measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The leaf material was collected and washed three times with 18.2 MΩcm Milli-Q water (Merck Millipore). The samples were placed in weighted Pyrex digestion tubes and dried at 88 ºC for 20-h. After cooling, leaf samples were weighted on Mettler five-decimal analytical scale, and 1-3 mL (depending on the sample dry weight) of the concentrated trace metal grade nitric acid Primar Plus (Fisher Chemicals) was added to each tube. Prior to the digestion, 20 µg/L of Indium (In) was added to the nitric acid as an internal standard to assess putative errors in the dilution process, variations in sample introduction and plasma stability in the ICP-MS instrument. The samples were then digested in DigiPREP MS dry block heaters (SCP Science; QMX Laboratories) for 4-h at 115 ˚C. After cooling down, the digests were diluted to 10-30 mL (depending on the volume of the nitric acid added) with 18.2 MΩcm Milli-Q Direct water and elemental analysis was performed using an ICP-MS, PerkinElmer NexION 2000 equipped with Elemental Scientific Inc 4DXX FAST Dual Rinse autosampler, FAST valve and peristaltic pump. The instrument was fitted with a PFA-ST3 MicroFlow nebulizer, baffled cyclonic C3 high sensitivity glass spray chamber cooled to 2 ºC with PC3X Peltier heated/cooled inlet system, 2.0 mm i.d. quartz injector torch and a set of nickel cones. Twenty-four elements were monitored including following stable isotopes: ^{7}Li, ^{11}B, ^{23}Na, ^{24}Mg, ^{31}P, ^{34}S, ^{39}K, ^{43}Ca, ^{48}Ti, ^{52}Cr, ^{55}Mn, ^{56}Fe,

^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{82}Se, ^{85}Rb, ^{88}Sr, ^{98}Mo, ^{111}Cd, ^{208}Pb and ^{115}In. Helium was used as a collision gas in Kinetic Energy Discrimination mode (KED) at a flow rate of 4.5 mL/min while measuring Na, Mg, P, S, K, Ca, Ti, Cr, Mn, Fe, Ni, Cu, Zn, As, Se and Pb to exclude possible polyatomic interferences.

The remaining elements were measured in the standard mode. The instrument Syngistix™ software for ICP-MS v.2.3 (Perkin Elmer) automatically corrected any isobaric interferences. The ICP-MS measurements were performed in peak hopping scan mode with dwell times ranging from 25 to 50 ms depending on the element, 20 sweeps per reading and three replicates. The ICP-MS conditions were as follow: RF power – 1600 Watts, auxiliary gas flow rate 1.20 L/min. Torch alignment, nebuliser gas flow and quadrupole ion deflector (QID) voltages (in standard and KED mode) were optimized before analysis for highest intensities and lowest interferences (oxides and doubly charged ions levels lower than 2.5 %) with NexION Setup Solution containing 1 µg/L of Be, Ce, Fe, ln, Li, Mg, Pb and U in 1 % nitric acid using a standard built-in software procedure. To correct for variation between and within ICP-MS analysis runs, liquid reference material was prepared using pooled digested samples and run after the instrument calibration and then after every nine samples in all ICP-MS sample sets. Equipment calibration was performed at the beginning of each analytical run using seven multi-element calibration standards (containing 2 µg/L In internal standard) prepared by diluting 1000 mg/L single element standards solutions (Inorganic Ventures; Essex Scientific Laboratory Supplies Ltd) with 10 % nitric acid. As a calibration blank, 10 % nitric acid containing 2 µg/L In internal standard was used, and it was run throughout the analysis. Sample concentrations were calculated using the external calibration method within the instrument software. Further data processing, including calculation of final elements concentrations, was performed in Microsoft Excel.

Ionome analysis

We created a matrix (samples x ion) in which each cell was filled with the calculated element concentration in a given sample. Afterwards, we applied a *z*-score transformation of each

individual ion across the samples in the matrix. We compared the concentration of each ion across the three temperature treatments by applying a linear model with the following design:

$$\text{Ion Concentration} \sim \text{Temperature Condition}$$

After fitting the model, we determined statistical significant differences between conditions by performing pairwise comparisons and visualizing the results of the comparisons using the compact letter display (CLD) as implemented in the in the multcomp v.1.4-12 R package.

The abundance profiles of each ion across temperature conditions was visualized using a heatmap created by the ggplot2 v.3.2.1 R package [6] using the standardized (z-score) ion abundance.

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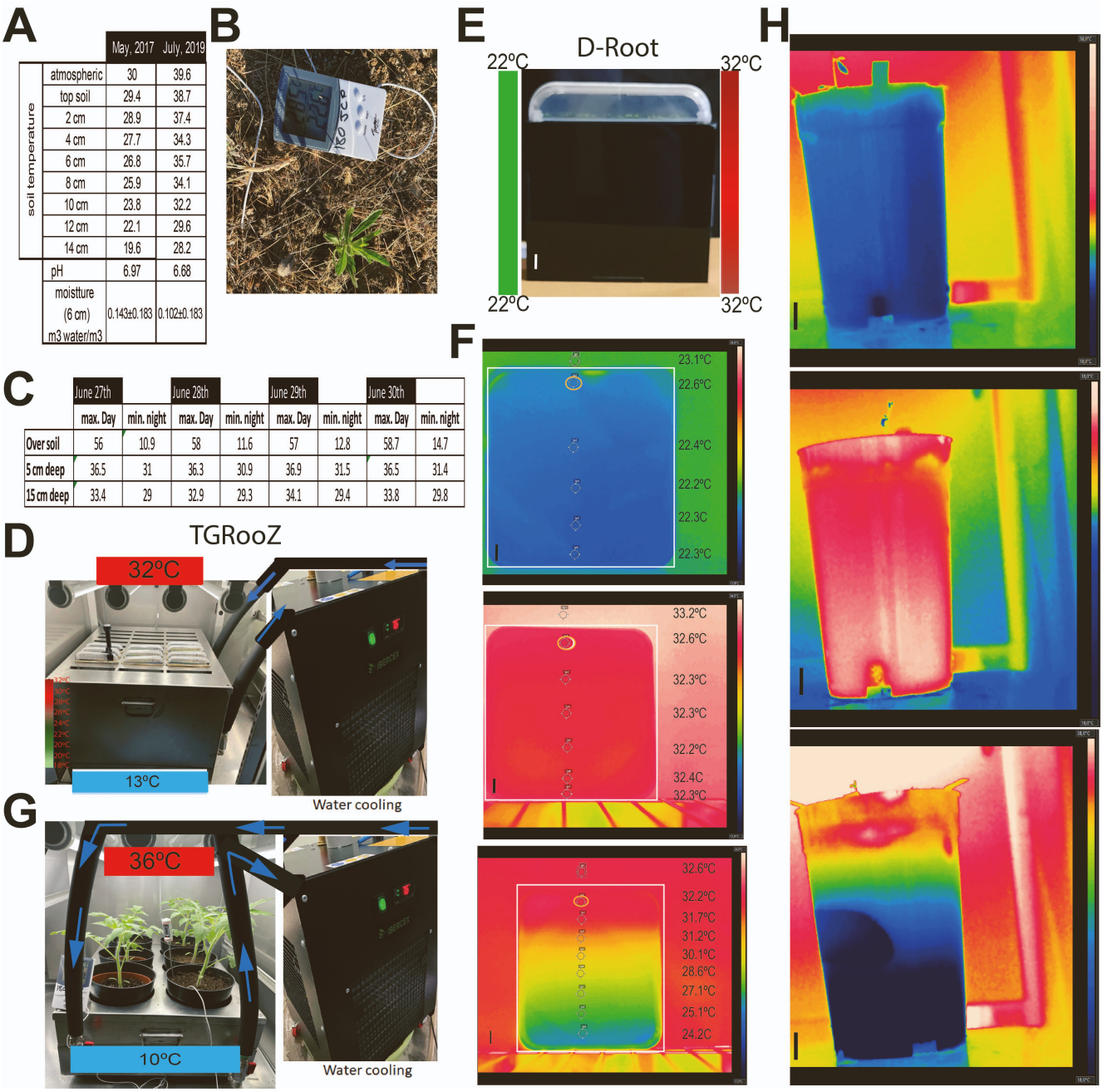
Supplemental Figures

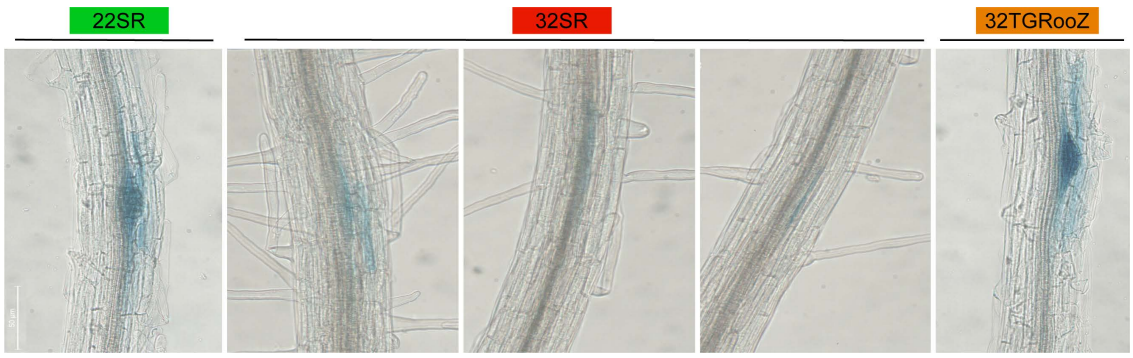


		May, 2017	July, 2019
soil temperature	atmospheric	30	39.6
	top soil	29.4	38.7
	2 cm	28.9	37.4
	4 cm	27.7	34.3
	6 cm	26.8	35.7
	8 cm	25.9	34.1
	10 cm	23.8	32.2
	12 cm	22.1	29.6
	14 cm	19.6	28.2
	pH	6.97	6.68
	moistture (6 cm) m3 water/m3	0.143±0.183	0.102±0.183

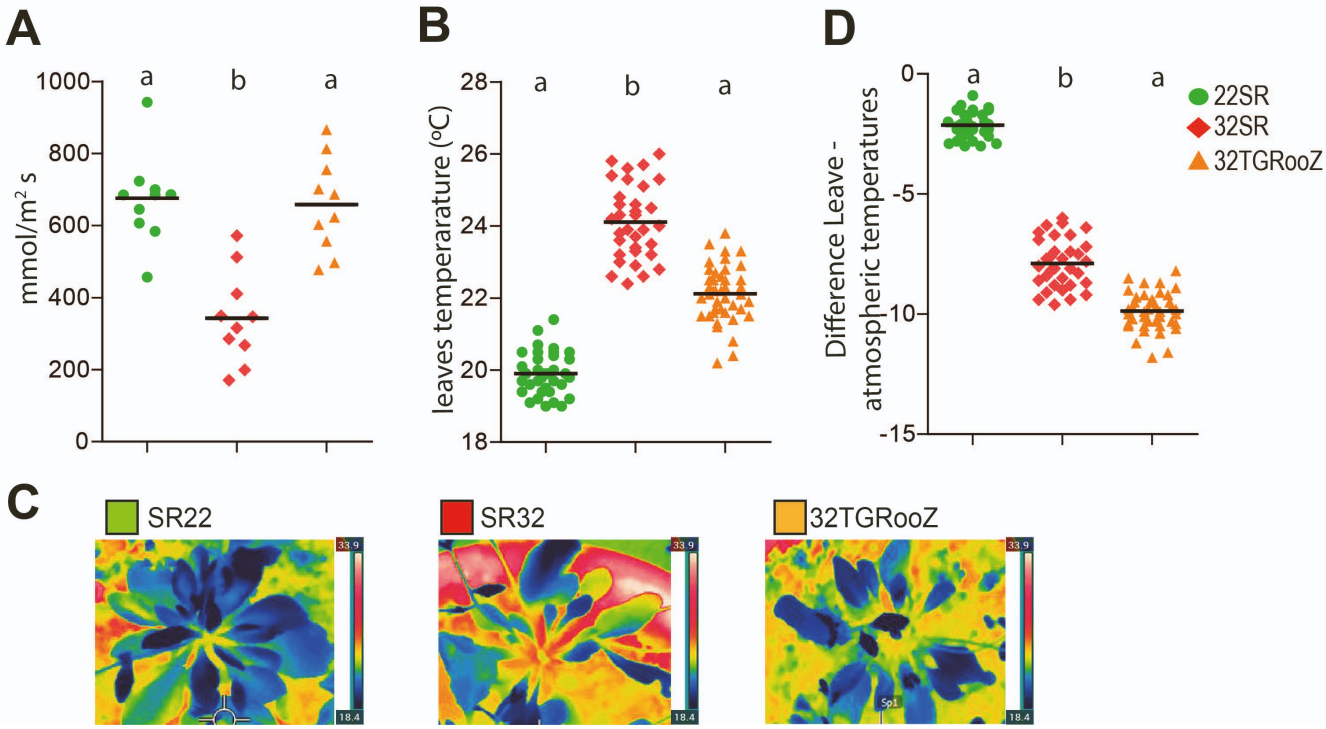
	June 27th		June 28th		June 29th		June 30th	
	max. Day	min. night	max. Day	min. night	max. Day	min. night	max. Day	min. night
Over soil	56	10.9	58	11.6	57	12.8	58.7	14.7
5 cm deep	36.5	31	36.3	30.9	36.9	31.5	36.5	31.4
15 cm deep	33.4	29	32.9	29.3	34.1	29.4	33.8	29.8

Supplemental Figure 1: TGRooZ device. (A) Temperature of the soil (GPS´s coordinates 40.40535848787632, -3.831371201424853) in May and June 2019 using a digital thermometer containing a metal probe. Measured were taken every 2 cm and the temperatures correspond to the average of 3 different measures. **(B)** Digital thermometer with an external probe to measure maxima and minima temperatures at 5 or 15 cm deep. **(C)** Maxima and minima temperatures during 4 days in natural soil (GPS´s coordinates 40.40535848787632, -3.831371201424853) between June 27th and 30th, 2022. **(D)** TGRooZ device to cultivate seedlings in 12x12 cm petri dishes containing agar-based

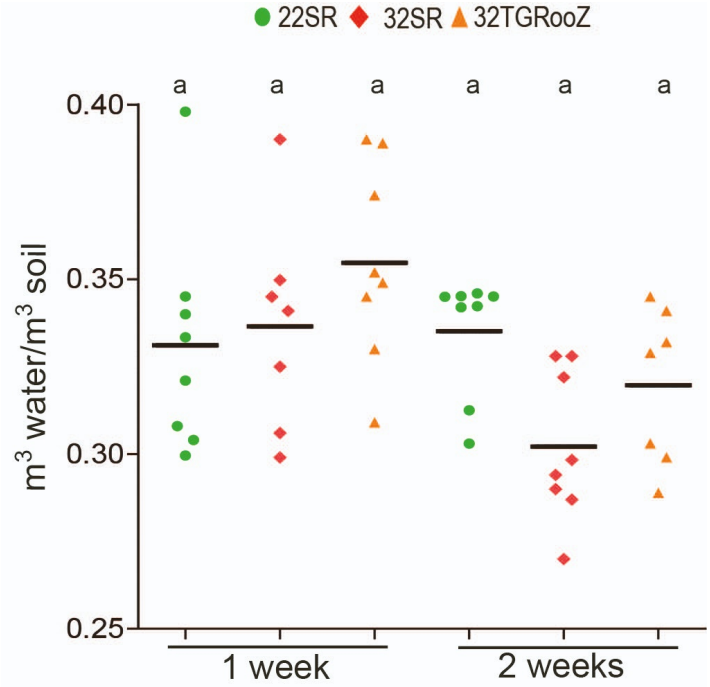
medium. To generate a temperature gradient in the root zone the base of the device is refrigerated by circulating cold water at 10-13 ºC, depending on the gradient desired, using a water-cooling machine. **(E)** D-Root system to preserve the roots from direct illumination. Seedling in the D-Root were cultivated to homogeneous 22ºC (22SR) or 32ºC (32SR). Scale bar corresponds to 1 cm. **(F)** Thermal pictures of 22SR, 32SR or 32TGRooz Petri 12x12 plates Notice the temperature gradient formed in the 32TGRooZ. Circles named as SP were used to calculate the temperature in the area using the FLIR studio software. The top SP corresponds to the chamber temperature. Yellow circles indicate the position of the shoots in the plate. seedlings. Scale bars correspond to 1 cm. **(G)** Modified TGRooZ device to cultivate plants in pots containing soil. Similarly, the base of the device was refrigerated by circulating cold water using a water-cooling machine at 10ºC. Right picture corresponds to pots cultivate at high-homogeneous temperature of 36ºC (36SR). **(H)** Thermal pictures of pots cultivated at 22ºC (22SR), 36ºC (36SR) or 36TGRooZshowing the homogeneous temperature or the gradient formed. Scale bars correspond to 5 cm.



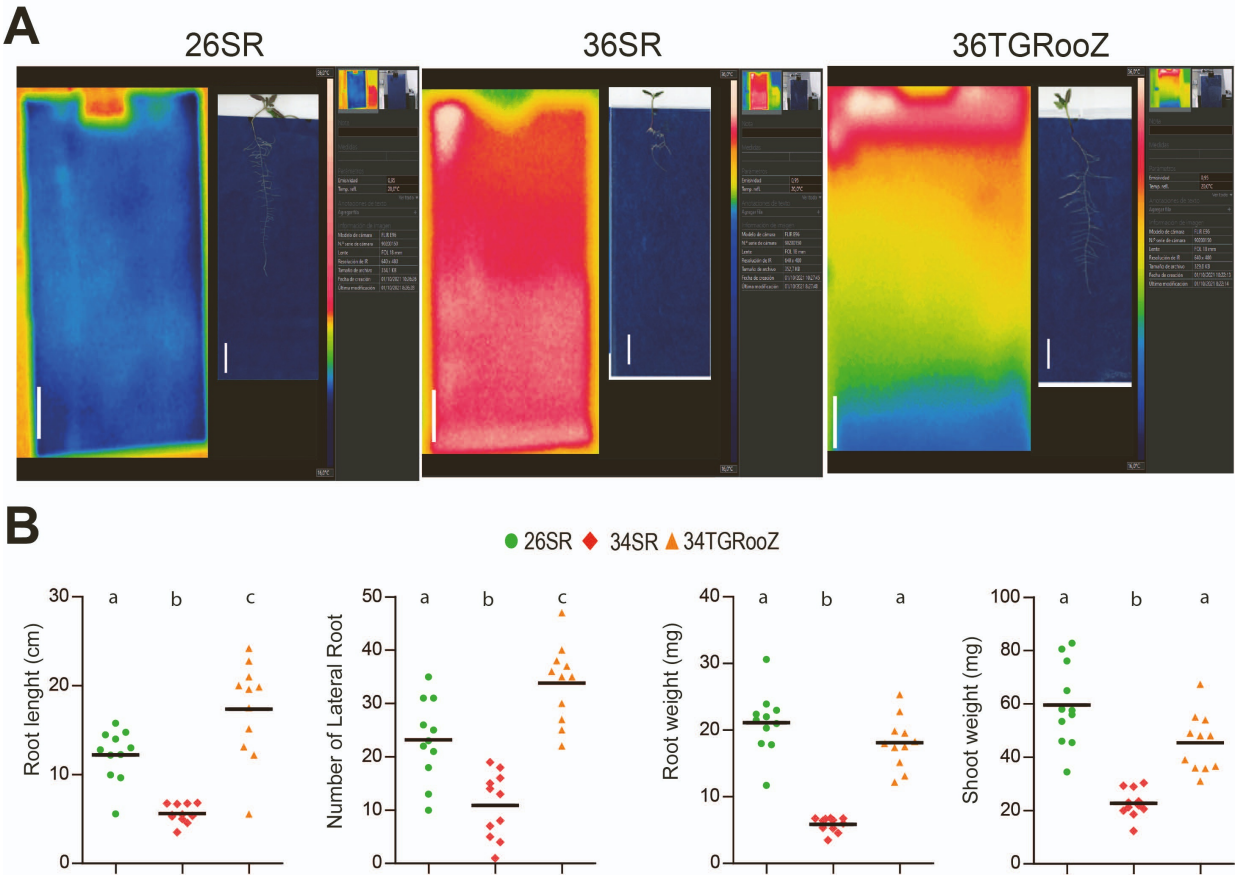
Supplemental Figure 2: High temperature blocks lateral root primordia formation. SKP2Bp::GUS seedlings were germinated at 22ºC from 4 days. Afterwards, they were transferred to 22ºC (SR22), 32ºC (SR32) or 32TGRooZ for 6 extra days. Then, these seedlings were stained for GUS activity directly on the plate to analyze the effect of high temperature after the transference point.



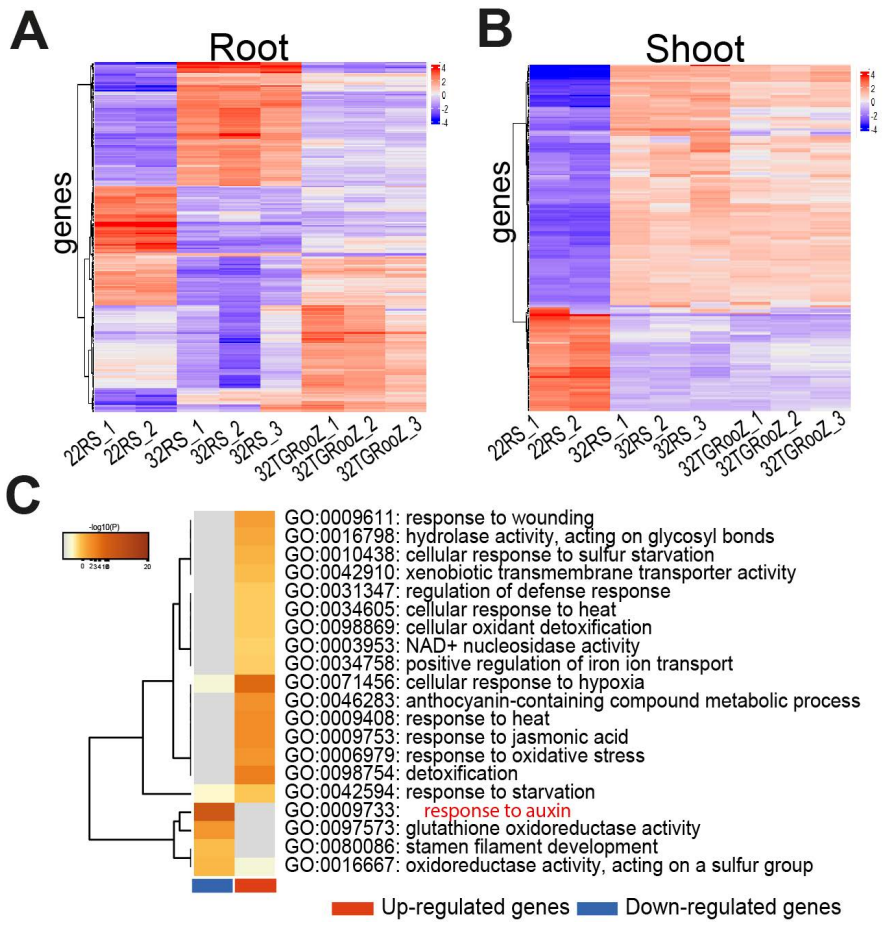
Supplemental Figure 3: Temperature in the root zone affects leave conductance and leave temperature. (A) Conductance (mol / m^2 s) of leaves of Arabidopsis plants grown in pots containing soil at 22ºC (22SR), 32ºC (32SR) or the modified 32TGRooZ for 2 weeks in the modified. n = 8. **(B)** Leaves temperature of plants grown as in the conditions explained in a. n≥33. **(C)** Representative thermal pictures of plants analyzed in b. Error bars correspond to standard deviation. **(D)** Temperature difference between atmosphere and the leaves of 22SR, 32SR or 32TGRooZ. Significance was analyzed by ANOVA and Tukey HSD post-test. $P < 0.05$. Different letters indicate significant differences.



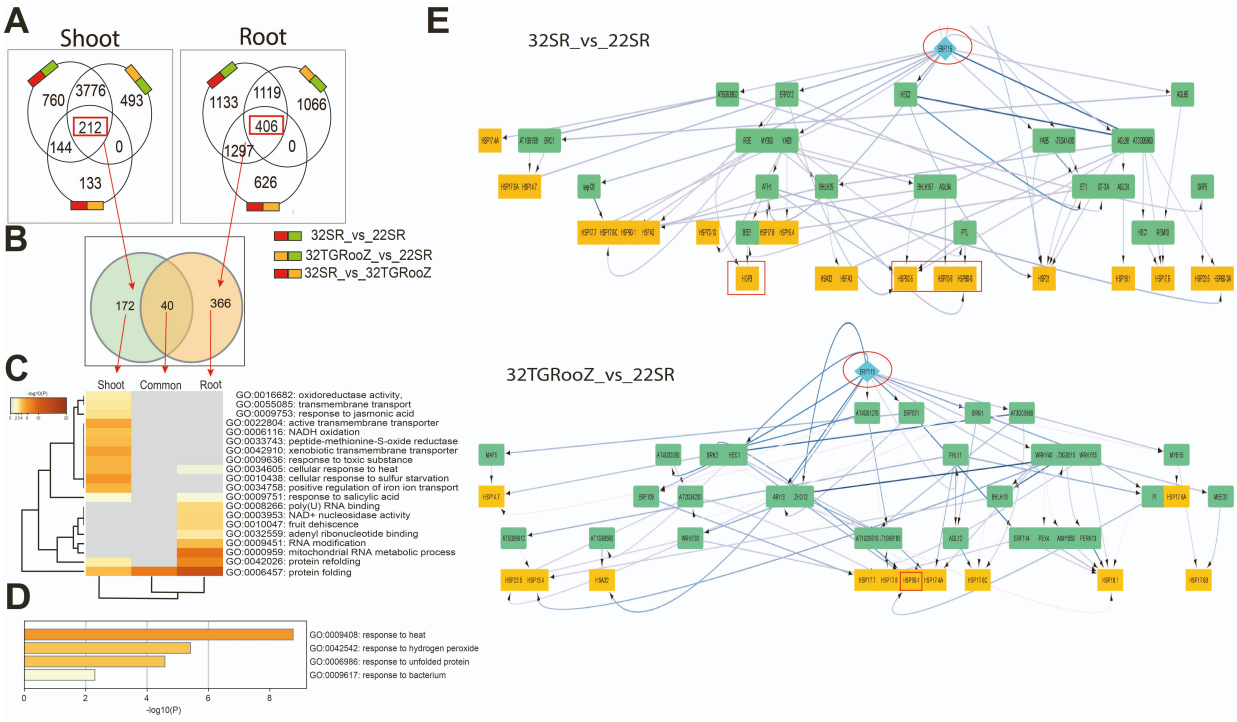
Supplemental Figure 4: Water content. Quantification of water content in the soil through all temperatures analyzed (22ºC, 22SR; 32ºC, 32SR and TGRooZ, 32TGRooZ). The graphs represent the measures taken by different probes at 1 week or 2 weeks after the Arabidopsis seedlings were transplanted to soil. We used the Teros 10 probe (Meter group) and, using the Zentra Z6 datalogger, the water content was recorded every 8 hours for an entire period of 2 weeks. Significance was analyzed by ANOVA and Tukey HSD post-test. $P < 0.05$. Different letters indicate significant differences.



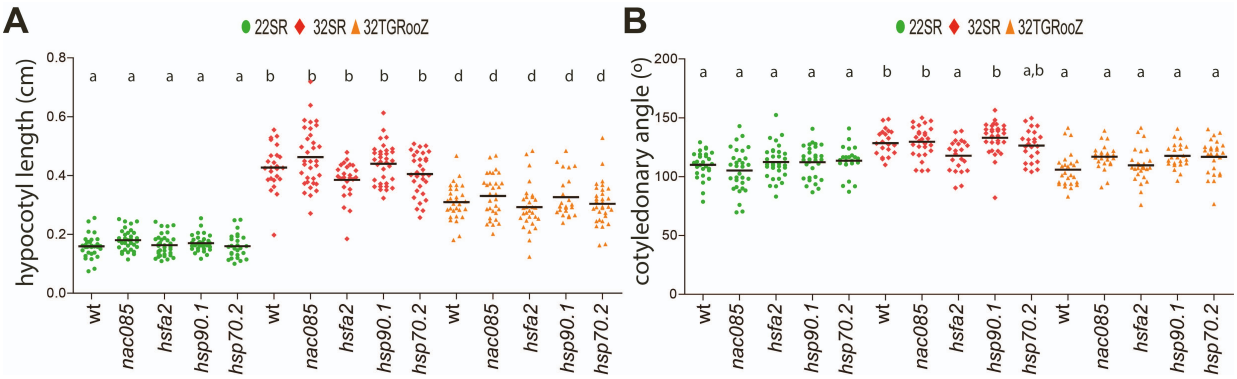
Supplemental Figure 5: Effect of TGRooZ in tomato seedlings. (A) Optical and thermal pictures, taken with a FLIRE96, of the germination paper cultivated at 26ºC, 36ºC or 36TGRooZ showing the homogeneous temperature or the gradient formed. Right photographs correspond to representative pictures of tomato seedling grown in those conditions. Tomato seeds were stratified for 4 days at 4ºC and then they were germinated in darkness for 5 days. Afterwards, seedlings were transferred to germination paper into a zip bag for 7 days. The paper was wetted with one-fourth of MS salts plus 1 mM of MES at pH=5.8. Scale bars correspond to 5 cm. **(B)** Root length, lateral root number, root and shoot fresh weight of 26SR, 36SR or 36TGRooZ tomato seedlings grown for 7 days after transplanting. Significance was analyzed by ANOVA and Tukey HSD post-test. $P < 0.05$. Different letters indicate significant differences.



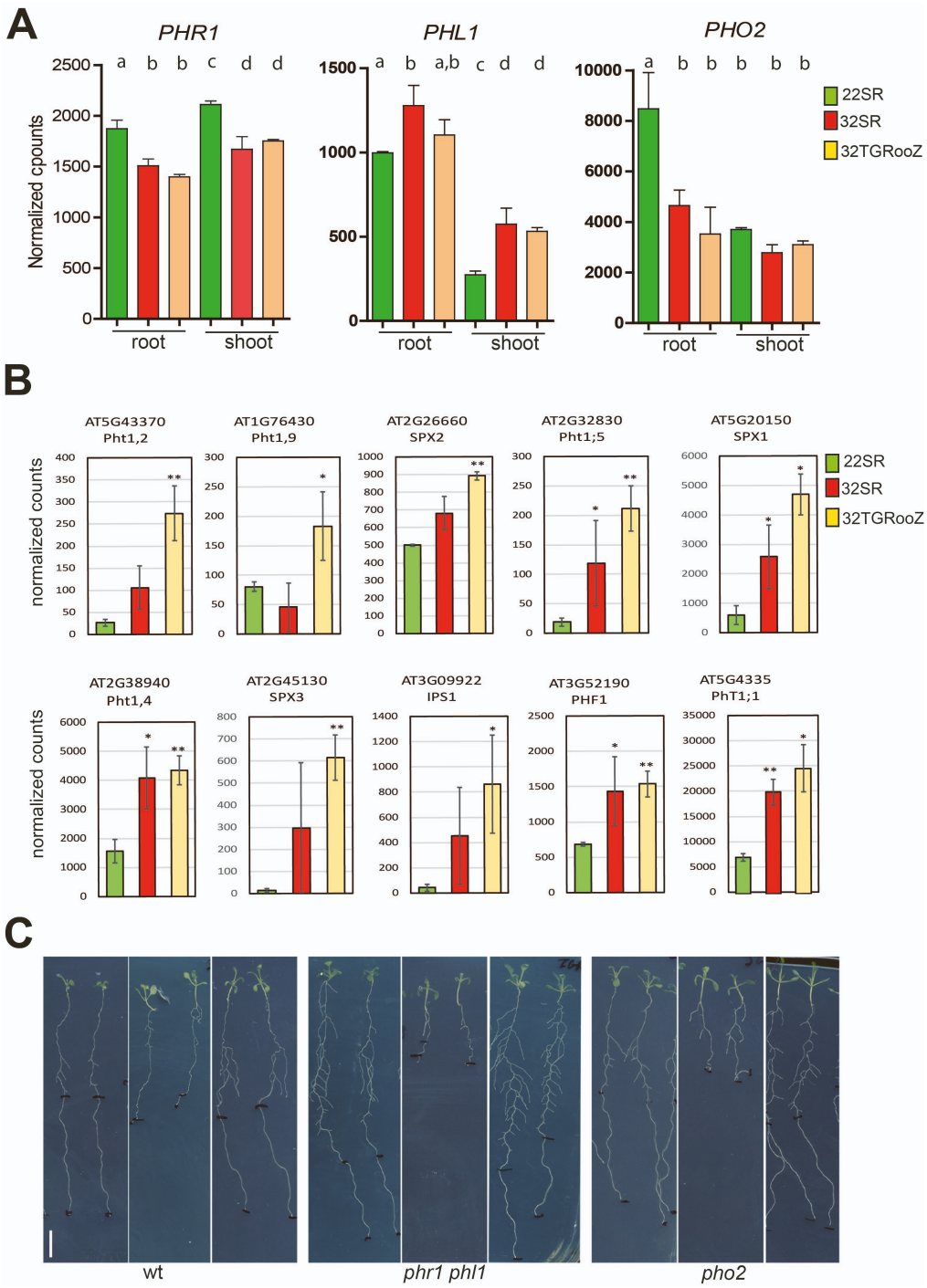
Supplemental Figure 6: Temperature gradient in the root zone modifies gene expression in the shoots under heat stress affecting the auxin response. (**A-B**) Clustering of the genes showing the highest variance in root **(A)** or shoot **(B)** samples of seedlings grown a 22RS, 32SR or 32TGRooZ. **(C)** Gene ontology heat map of shoots differentially expressed genes (DEG) from the 32SR vs 32TGRooZ (32SR_TGRooZ) comparisons. Red and blue boxes correspond to up- or down-regulated genes respectively.



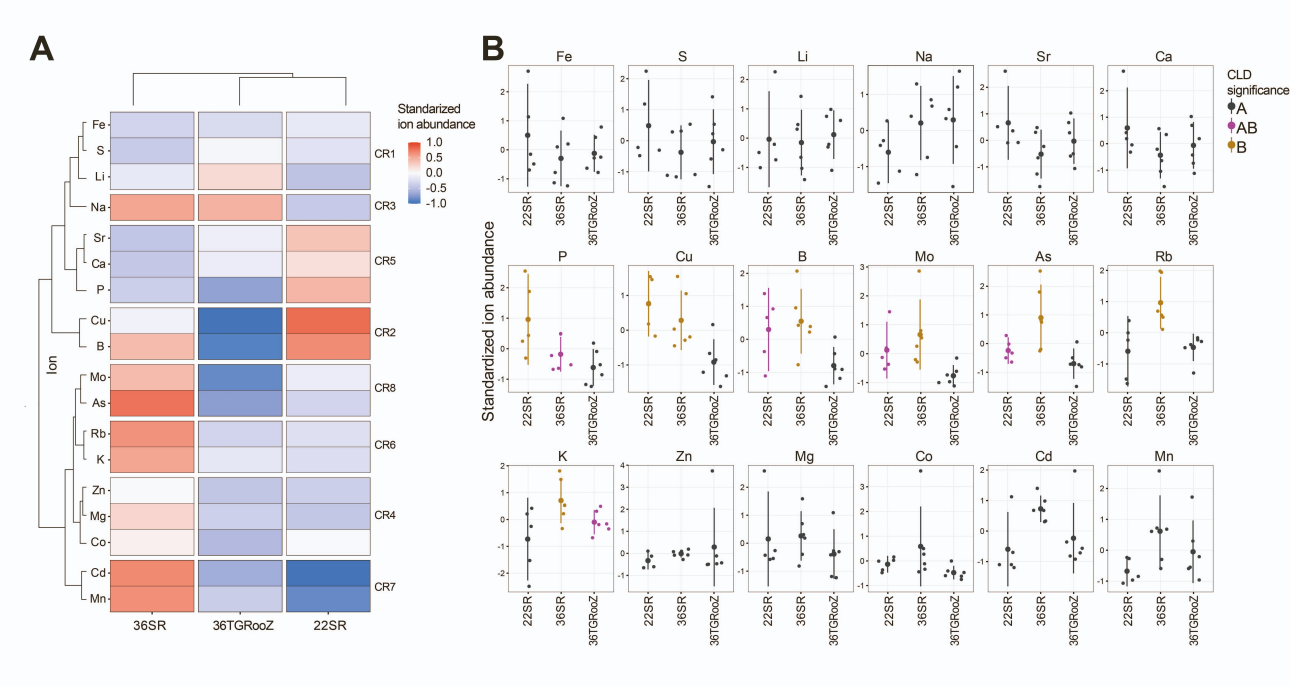
Supplemental Figure 7: Identification of genes regulated by high temperature. (**A**) Venn diagrams of genes upregulated by the effect of temperature in root and shoot in all the treatments (32SR vs 22SR, 32SR vs 32TGRooZ and 32TGRooZ vs 22SR). (**B**) Venn diagram of common upregulated genes identified in a. (**C**) Gene ontology heat map of DEG analyzed in b (shoot specific, root specific or common). (**D**) Gene ontology heat map of common DEG identified in B, analyzed in Metascape. (**E**) Gene network connecting *ERF115* transcription factor (red ellipse) with genes deregulated in the comparison between 32SR versus 22SR or 32TGRooZ versus 22SR. Red rectangles indicate HSP70 and HSP90 genes.



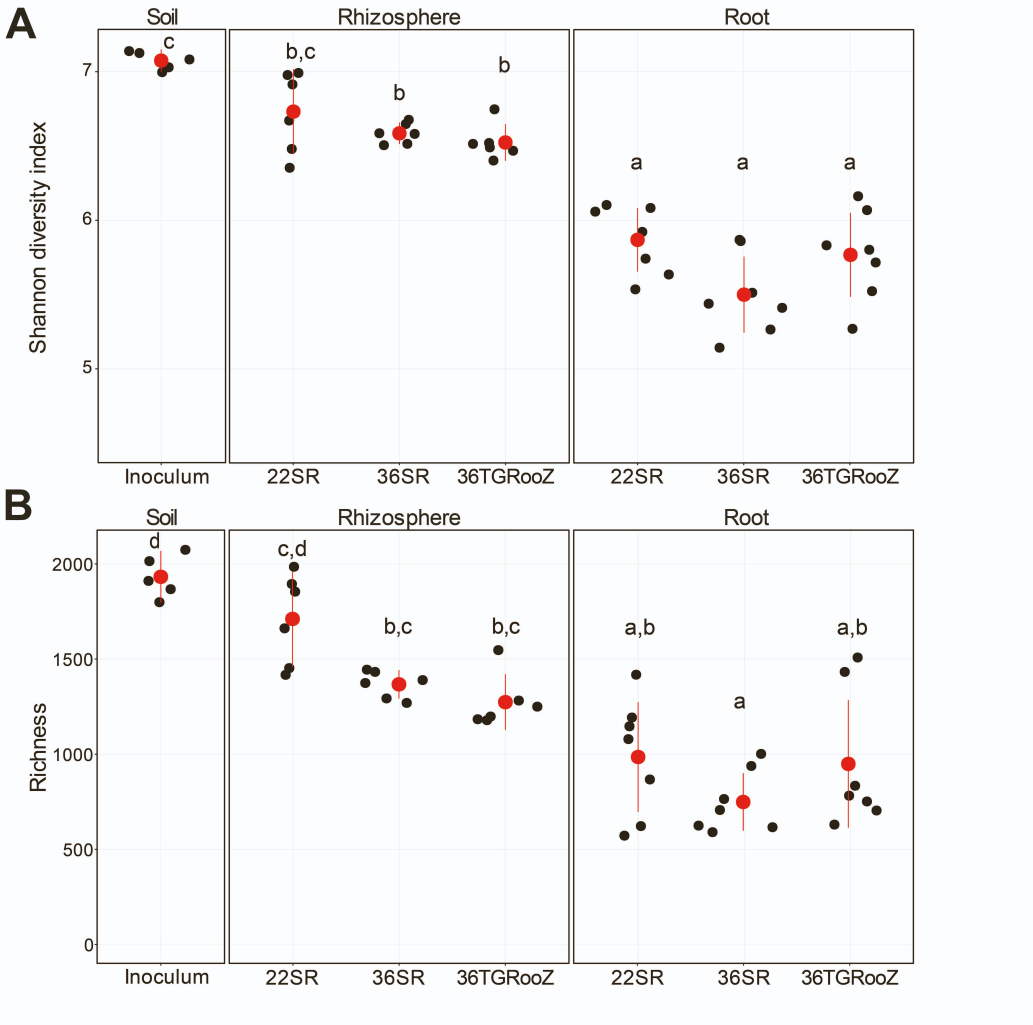
Supplemental Figure 8: Effect of high temperature and the temperature gradient in the root zone on heat response mutants. **(A)** Hypocotyl length of 22SR, 32SR or 32TGRooZ wild type (wt) and mutant seedlings. **(B)** Cotyledonary angle of 22SR, 32SR or 32TGRooZ wt and mutant seedlings. n≥24. Significance was analyzed by ANOVA and Tukey HSD post-test. Different letters indicate significant differences.



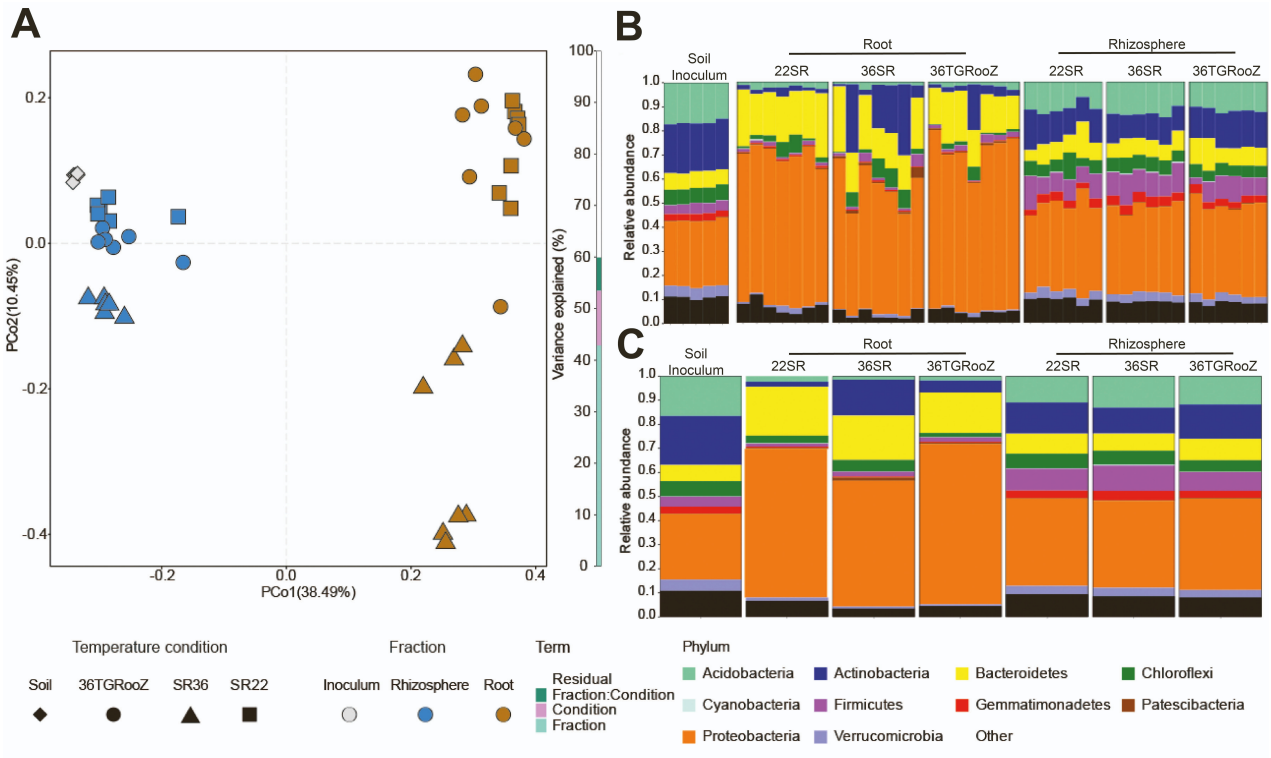
Supplemental Figure 9: Heat stress alters the expression of phosphate starvation response genes. **(A)** Effect of root temperature in the expression on *PHR1, PHL1* and *PHO2* genes. Values correspond to normalized counts from our RNAseq experiments in both shoot and root. Significance was analyzed by ANOVA and Tukey HSD post-test. **(B)** Effect of root temperature in the expression of genes involved in the Pi starvation response. Values correspond to normalized counts from our RNAseq experiments in roots. *, $P < 0.05$; **, $P < 0.01$ by T-test. **(C)** Representative pictures of wild type (wt) phr1 phl1 double mutant or pho2 mutant grown at 22ºC for 4 days and then transferred to 22ºC, 32ºC or 32TGRooZ for 6 extra days.



Supplemental Figure 10: High temperature in the root affects mineral nutrient accumulation in tomato leaves. **(A)** Clustered heatmap showing the standardized mineral nutrient concentration in leaves of tomato plants grown at 22ºC (22SR), 36ºC (36SR) or 36ºC with a temperature gradient in the root zone (36TGRooZ) for 4 weeks. C letter represents different clusters. **(B)** Individual standardized mineral nutrient abundances across the temperature treatments used in A**.** Significance was determined via pairwise comparisons and the results visualised using the compact letter display (CLD).



Supplemental Figure 11: Soil and rhizosphere support a higher bacterial alpha diversity as compared with roots in tomato. (A) Bacterial alpha diversity estimated using the Shannon Diversity. **(B)** Richness indexes in soil, rhizosphere, and root-associated samples of tomato plants grown at 22ºC (22SR), 36ºC (36SR) or 36TGRooZ for 4 weeks. Letters represent post hoc test results, based on a full factorial ANOVA model.



Supplemental Figure 12: Bacterial composition profiles of tomato plants exposed to different temperature treatments. (A) Principal coordinates analysis (PCo) based on Bray-Curtis dissimilarities between bacterial communities in soil, rhizosphere, and roots of tomato plants grown at 22ºC (22SR), 36ºC (36SR) or 36TGRooZ for 4 weeks. The bar graph to the right of the PCo shows the percentage of variance explained in a PERMANOVA model ($p < 0.05$). **(B,C)** Bars show individual **(B)** or Mean relative **(C)** abundance profiles of the ma.in bacterial phyla found in soil, rhizosphere, and roots of tomato plants from **A**.