Identification of core, conditional and crosstalk components of tomato heat stress response using integrative transcriptomics and orthology

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Abstract

Heat stress significantly affects global agricultural yield and food security and as climate change is expected to increase the frequency and severity of heatwaves, this is a growing challenge. Tomato plants are prone to heat stress exposure both in the field and in greenhouses, making heat stress resilience a key trait for breeding. While the identification of heat-associated genes has been addressed in multiple individual studies, the quantitative integration of data from these studies holds potential for low-cost, high-value knowledge gain about the complex network of actors involved in heat stress response mechanisms. To address this challenge, we have compiled a comprehensive data resource containing both novel and publicly available RNA-seq data on tomato in heat stress spanning multiple tissues, genotypes, and levels and durations of stress exposure. We show that in each individual dataset the large majority of responses originates from an interaction between the stimulus and the specific experimental setup. Conversely, by intersecting differentially expressed genes across experiments, we identify a tomato-specific core response of only 57 genes encoding heat shock proteins, transcriptional regulators, enzymes, transporters and several uncharacterized proteins. 17 of these genes lie within previously identified genetic loci associated with heat tolerance traits. Applying the same approach to all publicly available RNA-seq data on drought and salt stress in tomato, we find large overlaps in the conditional parts of the stress responses but the robust and sustained core responses are mostly stress-specific. Finally, we show that the core responses to these stresses are enriched with evolutionarily ancient genes with orthologs across all domains of life and that the heat core response genes form identifiable co-evolving clusters within the Streptophyta. Our study exemplifies the importance and advantage of using FAIR public data to interpret results of new stress experiments, and provides tools to perform such analyses in a relatively short time.

Introduction

As temperatures increase globally in the course of climate change, heat stress is one of the biggest challenges plants face. Crop productivity is significantly influenced by temperature, and in many regions, crops are already cultivated under conditions close to their maximum tolerable temperature. It has been projected that each 1°C rise in temperature above the optimum will lead to an approximate 10% reduction in crop yield (Battisti and Naylor 2009; C. Zhao et al. 2017). Tomato (*Solanum lycopersicum*) exhibits strong responses to temperature across its life cycle from germination to fruit formation (Bita and Gerats 2013) and is often grown in conditions prone to overheating, either in the greenhouse or in countries experiencing frequent heat waves such as Spain or Israel (Alsamir et al. 2021). Therefore, understanding the molecular mechanisms underlying the heat stress responses is crucial for targeted breeding of cultivars with improved heat tolerance and faster recovery.

When plants experience heat stress, it causes a spectrum of morphological damage including wilting and burning of leaves and branches, suppression of shoot and root growth, premature aging and shedding of leaves, and discoloration of plant tissues (Wahid et al. 2007). In tomato, for example, mean daily temperatures above 29 ºC greatly reduce the number and size of fruit. Viable pollen formation and

anther development is particularly affected by high temperatures (Pressman, Peet, and Pharr 2002). These phenotypic effects are a consequence of complex molecular changes. Elevated temperatures affect protein folding and the formation of misfolded protein aggregates, leading to cellular damage or even cell death (Goraya et al. 2017). Heat disturbs photosynthesis by reducing Rubisco activity and damaging photosystem II, resulting in a decrease in CO2 fixation and thus, in prolonged cases, a starvation response (Hu, Ding, and Zhu 2020; Nievola et al. 2017). Heat also increases the concentration of reactive oxygen and nitrogen species leading to oxidative stress which can damage proteins, lipid membranes, and nucleic acids (Li et al. 2020).

To combat these effects, the plant responds with a range of protective mechanisms. The rapid increase in the concentration of reactive species in the initial stage serves as a signal to activate a systemic response (Suzuki and Mittler 2006). This response entails the rapid expression of heat shock proteins (HSPs) which act as chaperones to prevent protein denaturation (Vierling 1991). To counteract the increased membrane fluidity and prevent ion leakage, plants also stabilize their membranes by incorporating more saturated fatty acids which increases the membrane's melting point (Źróbek-Sokolnik 2012). To neutralize the reactive oxygen and nitrogen molecules, plants produce a range of secondary compounds acting as antioxidants. These metabolites, among other functions, protect photosynthetic apparatus from oxidative damage and enable stress recovery (Nievola et al. 2017). In tomato, heat stress has been shown to induce the production of phenolic (Rivero et al. 2001) and terpenoid (Pazouki et al. 2016) compounds as well as steroids (Paupière et al. 2020).

In nature, heat stress is often accompanied by other stresses, and thus the regulation of heat response is involved in considerable crosstalk with elements of drought and salinity stress signaling (Nakashima, Yamaguchi-Shinozaki, and Shinozaki 2014). At the same time, unlike water limitation which tends to build up gradually under field conditions, heat acts immediately on the molecular and supramolecular structures of the whole plant, leading to the very rapid onset of symptoms but also allowing for very rapid detection and signaling response (Ruelland and Zachowski 2010). In multi-stress experiments, heat has been described as having a dominant and orthogonal effect on plant stress response compared to salt or osmotic stress (Sewelam et al. 2020). In this study, we therefore aim to examine and quantify the amount of overlap and specificity in the responses to heat, drought, and salt stress in tomato.

Furthermore, the complex interaction between damage-induced processes, regulated multi-level protective response and plant development results in a large number of molecular mechanisms being involved in the heat stress response which has been shown to be specific to tissue (Cooper, Ho, and Hauptmann 1984; Zhang et al. 2017), duration and extent of the stress as well as the developmental stage of the plant (Balla et al. 2019; Sarkar et al. 2021). The response also depends on the circadian rhythm because there is substantial interplay between heat and light regulation (Franklin 2009; Legris et al. 2017). We therefore secondly aim to identify which parts of the tomato heat stress response are consistent across tissues, developmental stages and duration and intensity of stress (i.e. the *core response*) and which parts depend on additional factors.

Since heat is not a plant-specific threat, and at least part of the stress response has been shown to be ubiquitous in all living organisms across kingdoms (Schlesinger 1990), we lastly aim to determine whether the genes involved in this core response encode evolutionarily widely conserved proteins and whether they form tightly co-evolving clusters.

To address these questions, we performed a systematic curation, reevaluation and comparison of all publicly available RNA-seq transcriptomic data on NCBI for heat stress, drought stress, and salt stress in tomato mapped to the latest reference genome, also incorporating three previously unpublished heat stress data sets from different tissues and experimental setups. By integrating data from multiple sources, the major actors in the process of interest can be more easily identified and so transcriptomic meta-analyses have recently been used to shed light on the response to different biotic and abiotic stresses in plants (Biniaz et al. 2022; Saidi, Mahjoubi, and Yacoubi 2023; Bano et al. 2022). In the following sections, we give an overview of the current status of available RNA-seq data for heat, drought, and salt stress in tomato (Fig. 1A) and examine the dominating factors of variation of gene expression within them (Fig. 2). We then examine the complexity, scale and diversity of the heat stress response and compare it to drought and salt stress response (Fig. 3). Next we develop a method for distilling the core response gene set and apply it to identify the tomato heat, drought, and salt stress core sets (Fig. 4). We also overlay the heat stress core genes with already known QTLs in the literature and thereby show potential areas of interest for breeding (Fig. 4E). Finally, we examine the evolutionary history of the core gene sets of the three stresses using the Orthologous Matrix (OMA) Database (Altenhoff et al. 2021) and the co-evolution patterns of the heat stress response using the HogProf algorithm (Moi et al. 2020) (Fig. 5).

We provide quantified transcripts for all 338 re-mapped samples and differential gene expression and GO term analyses for each examined experiment as a comprehensive data resource on heat, drought, and salt stress in tomato. All code for our analyses is publicly available to be used as a template for similar studies at https://github.com/NAMlab/tomato-rna-meta/. We also created an automated, scalable pipeline for downloading and mapping large transcriptomic data sets which is freely available at https://github.com/NAMlab/rnaseq-mapper. Many of the results can be explored interactively at http://szymanskilab.shinyapps.io/tomato-rna-meta .

Results

Data Set

To generate a comprehensive data resource combining experiments involving heat, drought and salinity stress on tomato cultivars, we searched the NCBI Sequence Read Archive (SRA) for "*Solanum lycopersicum* AND heat", "*Solanum lycopersicum* AND drought", "*Solanum lycopersicum AND* salt", respectively, and filtered the resulting entries according to the following criteria: reads contain total mRNA, samples were taken from non-mutant/wildtype plants, growth, treatment, and sampling conditions were sufficiently described to determine the genotype and tissue, plants were not subjected

to a combination of multiple stresses, for heat stress: The heat stress temperature was at least 32°C and plants were grown in (semi-)controlled conditions to ensure consistent exposure to the set temperature. 23 data sources fulfilled the criteria, to which we added 3 previously unpublished heat stress datasets from 3 different experiments: a comparison of different leaf ages and temperature levels performed at the IPB Halle, a time series of seedling grown on media including day and night cycles performed at the IGZ Großbeeren, and an experiment on ovaries in the greenhouse performed at the Hebrew University of Jerusalem (HUJI); growth and sampling conditions for these datasets are described in the methods section. An overview of the used data sources can be found in Table 1.

Table 1
Data Sources used for the meta analysis. Journal Publications were frequently not linked to the data set on NCBI, they were manually added.

Data Source ID	NCBI BioProject Accession	GEO Accession	Reference
DS-1	PRJNA657834	GSE156402	-
DS-2	PRJNA484882	-	(Zhou et al. 2019)
DS-3	PRJNA376497	-	(Liu et al. 2017)
DS-4	PRJNA644865	-	(Diouf et al. 2020)
DS-5	PRJNA869132	-	-
DS-6	PRJNA635375	GSE151277	(Q. Wang et al. 2021)
DS-7	PRJNA800740	-	-
DS-8	PRJNA745152	-	(Nie et al. 2023)
DS-9	PRJNA746070	-	-
DS-10	being submitted to NCBI	(data from HUJI)	this publication
DS-11	being submitted to NCBI	(data from IPB)	this publication
DS-12	PRJNA624892	GSE148530	(T. Zhao et al. 2020, 2021)
DS-13	being submitted to NCBI	(data from IGZ)	this publication
DS-14	PRJNA560638	-	(Wen et al. 2019)
DS-15	PRJNA603594	-	(Almeida, Perez-Fons, and Fraser 2021)
DS-16	PRJNA562700	-	(Batista et al. 2020)
DS-17	PRJNA639840	GSE152620	(Lopez-Delacalle et al. 2021)
DS-18	PRJNA753098	-	-
DS-19	PRJNA769714	GSE185583	(Jansma et al. 2022)
DS-20	PRJNA730730	GSE174607	-
DS-21	PRJNA319398	GSE80556	(Keller et al. 2017)
DS-22	PRJNA559982	-	(Kashyap et al. 2020)
DS-23	PRJNA476376	-	(Keshishian et al. 2018)
DS-24	PRJNA888477	-	(B. Wang et al. 2023)
DS-25	PRJNA282940	GSE68500	(Fragkostefanakis et al. 2016)

Data Source ID	NCBI BioProject Accession	GEO Accession	Reference
DS-26	PRJEB42497	-	(Mellidou et al. 2021)

The data set contains 338 samples: 92 from heat stressed plants, 72 from drought, 37 from salt, and 137 from respective controls. They stem from 8 different tissues and 22 different genotypes. A complete list of the used samples and their annotated metadata can be found in Table S1 and the interactive shiny app accompanying this manuscript. After comparing stress treated replicates with their respective controls and calculating fold-changes and p-values for differential expression, we end up with 88 contrasts, 39 of which pertain to heat, 36 to drought, and 13 to salt stress (see Figure Fig. 1A). Data is dominated by leaf contrasts (58) followed by seedling and fruit with 10 contrasts each. Only a minority of data sources represent anther, ovaries, pollen, seed, and root (Fig. 1A). In the following sections we will refer to the dataset of normalized log-transformed transcripts per million (TPM) as the logTPM data, and to the fold changes to respective controls as fold change data.

Data source is the strongest factor determining both gene expression and transcriptomic stress response

Analysing the variance components of the logTPM data revealed that the largest known source of variance was the experiment/data source, accounting for 28.6% of the variance in gene expression. An additional 11.9% of the gene expression in our data set was determined by the genotype, 7.7% by tissue and only 3.7% by the treatment (control, heat, drought, or salt stress). The remaining 48.1% of variance could not be explained by these factors. In the first two dimensions of a principal component analysis (PCA), representing 47.0% of the variance, most samples from the pollen, ovary and anther tissues placed distantly to the other samples, even from the same genotype, reflecting their different expression profile compared to vegetative tissues (Fig. 2A, this effect has also been observed in Arabidopsis (Klepikova et al. 2016) although not in pollen and ovary tissue specifically). Samples from seedling, root, fruit, and seed co-localised or formed a sub-cluster within the leaf samples. In contrast, we observed almost no separation by treatment, and no following principal component (PC) could be attributed to a general separation of the different treatments (Figure S1 and shiny app).

The same holds for the fold-change data, where the experiment/data source was still the largest known source of variation (27.6%), followed by treatment (10.9%), tissue (8.0%), and genotype (4.9%). The remaining 48.6% of data variance was not explained by the model. In the first two components of the PCA (representing 22.0% of the variance), the tissues formed tighter clusters but neither tissue nor treatments separated clearly (Fig. 2B). Accordingly, none of the following PCs could be associated with the treatment and tissue factor (Figure S2 and shiny app).

These results indicate that the large majority of responses observed in any individual heat, drought or salt stress experiment is specific to the given experimental setup.

Heat stress triggers surprisingly diverse transcriptomic responses

In general, heat stress led to extensive changes in gene expression, affecting more genes than drought stress but fewer than salt stress. The number of differentially expressed genes (DEG, using an FDR threshold of < 0.05) between treatment and control in heat varied between 2187 (contrast DS-10-1, equivalent to 6.3% of all quantified transcripts) and 14376 (contrast DS-11-5-1, equivalent to 41.44% of all quantified transcripts) with a median number of 8788 changes (Fig. 3A). This compares to a median of 4229 and 11347 DEGs in drought and salt stress, respectively. No correlation with the length of treatment or treatment intensity could be observed for the heat stress contrasts (Fig. 3A).

To describe the gene expression response on the functional level, we translated the DEG results into up- and down-regulation of functional groups (gene ontology terms; GO terms). GO term analysis for each contrast revealed enrichment of 151 to 1128 GO terms among the upregulated genes (median 656) and 437 to 1888 GO terms among the downregulated genes (median 1158; adjusted p-value < 0.05). Again, no correlation with the length of treatment or treatment intensity could be observed (Fig. 3B). The GO term “response to heat” (GO:0009408) was enriched in the upregulated genes of 26 out of 28 contrasts so the criteria for compiling the dataset were successful in selecting experiments which caused heat stress. Also consistently enriched were the terms “protein folding” (GO:0006457), “response to high light intensity” (GO:0009644; both in 28 out of 28 contrasts), “post-embryonic development” (GO:0009791) and “reproductive process” (GO:0022414; each in 27 contrasts) and “response to oxidative stress” (GO:0006979), “response to ethanol” (GO:0045471) and “cellular catabolic process” (GO:0044248) in 26 out of 28 contrasts.

To establish the size of the total potential heat stress response, we selected all genes significantly affected in at least one of the examined heat stress contrasts (p-value < 0.05, adjusted for all comparisons). Surprisingly, this yielded 26,133 genes, more than two thirds of the 34,688 protein coding genes in the genome. The genes cover a variety of biological processes and mechanisms, of which some but not all are directly linked to heat stress (Table S2). The majority of the affected genes (16,788 of 26,133) were significantly upregulated in some contrasts but downregulated in others.

Examining the data on drought and salt stress experiments the same way, we obtained analogous results and found considerable overlaps with the heat stress gene set as well as the enriched GO terms, representing potential targets downstream of the crosstalk between the stresses (Fig. 3C and D).

These results confirm that a majority of tomato stress responses is activated in interaction with additional factors, such as the tissue, the genotype, the duration or the intensity of the stress. Therefore, extraction of core stress-specific responses is not straightforward. Thus in the next step, we searched for a strategy to identify the most likely elements of such a core stress response and evaluated the number of independent experiments and statistics needed to provide a reasonable estimate.

Combining experiments selects more meaningful candidate genes than ordering by p-value or π score

When faced with a large number of differentially expressed genes, one often has to prioritize and filter out genes for follow-up experimental functional characterisation. One simple strategy is ranking DEGs by

p-value and choosing from among the genes with the lowest p-value. But statistical significance does not always reflect biological impact. To alleviate this, some methods also take into account the magnitude of the fold change such as the π significance score developed in (Xiao et al. 2012) which multiplies the $\log_{10}$-transformed p-value with the absolute fold change value. In terms of meta-analyses, gene candidate selection relates to identification of responses that are most likely to be confirmed in multiple independent experiments.

To explore these strategies for this dataset, we calculated the number of genes selected for a given p-value and π score cutoff for each contrast in our dataset (Fig. 4A 1–2). Expectedly, in both cases the number of potential candidates quickly decreased with decreasing p (or π) value cutoff irrespective of whether heat, drought or salt stress was examined. As a benchmark for how representative the selected genes are for the phenomenon of interest, we then determined the proportion of those candidates that are annotated with the primary GO term for this stress type or any of its children terms (GO:0009408 response to heat, GO:0009414 response to water deprivation, GO:0009651 response to salt stress). For comparison, we did the same with an nonspecific term which was comparably enriched in the union set (GO:0009203 ribonucleoside triphosphate catabolic process, GO:0042454 ribonucleoside catabolic process, GO:0098542 defense response to other organism), Fig. 4A 4–5. For the drought stress and salt stress treatments, the proportion of genes representative of the stress response did not change much, showing that this strategy was not very useful to identify the characteristic “core response” to that stress. The enrichment with the nonspecific term did not change either. In contrast, for heat stress, both strategies increased the proportion of representative genes. However, the confidence intervals of these curves are quite large, indicating that this observation was true for some but not consistent across all experiments. Overall, lowering the p-value cutoff reduced likely false positives at least in some heat stress experiments but was not a generally reliable strategy and while including the fold-change via a π score smoothed out the curves, it did not improve the results by much.

As an alternative approach for meta-analyses, we tested intersecting DEGs from multiple experiments by selecting only those genes which are significantly over- or under-expressed in at least 80% of the examined contrasts of the respective stress type (Fig. 4A 3). As before, the number of selected genes quickly decreased as more contrasts were added with most genes being eliminated in the first 5–10 contrasts. However, unlike the previously described strategies, the selected genes became more and more enriched in the respective stress-describing GO term for all three examined stresses with relatively small confidence intervals, meaning the specificity in the gene sets was increasing with more data (Fig. 4A 6). This suggests that carrying out a treatment repeatedly in different laboratories under slightly varying conditions facilitates the identification of a more representative core response, addressing the limitations associated with a single-experiment analysis, which were especially visible in our drought and salt stress datasets.

Defining the core heat stress response as differentially abundant genes in ≥ 80% of our 28 contrasts, we find 46 up- and 11 downregulated genes which are responsive consistently across the different tissues, genotypes, and data sources (Table 2, Fig. 4B). The biological processes enriched in these upregulated

genes are much more specific: response to heat (GO:0009408), protein folding (GO:0006457), response to oxidative stress (GO:0006979), and response to light intensity (GO:0009642) are the top hits (Table S3), the last probably because there is substantial cross-talk between heat and light regulation (Franklin 2009; Legris et al. 2017).

About one third of the upregulated genes encode heat shock proteins and other chaperones (e.g. *HSP21* and *BAG6-like**; see Table 2 for full gene description and ID), on top of that there are genes encoding regulatory proteins and transcription factors (*SlC3H68*, *VA1**), enzymes (*GGCT**, *APX**), and transmembrane transporters (*COPT**, *NPF1.2**), as well as some hypothetical and previously uncharacterized proteins (labeled as *unknown*; BLASTp results for them in Table S4). The downregulated genes include those for fatty acid desaturases and enzymes important for cell wall biosynthesis (*FAD**, *UGE4**), transporters (*SlVGT2*, *MGTRNIPA**) and regulatory proteins (*CBP**, *RSMG**). Twelve of the upregulated genes have a predicted binding site for heat shock factors (Fig. 4B). Eight of them encode heat shock proteins, two are unknown proteins and the last two are *ZFP** and *GST**.

There are few overlaps between the genes in this core set and the analogously derived core sets for drought and salt stress (Fig. 4C). The up- and down-regulated genes for each stress are clearly separated and the stresses largely distinct. Of the three stress types, drought and salt stress seem to share more of their transcriptional response with each other than with heat stress.

Table 2

Heat Stress Core genes (transcript IDs and gene descriptions from the ITAG4.1 annotation). Abbreviations were chosen to more easily refer to genes within this manuscript. Where possible, existing symbols from UniProt or Solgenomics were used. Otherwise abbreviations were chosen based on the gene description and marked with an asterisk throughout (*) to show they do not represent confirmed gene names.

Effect of HS	Transcript ID	Gene Description	Abbreviation (Source)
UPREGULATED	Solyc01g095320.4.1	Bag family molecular chaperone regulator 6-like protein	*BAG6-like**
UPREGULATED	Solyc01g102960.3.1	22.0 kDa class IV heat shock protein	*HSP22-1**
UPREGULATED	Solyc02g080130.5.1	Chaperone protein dnaJ 50	*DnaJ50**
UPREGULATED	Solyc03g082420.3.1	Small heat shock protein, chloroplastic	*HSP21* (UniProt)
UPREGULATED	Solyc03g113930.3.1	22.0 kDa class IV heat shock protein	*HSP22-2**
UPREGULATED	Solyc04g011440.4.1	ethylene-responsive heat shock protein cognate 70	*ER21* (Solgenomics)
UPREGULATED	Solyc04g014480.3.1	15.7 kDa heat shock protein, peroxisomal	*HSP15.7**
UPREGULATED	Solyc05g014280.4.1	small heat shock protein 1	*VIS1* (Solgenomics)
UPREGULATED	Solyc06g011370.4.1	Chaperone protein ClpB	*ClpB**
UPREGULATED	Solyc06g036290.3.1	heat shock protein 90	*HSP90* (Solgenomics)
UPREGULATED	Solyc06g076520.1.1	Class I heat shock protein	*HSPI-1**
UPREGULATED	Solyc06g076560.2.1	Class I heat shock protein	*HSPI-2**
UPREGULATED	Solyc08g062340.3.1	17.9 kDa class II heat shock protein	*HSP17.9**
UPREGULATED	Solyc08g078700.3.1	Mitochondrial small heat shock protein	*MTSHP* (Solgenomics)
UPREGULATED	Solyc09g015000.4.1	Class I heat shock protein	*HSPI-3**
UPREGULATED	Solyc11g020040.2.1	heat shock protein 70	*HSP70* (Solgenomics)
UPREGULATED	Solyc11g066100.2.1	Heat shock protein 70	*HSP70-2**
UPREGULATED	Solyc11g071830.2.1	DnaJ protein like	*DnaJ-like**
UPREGULATED	Solyc01g104740.3.1	Multiprotein-bridging factor 1c	*MBF1c-ER24*

Effect of HS	Transcript ID	Gene Description	Abbreviation (Source)
			(Solgenomics)
UPREGULATED	Solyc02g030450.3.1	Protein ROOT HAIR DEFECTIVE 3 homolog	*RHD3**
UPREGULATED	Solyc02g079020.4.1	B3 domain-containing transcription repressor VA.1	*VA1**
UPREGULATED	Solyc03g063760.4.1	DNA-binding protein SMUBP-2	*SMUBP-2**
UPREGULATED	Solyc03g083190.3.1	gamma-irradiation and mitomycin c induced.1	*GMI1**
UPREGULATED	Solyc03g117440.3.1	RNA helicase DEAD14	*SIDEAD14* (Solgenomics)
UPREGULATED	Solyc04g054290.3.1	Pentatricopeptide repeat-containing protein	*PCMP**
UPREGULATED	Solyc05g055010.4.1	RNA-binding protein 42	*RBP42**
UPREGULATED	Solyc06g076670.3.1	RNA-binding (RRM/RBD/RNP motifs) family protein	*RNBP**
UPREGULATED	Solyc08g005420.3.1	cell division cycle 20.2, cofactor of APC complex-like	*CDC20.2**
UPREGULATED	Solyc08g067960.3.1	CHY-type/CTCHY-type/RING-type Zinc finger protein	*ZFP**
UPREGULATED	Solyc10g006610.5.1	Auxin-responsive GH3 family protein	*AUXGH3**
UPREGULATED	Solyc11g066830.2.1	Zinc finger transcription factor 68	*SlC3H68* (Solgenomics)
DOWNREGULATED	Solyc06g082710.5.1	KH domain-containing protein	*KHP**
DOWNREGULATED	Solyc07g006830.4.1	ribosomal RNA small subunit methyltransferase G	*RSMG**
DOWNREGULATED	Solyc12g055930.3.1	Calmodulin-binding protein	*CBP**
UPREGULATED	Solyc01g065840.4.1	F-box/RNI-like superfamily protein	*F-box-like**
UPREGULATED	Solyc02g062130.5.1	Ferredoxin-NADP reductase, chloroplastic	*FNR**
UPREGULATED	Solyc03g113590.3.1	5-formyltetrahydrofolate cycloligase	*AF516366* (Solgenomics)
UPREGULATED	Solyc08g006150.3.1	Gamma-glutamylcyclotransferase	*GGCT**

Effect of HS	Transcript ID	Gene Description	Abbreviation (Source)
UPREGULATED	Solyc09g007270.3.1	Ascorbate peroxidase	*APX**
UPREGULATED	Solyc10g084400.2.1	Glutathione S-transferase	*GST**
DOWNREGULATED	Solyc01g102950.3.1	Lycopene beta/epsilon cyclase protein	*LCYB**
DOWNREGULATED	Solyc04g080850.3.1	Thioredoxin	*TXN**
DOWNREGULATED	Solyc08g063090.2.1	Fatty acid desaturase	*FAD**
DOWNREGULATED	Solyc09g091510.3.1	chalcone synthase 1	*CHS1* (Solgenomics)
DOWNREGULATED	Solyc10g011810.4.1	Delta(8)-fatty-acid desaturase	*D8FAD**
DOWNREGULATED	Solyc10g012410.3.1	UDP-glucose 4-epimerase 4	*UGE4**
UPREGULATED	Solyc01g101070.3.1	ABC transporter-like	*ABCTR-like**
UPREGULATED	Solyc08g066940.4.1	Protein NRT1/ PTR FAMILY 1.2	*NPF1.2**
UPREGULATED	Solyc09g011410.4.1	Copper ion transmembrane transporter	*COPT**
DOWNREGULATED	Solyc01g103760.3.1	magnesium transporter NIPA (DUF803)	*MGTRNIPA**
DOWNREGULATED	Solyc03g096950.4.1	Vacuolar glucose transporter 2	*SlVGT2* (Solgenomics)
UPREGULATED	Solyc03g097960.1.1	Cysteine/Histidine-rich .1	*unknown-1**
UPREGULATED	Solyc06g150138.1.1	Unknown protein	*unknown-2**
UPREGULATED	Solyc07g065410.1.1	hypothetical protein	*unknown-3**
UPREGULATED	Solyc09g008970.1.1	plant/protein	*unknown-4**
UPREGULATED	Solyc10g081480.2.1	maltase-glucoamylase, intestinal protein	*unknown-5**
UPREGULATED	Solyc11g070060.2.1	hypothetical protein	*unknown-6**

Tissue specific responses

After establishing the core heat stress response with 57 genes in total, we hypothesized that genes in sub-functionalized gene families escape the intersect criterion but cannot be considered noise. To investigate this, we employed Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) with the aim of identifying genes whose expression profiles most effectively discriminate between different tissues under heat stress. However, the analysis failed to identify any orthogonal components with

significant predictive capability, indicating that the data set does not contain enough data to determine the tissue specific variance component of the heat response.

Potential for breeding and the heat stress core response

The core genes involved in the tomato heat stress response are spread across all 12 chromosomes, with notable clustering in certain regions, particularly towards the end of chromosome 2 and the start of chromosome 9 (Fig. 4E). (Ayenan et al. 2019) reviewed four mapping studies on heat tolerance traits and combined them into 13 meta quantitative loci (from now on referred to as QTLs for simplicity). These traits include heat stress effects on pollen viability, flower and inflorescence development as well as photosynthetic parameters. Only 17 of our identified core heat stress response genes co-localized with these QTLs. Individual examples include: *BAG6-like**, putatively a modulator of chaperone activity of HSP70 (Corduan et al. 2009), in MQTL 1.4 (chromosome 1) for the anther length and heat-induced membrane damage (measured as relative electrical conductivity (Wen et al. 2019)) traits, and *VA1** and *DnaJ50** in the region of MQTL2.2 (chromosome 2) for the same two phenotypes. The DnaJ proteins indeed have been indicated to increase heat tolerance in tomato while overexpressed (Kong, Deng, Wang, et al. 2014) and affect the efficiency of Photosystem II in chilling stress (Kong, Deng, Zhou, et al. 2014). The remaining chromosomal regions enriched with core genes could be valuable targets for future research into heat stress resilience mechanisms in tomato.

Heat Stress Response Genes are Ancient

To verify whether heat stress response is, as we hypothesized, an evolutionarily conserved process, we determined the most recent common ancestor (MRCA) of all orthologs found in the Orthologous Matrix (OMA) database for each of our heat stress core genes. Genes whose orthologs have a more recent MRCA likely diverged more recently in evolutionary history, i.e. are evolutionarily younger and potentially less conserved across diverse species. We performed the same analysis for the drought and salt stress core genes as well as a baseline for comparison of 100 genes randomly sampled from the tomato genome (repeated 5 times, Fig. 5A). In comparison to the baseline, all core responses are enriched in genes which are evolutionarily older and likely more conserved across the tree of life. Out of the three stress responses, the heat stress genes seem to have the deepest evolutionary history with more than half of the heat core stress genes having orthologs outside of the Viridiplantae. Heat shock proteins have their predicted root at many different levels (from LUCA up until the Mesangiospermae) while several regulators and transporters (*COPT**, *VA1**, *NPF1.2**), as well as the unknown genes mostly appear later in evolutionary history.

If the genes involved in heat stress response are conserved and ancient, it could be expected that their functional interactions, i.e. the heat stress response process, is equally conserved across the tree of life. To test this, we analyzed the co-evolution of the heat stress core response genes using the HogProf algorithm for phylogenetic profiling (Moi et al. 2020) first only within the Streptophyta and then across all of life (Fig. 5B). Strong co-evolution between two proteins (whenever one is present, the other is present as well) indicates involvement in the same biological process and a mutual functional dependency of the

two proteins. Within the Streptophyta clade, four small clusters form with a perfect co-evolution score: *FAD** and *D8FAD** are predicted to be paralogs with the duplication event having happened on the level of the Spermatophyta, *HSP22-1** and *HSP22-2** are paralogs duplicated at the level of lamiids, and *ER21* and *HSP70-2** paralogs duplicated at the level of Viridiplantae. *HSP70* is not marked as a paralog of *ER21* or *HSP70-2** and *HSPI-1** and *HSPI-2** map to the same entry on OMA, hence they have identical co-evolution with other genes and perfect co-evolution with each other. Expectedly, these clusters are equally strong across the whole tree of life. However, within the Streptophyta, two larger clusters emerge linking also non-paralogous genes (e.g. *D8FAD** to *CHS1*, *GGCT** to *VIS1*). These clusters only partially hold up when examining the co-evolution across all of life, indicating a part of core stress response genes evolves under a plant clade-specific selective pressure. Interestingly, the uncharacterized genes 4 and 5 are part of such a cluster as well.

Tools for meta analyses

We provide this analysis as a template for researchers interested in performing similar transcriptomic meta analyses; therefore all code for the analyses used here is designed in an easily reusable way and freely available at https://github.com/NAMlab/tomato-rna-meta. We also provide an rnaseq-mapper tool to quantify the transcript abundances of many RNA sequencing samples by mapping to a common reference transcriptome. The rnaseq-mapper is able to process a combination of both local FASTQ files and published sequence files on the NCBI Sequence Read Archive (single- or paired-end each) which it automatically downloads and combines with the local files to produce one coherent dataset. The tool is designed to be scalable also for large datasets by utilizing Nextflow to automatically parallelize tasks across CPU cores on a local machine or nodes in a computing cluster, and it is economical with disk space use by deleting data that is no longer required while running. The tool is available at https://github.com/NAMlab/rnaseq-mapper and includes all required software dependencies in an Apptainer/Singularity container which simplifies installation and execution to one line each.

Discussion

In our study, the data source (i.e. BioProject on NCBI) emerged as the strongest variance component of gene expression, accounting for more variance than the controlled factors of treatment, genotype, and tissue. This indicates that abiotic stress response in plants is a highly complex mechanism in which many parts of the response depend on the interaction with additional factors which are different from experiment to experiment, even if genotype and tissue type are the same. This simple observation highlights the limited scope of inference when reporting differentially expressed genes from a single experiment: which genes are representative of the phenomenon in question and which are specifically responsive to the unique conditions of the experiment can only be evaluated in light of other publications or experiments. This finding is also underpinned by the fact that two thirds of all protein coding genes significantly responded to heat in at least one experiment but most of them were found both up- and downregulated, depending on the experiment. The variance component proportions are subject to the limited amount of data and metadata: 17 out of 20 examined genotypes were only present in one data

source so it is challenging to differentiate genotype from data source effects. We also did not account for treatment details such as the duration or the intensity of stress which might account for parts of the differences between experiments. While a systematic study of intra-lab reproducibility of plant growth parameters found the transcriptome to be more replicable than physiological and metabolic parameters under control conditions (Massonnet et al. 2010), we show that intra-lab differences can have a strong transcriptomic effect in stress experiments. The authors highlight the importance of detailed growth protocols to maximize replicability of results between experimental sites. More recently, elaborate guidelines for such protocols have been developed, such as the Minimum Information About Plant Phenotyping Experiments standard (MIAPPE), which can serve as a guideline for describing the growth, treatment, and sampling conditions of plant experiments (Papoutsoglou et al. 2020). One addressed factor which is rarely otherwise reported in experimental metadata but has been shown to have significant influence on the response to many abiotic stresses, including heat stress, is the time of day at sampling (Xu, Yuan, and Xie 2022; Blair et al. 2019). The process of measuring gene expression can be documented following the Minimum information about a microarray experiment (MIAME, (Brazma et al. 2001)) or Minimum Information About a Next-generation Sequencing Experiment (MINSEQE, https://www.fged.org/projects/minseqe/) standards. If transcriptomic data are documented in this way and published in an open access database such as the NCBI SRA, they can be made findable, accessible, interoperable and reusable (FAIR, (Wilkinson et al. 2016)) and thereby have a bigger impact for scientific research. As the main limitation in our study was sufficiently well annotated experimental data, this would greatly help to disentangle conditional interactions in future meta-analyses.

To determine the subset of representative differentially expressed genes we compared three methods: lowering the p-value cutoff, a π score cutoff which also explicitly includes the fold-change, and combining differentially expressed genes from multiple data sources using an 80% intersect criterion. The latter strategy was the most successful and analyzing our heat stress data this way, we found a core set of 46 and 11 genes which are consistently up- and downregulated across the different data sources, tissues and genotypes. These genes consist to a large part of the expected heat shock proteins and chaperones but also include regulators, transporter proteins and enzymes; two of the downregulated enzymes being predicted fatty acid desaturases (*FAD**, *D8FAD**) which presumably play a role in stabilizing the cell membranes (Nievola et al. 2017). Among the upregulated genes there are also 6 which encode hypothetical or currently uncharacterized proteins (Table S4) as well as one encoding an auxin responsive GH3 family protein (*AUXGH3**) which is interesting for a suite of developmental responses to heat (thermomorphogenesis, (Bianchimano et al. 2023). This core heat stress response gene set provides a promising target for understanding the genetic basis of heat stress response and breeding or genetically engineering tomato varieties that are more resilient to heat stress. Because of its robust response, the core set can furthermore serve as a positive control or benchmark for heat stress experiments in tomato. A recent study in Arabidopsis combining 9 data sources on heat stress and intersecting the differentially expressed genes in a similar fashion (Chen et al. 2023) found seven genes which were consistently differentially expressed across different tissues and data sources and highly correlated: AT1G30070, AT1G54050 (*HSP17.4B*), AT1G74310 (*HSP101*), AT2G20560 (*DNAJ*),

AT3G12580 (*HSP70-4*), AT4G12400 (*HOP3*), and AT5G25450. Like most genes in our core set, they all operate as molecular chaperones or serve heat regulatory roles. However, remarkably, none of the seven genes are direct orthologs of any of our core genes, according to OMA. This suggests it is important to perform species-specific analyses and not only use direct orthologs as a proxy for candidate genes. Surprisingly, neither the seven genes in Arabidopsis nor our core set included heat shock factors. A possible explanation is that heat shock factors are only transiently expressed: (Arofatullah et al. 2018) found that the upregulation of HsfA2 and HsfB1 peaked 6 hours after exposure to heat and started declining after 12 hours. As many of our samples were taken in later stages of the heat stress response, this is likely to filter out such transient effects. Heat shock factors may also be regulated post-transcriptionally. Twelve of our core set genes did however have a predicted binding site for heat shock factors, confirming the key role of heat shock factors in the core heat stress response.

Comparison of the overall, non-core heat, drought and salt stress responses indicated multiple genes involved in shared response to different stress types. Indeed, heat, drought, and salt stress are all known to trigger the accumulation of reactive oxygen species (Hasanuzzaman et al. 2020) and thereby the corresponding genetic responses and some ABA-induced transcription factors which function in osmotic stress have been reported to function in cross-talk with other abiotic stresses including heat stress (Nakashima, Yamaguchi-Shinozaki, and Shinozaki 2014). Conversely, the genes in the core response sets identified by the intersection of multiple experiments however show only few overlaps which suggests that even though a part of the signaling pathways to the stresses are shared, the sustained response to them is quite specific. Among the overlaps in that response, salt and drought stress share more common genes which is in line with (Sewelam et al. 2020) who found the transcriptomic heat stress response to be orthogonal to drought and salt stress.

One striking property of the core stress response genes is their evolutionary conservation. We found evidence that the core heat, drought, and salt stress responses are enriched in ancient genes and that among the three stresses, the genes involved in heat stress response have the oldest origin. A systematic analysis of genes annotated with stress-response GO terms in Arabidopsis showed a similar enrichment and suggested its origin as the stabilizing selection pressure exerted on functionally important genes (Mustafin et al. 2019). The authors also noted that genes of a similar age tend to functionally interact with each other. A similar trend emerged from our co-evolution analysis: An exponential decay model fitted on the jaccard index of two genes based on their MRCA level difference showed a clear correlation (p-value < 2e-16) but with an RMSE of only 0.1494063. Thus, while similar age is an important factor, it does not explain the observed co-evolution patterns fully. We also found that co-evolution patterns within the Streptophyta clade were mostly not present in the whole tree of life. Examples of such plant clade specific co-evolution events include a cluster of *MBF1c-ER24*, *unknown-6**, *RSMG**, *AF516366* and *SMUBP-2** showing an interaction with *HSP21*, *MTSHP* and *GMI1** genes only in Streptophyta. Another example includes co-evolution of chalcone synthase *CHS1* with *SIVGT2* and *HSPI-3** becoming apparent in the plant-clade only. This could indicate specialized (additional) functions and interactions of the heat stress response genes within land plants, perhaps caused by unique evolutionary pressures which animals, fungi or prokaryotes are not exposed to.

It is also important to mention some general limitations of our approach: In our findings we interpret changes in mRNA levels as targeted regulation events, which is not necessarily the case as temperature might influence the degradation of mRNA and proteins as well as translation (Krantz et al. 2021). Furthermore, we mapped all samples to the tomato reference genome which comes from the Heinz 1706 genotype. Although we only used the coding sequence which is likely more conserved than the whole transcriptome in order to reduce wrong mappings, there could be errors in quantification with non-Heinz genotypes. Finally, the GO term annotations, used here as an orthogonal ground truth, are based on predictions and might be erroneous or incomplete; as different biological processes are annotated with varying level of quality and specificity, there might also be a bias in the completeness and quality of the GO term annotations these predictions are based on.

Conclusion

The inter-lab variety in experimental design, experimental conditions and sample preparation has a large impact on differentially expressed genes and depending on these effects the heat stress response in tomato can cover two thirds of all protein coding genes in the genome. However, it is possible to reliably extract a core set of responsive genes by combining data from multiple data sources but this requires authors to publish and document their transcriptomic data in a FAIR way. This heat stress core response set consists of mostly expected players (heat shock proteins, regulators, enzymes and transport proteins) but also several unexpected genes not previously annotated as heat-responsive as well as some genes encoding uncharacterized proteins. Core responses derived this way for different stresses (heat, drought, salt) are mostly specific to the stress and even though they are all enriched in genes with an ancient origin, the exact genes involved in the core response are specific to the species.

With this work, we provide a comprehensive overview and resource over transcriptomic data for heat, drought, and salt stress in tomato. All our code and tools are published according to FAIR principles and may serve as a template for similar studies in other stresses or species.

Materials and Methods

Growth, sampling and sequencing protocols for novel data DS-10

Tomato plants of cultivar M82 were grown in two temperature-controlled greenhouses, one with normal temperatures (28°C/22°C, day/night) which used as control and the other with high temperatures in which plants were subjected to at least 32°C for 50 days (see supplementary table S5). At least 4–5 Ovaries at stage S2 were then harvested into liquid nitrogen for RNA extraction (NucleoSpin RNA Plant, Macherey-Nagel, Germany). Each replicate was pooled from at least 5 plants. RNA-seq libraries were prepared using KAPA Stranded mRNA-Seq Kit (Kapa Biosystems, Wilmington, MA) and sequenced using Nextseq 2000 (Illumina, USA) for single-end 50bp reads.

DS-11

Plants of tomato *Solanum lycopersicum* cultivar LA4024 were grown on soil in phytocabinets (CLF Plant Climatics, Emersacker, Germany; model AR-66-L) under long-day (16h light, 8 h darkness) conditions. In control conditions (CN), the daytime temperature was 23°C and in heat stress conditions (HS) it was 32°C or 37°C respectively. In both conditions the temperature at night was set to 23°C, the relative humidity was 65% and the light intensity between 150 and 200 pmol s-1 m2. The sampling was done by immediately freezing the plant material in liquid nitrogen. After the material was grinded in a mortar the material was stored at − 80°C until further processing.

Total RNA was extracted from tomato leaves using the RNeasy© Plant Mini Kit (Qiagen United States) following the manufacturer's protocol. For each sample, one microgram of total RNA was treated with DNA-freeTM DNA Removal Kit (AM1906, Invitrogen) and cDNA was synthesized using the Proto-Script first strand cDNA synthesis kit (New England Biolabs). It was then Illumina sequenced with 150 bp paired-end sequencing.

DS-13

Seedlings of Moneymaker were grown in a phytocabinet at 25°C 12h day/night cycle for 7 days. Then heat stress treated plants were exposed to 37°C at the beginning of a light phase and sampled after 1, 4, 8, 12, 13, 16, 20, 24, and 25 hours (hence time points 13–24 were sampled in the dark). The whole seedling was then harvested and sequenced using paired-end sequencing.

Data Collection and Processing

The NCBI SRA was searched on November 16th 2022 and results filtered according to the above mentioned criteria. Reads were downloaded using fasterq-dump from the NCBI SRA Toolkit version 3.0.0. Transcript abundance was quantified using kallisto version 0.46.1 (Bray et al. 2016) using the coding sequence of the protein coding gene models from the ITAG4.1 tomato genome as reference (https://solgenomics.net/ftp/tomato_genome/annotation/ITAG4.1_release/).

Differentially expressed genes were identified using edgeR version 3.24.3 (Robinson, McCarthy, and Smyth 2010). For time series experiments, treatment samples were compared to control samples of the same time point where possible, otherwise to the time point before the stress started. The exact comparisons for each contrast can be seen in the "sample.group" and "respective.control" columns in table S1 in the supplement

Analyses

Unless mentioned otherwise, analyses were carried out using R, version 4.3.2. For exact details, see code at https://github.com/NAMlab/tomato-rna-meta. Variance components were estimated fitting a REML model on the logTPMs/fold changes depending on stress type, tissue, data source, and genotype (no interactions) using the VCA package version 1.4.5. Reported values are averaged across all genes (mean). 95% confidence intervals of estimates were always < 1%, hence only the mean is reported.

HSF binding sites in the heatmap in Fig. 4B were predicted using FIMO (Grant, Bailey, and Noble 2011) (meme suite version 4.12.0) on the 500bp promoters of all protein coding genes in the genome (extracted using https://github.com/RimGubaev/extract_promoters). The search motif is a manually created consensus sequence of the motifs found at http://neomorph.salk.edu/dap_web/pages/browse_table_aj.php?AGI=HSF (see repository for motif file).

Meta QTLs were taken from (Ayenan et al. 2019) and their chromosomal locations visualized alongside the location of the heat stress core genes using the chromoMap package version 4.1.1 in Fig. 4E.

To determine the MRCA of core response genes (Fig. 5A), their amino acid sequences were searched against the OMA database using their REST API (https://omabrowser.org/oma/uses/#REST), the returned hits filtered for *Solanum lycopersicum* and then the found HOGs queried for their MRCA (see Ruby script in repository). For quantifying the co-evolution of heat stress response core genes (Fig. 5B), a simplified version of the HogProf Algorithm (Moi et al. 2020) was used (python script also provided in the repository).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The code and data for all analyses is freely available at https://github.com/NAMlab/tomato-rna-meta . The rnaseq-mapper tool to quantify the transcript abundances of many RNA sequencing samples by mapping to a common reference transcriptome is available at https://github.com/NAMlab/rnaseq-mapper and includes all required software dependencies in an Apptainer/Singularity container which simplifies installation and execution to one line each. Novel sequence data is currently being submitted.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

DP, AK, and LVA collected and curated data sets and performed exploratory analyses. DP wrote the code for and performed final analyses and made figures. DP, AB, and JS wrote the manuscript. YL, PW, NB, AT, AI, and NT performed experiments and contributed RNA-Seq data. All authors edited the manuscript.

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Figures

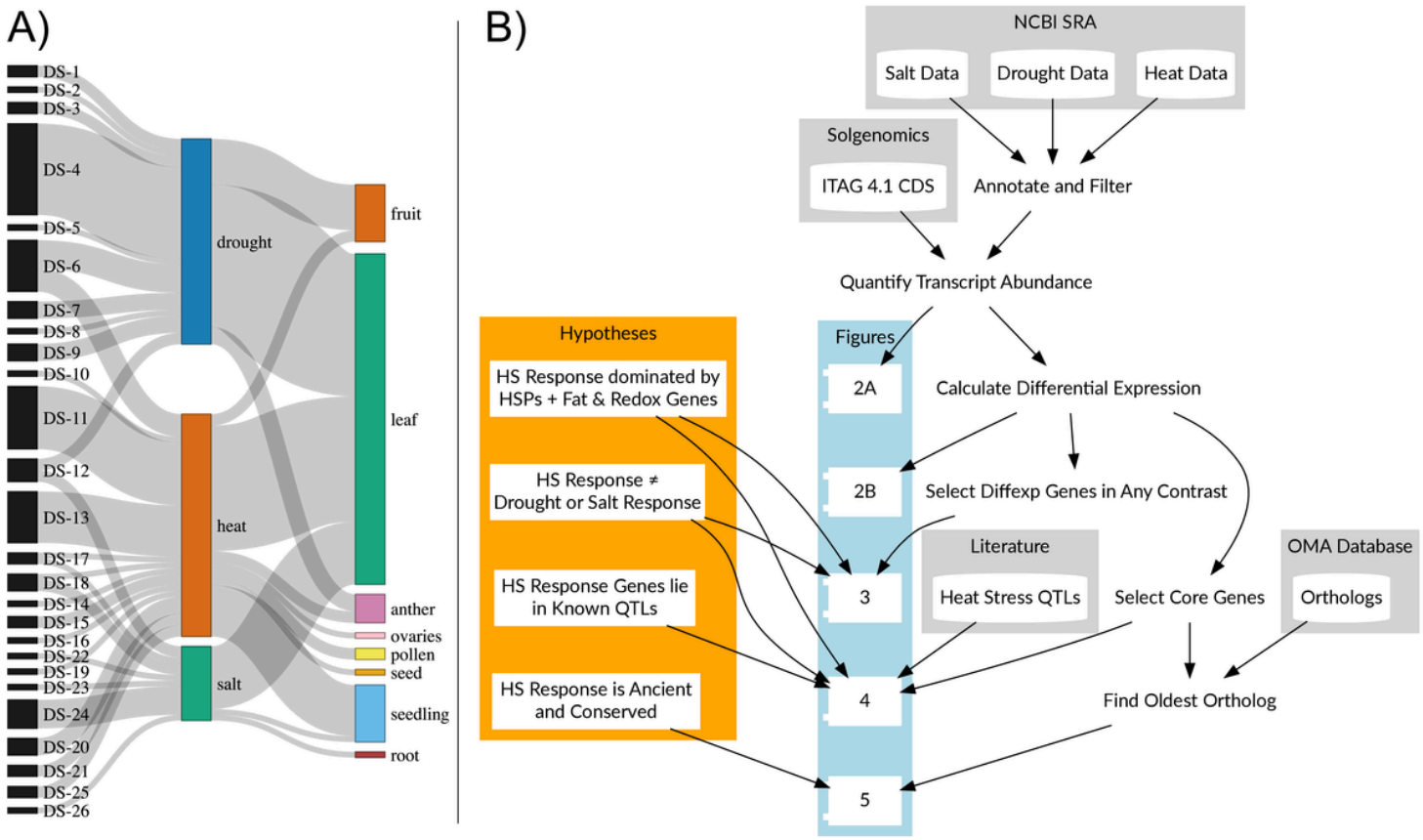


Figure 1

A) Sankey plot showing the number of contrasts available from each data source and for each stress type and tissue. Most data sources only examined one type of stress and the majority of samples were taken from the leaf tissue. B) Overview of the main steps of the analysis, which figures they produce, and which hypotheses these figures examine.

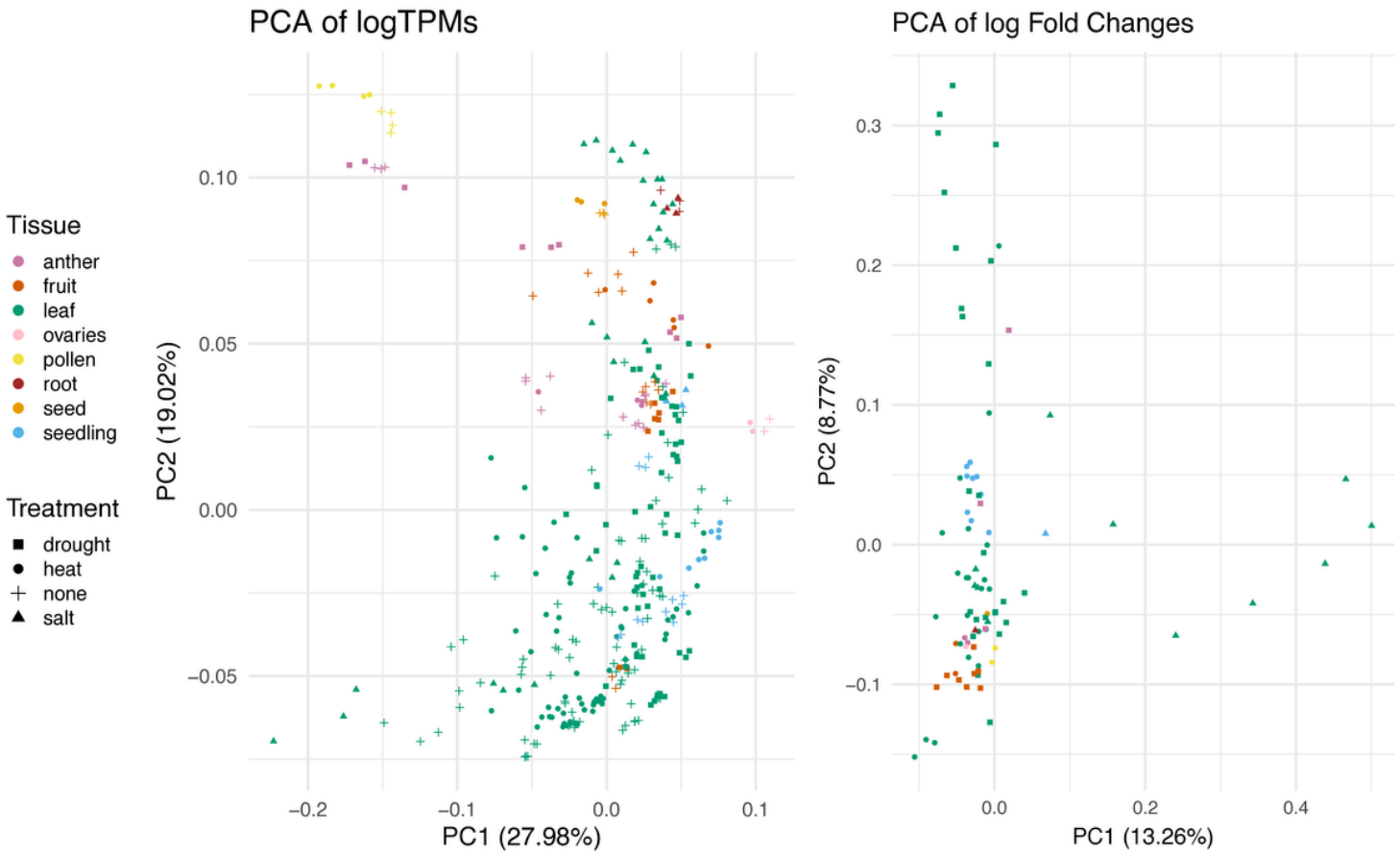


Figure 2

A) First 2 dimensions of a PCA on the logTPM values of gene abundance (every data point represents one sample). Colors and shapes represent tissue and stress types, respectively. B) First 2 dimensions of a PCA on the log-transformed fold-changes of gene abundance (every data point represents one contrast). Other components and factors can be visualized for both plots in the interactive shiny app accompanying this manuscript.

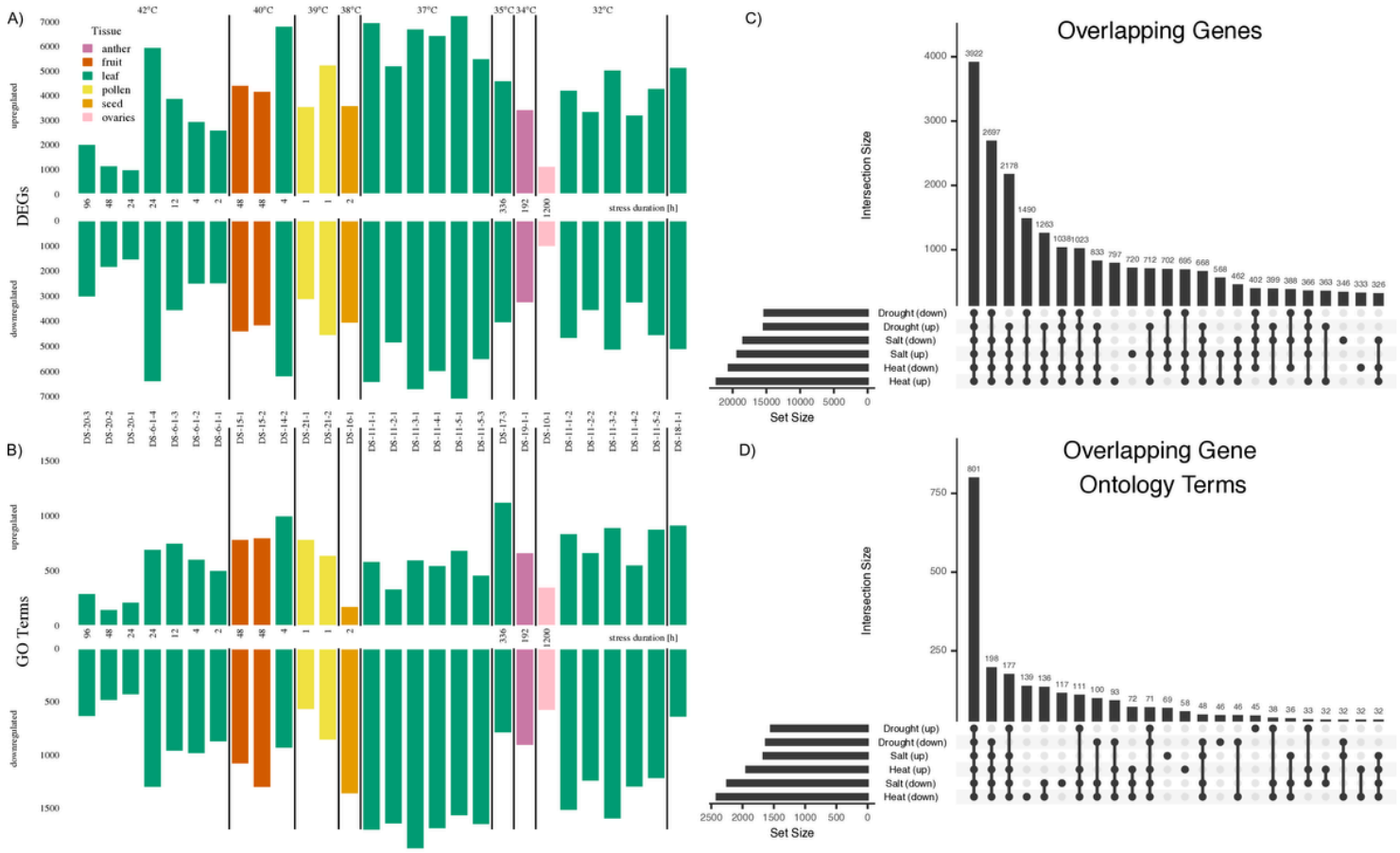


Figure 3

A) Number of differentially expressed genes in each contrast, contrasts ordered by temperature and within that duration of stress. No clear correlation or pattern is visible. B) Number of enriched GO Terms among the up- or downregulated genes in each contrast, contrasts in identical order. C) Upset plot of the union gene sets for heat, salt, and drought stress (significant differential expression in at least one contrast). The biggest intersection are genes which are up- and down-regulated in at least one contrast of each stress type, indicating low specificity of this approach. D) Upset plot of GO Terms enriched in each of the union response sets.

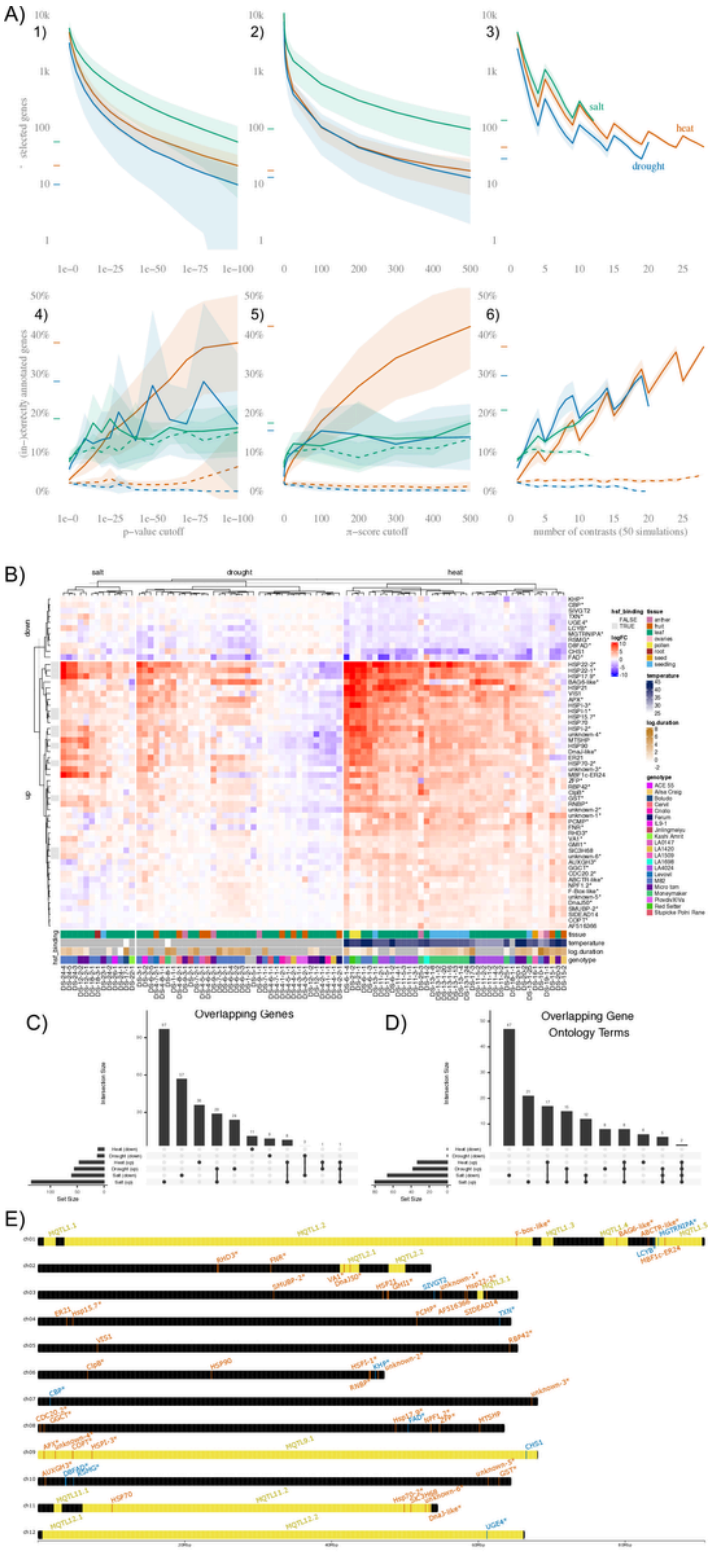
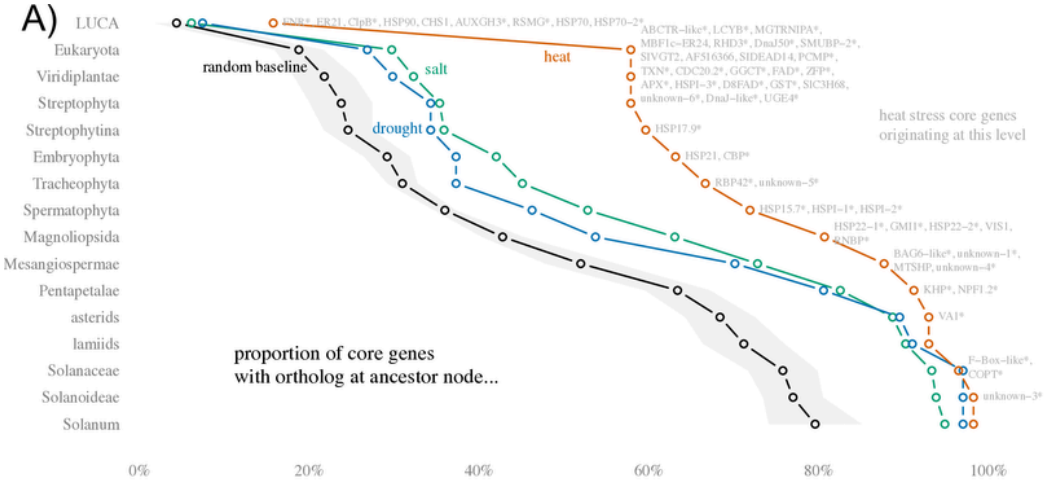


Figure 4

A) Comparison of three strategies to identify candidate genes from a set of differentially expressed genes. Both lowering the p-value/π score cutoff and using the 80% intersection of multiple contrasts quickly reduce the number of potential candidates but only the latter reliably enriches the candidate set with genes representative to the examined process. Dips in the intersection plots are an artifact of the 80% rule (80% of 4 is 3.2 which requires 4 contrasts and 80% of 5 is also 4 contrasts). B) Heatmap of the

heat stress core genes. Genes are selected and separated into up- and downregulated only on the basis of their heat stress response but their fold-change is shown for all contrasts. C) Upset plot of the core set genes for heat, salt, and drought stress (significant differential expression in at least 80% of contrasts). The gene which is upregulated in all three stresses is HspI-1. D) Upset plot of GO Terms enriched in each of the core response sets. E) Visualization of heat stress core response genes and known high-precision heat response QTLs on the tomato chromosomes. Upregulated genes in orange, downregulated genes in blue, and QTLs in yellow. QTLs were taken from (Ayenan et al. 2019).



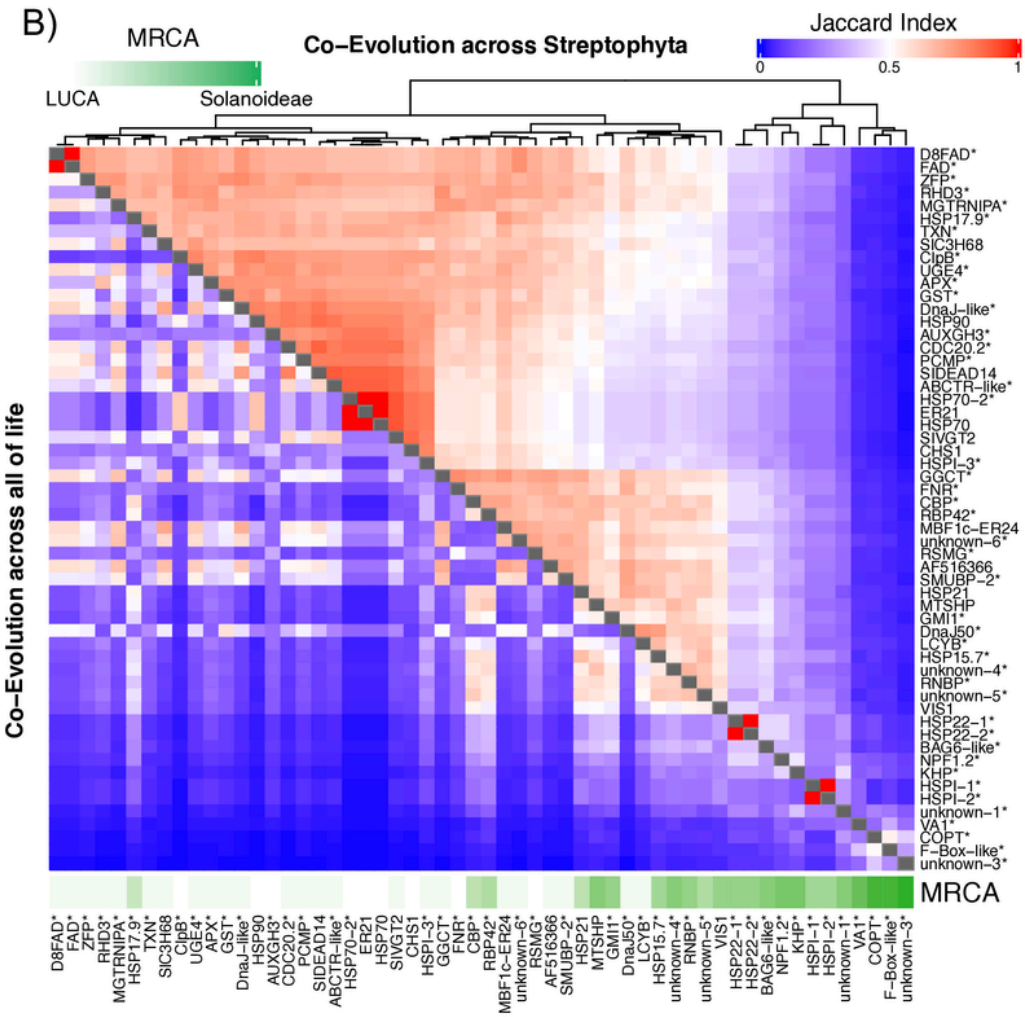


Figure 5

A) Evolutionary History of core stress response genes. LUCA = Last Universal Common Ancestor. Predicted point of origin (Most Recent Common Ancestor, MRCA) of heat stress core genes are labeled in gray. B) Co-Evolution of heat stress core response genes determined by phylogenetic profiling. High values correspond to strong-co-evolution indicating mutual functional dependency and involvement in the same biological process.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarydata.zip