Plant, Cell & Environment

Plant, Cell and Environment (2016) **39**, 1264–1278 doi: 10.1111/pce.12664

Original Article

Dissecting the proteome dynamics of the early heat stress response leading to plant survival or death in Arabidopsis

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ABSTRACT

In many plant species, an exposure to a sublethal temperature triggers an adaptative response called acclimation. This response involves an extensive molecular reprogramming that allows the plant to further survive to an otherwise lethal increase of temperature. A related response is also launched under an abrupt and lethal heat stress that, in this case, is unable to successfully promote thermotolerance and therefore ends up in plant death. Although these molecular programmes are expected to have common players, the overlapping degree and the specific regulators of each process are currently unknown.

We have carried out a high-throughput comparative proteomics analysis during acclimation and during the early stages of the plant response to a severe heat stress that lead Arabidopsis seedlings either to survival or death. This analysis dissects these responses, unravels the common players and identifies the specific proteins associated with these different fates. Thermotolerance assays of mutants in genes with an uncharacterized role in heat stress demonstrate the relevance of this study to uncover both positive and negative heat regulators and pinpoint a pivotal role of JR1 and BAG6 in heat tolerance.

Key-words: Acclimation; heat stress response; iTRAQ; proteomics; plants.

INTRODUCTION

The foreseen climate change involves, among other effects, increases of temperature in many areas of the globe, with heat waves occurring more often and lasting longer. These changes are expected to affect crop growth, especially in already susceptible regions (IPCC, 2014). In this scenario plant science should focus on understanding how plants behave in a situation of increasing temperature and apply this knowledge to improve plant performance under these conditions.

Heat stress is one of the most prominent and deleterious environmental factors affecting plants, as it adversely impacts almost all aspects of plant development, including growth, reproduction and yield (Wheeler *et al.*, 2000, Stone, 2001, Wahid *et al.*, 2007). Heat response is a complex trait and, therefore, the plant molecular, physiological and phenological processes triggered by this stress depend on different aspects as heat intensity and duration, the rate of increase in temperature or the plant developmental stage among others (Larkindale *et al.*, 2005, Wahid *et al.*, 2007, Larkindale & Vierling, 2008, Ahsan *et al.*, 2010). Upon a moderate heat stress, plants launch specialized gene expression programmes that may lead to the promotion of the stress protection and survival, a process known as acclimation. Alternatively, if the heat stress is severe enough, the extensive cellular injury can result in the death of the whole plant.

The response of plants to heat stress has been examined profusely for years at the transcriptional level (Rizhsky *et al.*, 2004, Lim *et al.*, 2006, Schramm *et al.*, 2006, Larkindale & Vierling, 2008, Zeller *et al.*, 2009, Yanguez *et al.*, 2013). In addition, recently, an increasing number of groups have also described the plant response to heat at the translational level (Matsuura *et al.*, 2010, Ueda *et al.*, 2012, Yanguez *et al.*, 2013), revealing that there is an important regulation of translation that should account in part for the lack of a perfect correlation of mRNA and protein levels during the heat treatment. This lack of correlation has pushed the researchers to explore proteomics, because this methodology provides a more direct assessment of the actual proteins performing the signalling, enzymatic, regulatory and structural functions encoded by the genome and transcriptome.

In this context, several groups have studied the proteome response to heat stress in different plant species (Lee *et al.*, 2007, Palmblad *et al.*, 2008, Ahsan *et al.*, 2010, Neilson *et al.*, 2010, Zou *et al.*, 2011, Li *et al.*, 2013, Rocco *et al.*, 2013, Ismaili *et al.*, 2014, Liao *et al.*, 2014, Liu *et al.*, 2014). These studies focused on the proteome changes in response to different episodes of heat but did not consider the effective acquisition of plant thermotolerance. In other words, these analyses have obviated the difference between a possible general stress response, which could be established independently from the final fate of the plants (in terms of plant death or survival), and the specific changes that lead to plant heat stress tolerance. Only a few studies have indirectly analysed this issue by comparison of the heat stress response in heat sensitive and tolerant varieties (Xu & Huang, 2008, Xu & Huang, 2010) or tissues (Ahsan *et al.*, 2010, Bokszczanin & Fragkostefanakis, 2013). In addition, almost all the proteomic studies were carried out during the heat

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treatment leaving the response during the recovery period almost completely unknown.

All these transcriptomic, translatomic and proteomic studies have allowed the identification of a large number of genes with potential roles in heat stress response. Indeed, in some cases, mainly in the case of heat shock factors (HSFs) and heat shock proteins (HSPs), their role in thermotolerance has been confirmed by genetic engineering (Yeh *et al.*, 2012). Although the identification of these factors has contributed substantially to our understanding of the molecular basis of the heat stress response, the mechanisms that assure the proper acclimation of plants in contrast to the general response to heat stress are still unclear because of the lack of a comprehensive analysis.

In the present study, we aimed to investigate by a quantitative multiplexed proteomics approach the intriguing question of how plants acclimate to heat stress, a process that allows the plants to survive to an otherwise lethal heat stress condition. To do so, we have established different stress regimes; a heat-induced acclimation at 38 °C followed by a heat challenge at 45 °C or a direct exposure to this latter 45 °C condition. These regimes conduct plants to a completely different fate although they have been exposed to the same severe heat stress. In the case of the plants that have been acclimated, the subsequent challenge to 45 °C does not constrain plants survival while in the case of the plants that have been directly exposed to the abrupt severe condition, this treatment causes plant death. Based on this difference, we have studied the proteome expression in control condition, during the acclimation priming event and at the early stages of the recovery periods that will lead plants either to survival or death. This analysis has allowed us to get a deeper insight of the acclimation process, to understand the general stress response to heat and to identify pivotal proteins involved in the survival or death plant fate.

MATERIALS AND METHODS

Plant material, treatments and sample collection

Arabidopsis thaliana ecotype Columbia-0 (Col-0) seeds were surface sterilized, sown on MS medium and stratified. Plants were grown on vertically oriented plates in a chamber at 22 °C (16 h light/8 h dark). Seven-day-old seedlings were subjected to heat treatments as follows: for the acclimation treatment (A), the plates were transferred to 38 °C for 1 h and set back to 22 °C for 1 h; for the CS (committed to survival) treatment, plants were subjected to the acclimation event followed by a challenge of 45 °C during 3 h and set back to 22 °C for 5 h; for the CD (committed to death) treatment, plants were directly incubated at 45 °C during 3 h and set back to 22 °C for 5 h. Heat treatments were performed in an oven (Memmert). Seedlings were harvested and snap-frozen in liquid nitrogen. For short and long-term symptom evaluation, photographs were taken 5 h and 7 days after the last heat stress challenge.

For the thermotolerance assays of the T-DNA mutants, 7-day-old seedlings were subjected to heat treatment by submersion of film-sealed plates into a temperature controlled bath at 45 °C during 30 min. After the heat treatment, plates were cooled down on a water bath at room temperature (21–22 °C) for 5 min, unwrapped and returned to control conditions. The thermotolerance phenotype was observed 4 days after the heat treatment. For these assays, the control and the mutant lines were grown in the same plate at equivalent positions.

Metabolic labelling of newly synthesized proteins

Metabolic labelling of *de novo* translated proteins was carried out every hour during the course of each treatment as described in Yanguez *et al.* (2013), using 20 plants for each time point and an 11% SDS-PAGE gel for electrophoresis.

Protein extraction, digestion and iTRAQ labelling

Two biological replicates for each condition were independently analysed. Plant material was ground with mortar and pestle using liquid nitrogen. Aliquots of 200 mg of ground tissue were made for every condition, and proteins were extracted as follows: 200 μL of a buffer containing 50 mM Tris–HCl (pH 8.5), 4% SDS and 50 mM dithiothreitol (DTT) was added to each aliquot and mix well with the plant tissue by vortexing. Samples were put in a shaker at 4 °C during 30 min and then centrifuged at 16 000 *g* for 15 min. Supernatants were transferred to a new tube. Extracts were cleaned up by trichloroacetic acid (TCA)-acetone precipitation. Briefly, 2 volumes of a solution containing 10% TCA and 0.07% DTT in acetone at −20 °C were added to each sample and vortexed. Proteins were precipitated overnight at −20 °C. Supernatant was discarded and pellets were washed four times with the same last solution lacking TCA.

Protein extracts were digested using the filter aided sample preparation (FASP) protocol (Wisniewski *et al.*, 2009). Briefly, pelleted samples were dissolved in 50 mM Tris–HCl (pH 8.5), 4% SDS and 50 mM DTT, boiled for 10 min and centrifuged. Protein concentration in the supernatant was measured by the Direct Detect® Spectrometer (Millipore). About 150 μg of protein was diluted in 8 M urea in 0.1 M Tris–HCl (pH 8.5) (UA), and loaded onto 30 kDa centrifugal filter devices (FASP Protein Digestion Kit, Expedeon, TN, USA). The denaturation buffer was replaced by washing three times with UA. Proteins were then alkylated using 50 mM iodoacetamide in UA for 20 min in the dark, and the excess of alkylation reagents was eliminated by washing three times with UA and three additional times with 50 mM ammonium bicarbonate. Proteins were digested overnight at 37 °C with modified trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate at 50:1 protein:trypsin (*w/w*) ratio. The resulting peptides were eluted by centrifugation with 50 mM ammonium bicarbonate (twice) and 0.5 M sodium chloride. Trifluoroacetic acid (TFA) was added to a final concentration of 1%, and the peptides were finally desalted onto C18 Oasis-HLB cartridges and dried down for further analysis.

For stable isobaric labelling, the resulting tryptic peptides were dissolved in triethylammonium bicarbonate (TEAB) buffer, and the concentration of peptides was determined by measuring amide bonds with the Direct Detect system. Equal amounts of each peptide sample were labelled using the 4-plex iTRAQ Reagents Multiplex Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol in Experiment 1 or labelled using four out of eight labels from the 8-plex iTRAQ Reagents Multiplex Kit in the biological replica Experiment 2. Briefly, each peptide solution was independently labelled at room temperature for 1 h with one iTRAQ reagent vial previously reconstituted with isopropanol. After incubation at room temperature for 1 h, reaction was stopped with diluted TFA, and peptides were combined. Samples were concentrated in a Speed Vac, desalted onto C18 Oasis-HLB cartridges and dried down for further analysis.

LC-MS/MS and quantitative analysis

Labelled peptides were loaded into the LC-MS/MS system for on-line desalting onto C18 cartridges and analysed by LC-MS/MS using a C-18 reversed phase nano-column (75 μm I.D. × 50 cm, 2 μm particle size, Acclaim PepMap RSLC, 100 C18; Thermo Fisher Scientific, Waltham, MA, USA) in a continuous acetonitrile gradient consisting of 0–30% B in 360 min, 50–90% B in 3 min (A = 0.5% formic acid; B = 90% acetonitrile, 0.5% formic acid). A flow rate of 200 nL min^{-1} was used to elute peptides from the RP nano-column to an emitter nanospray needle for real-time ionization and peptide fragmentation on a Q-Exactive mass spectrometer (Thermo Fisher). An enhanced FT-resolution spectrum (resolution = 70.000) followed by the MS/MS spectra from the 15 most intense parent ions was analysed along the chromatographic run. Dynamic exclusion was set at 30 s. For increasing proteome coverage, iTRAQ-labelled samples were also fractionated by cation exchange chromatography (Oasis HLB-MCX columns) into six fractions, which were desalted and analysed by using the same system and conditions described before.

For peptide identification, all spectra were analysed with Proteome Discoverer (version 1.4.0.29, Thermo Fisher Scientific) using SEQUEST-HT (Thermo Fisher Scientific). For database searching at the Uniprot database containing all sequences from *A. thaliana* and crap contaminants (February 28th, 2013; 31930 sequences), parameters were selected as follows: trypsin digestion with two maximum missed cleavage sites, precursor and fragment mass tolerances of 2 Da and 0.02 Da, respectively, carbamidomethyl cysteine as fixed modification and methionine oxidation as dynamic modifications. For iTRAQ labelled peptides, N-terminal and Lys iTRAQ modifications were selected as a fixed modification. Peptide identification was validated using the probability ratio method (Martinez-Bartolome *et al.*, 2008) with an additional filtering for precursor mass tolerance of 12e−03 mg. g^{-1}. False discovery rate (FDR) was calculated using inverted databases, and the refined method (Navarro *et al.*, 2014) was used to filter peptides for quantitation, as previously described (Bonzon-Kulichenko *et al.*, 2011). Protein quantification from reporter ion intensities and statistical analysis of quantitative data were performed using QuiXoT, based on a statistical model previously described (Jorge *et al.*, 2014, Navarro *et al.*, 2014). In this model protein log2-ratios are expressed in form of the standardized variables, that is in units of standard deviation according to their estimated variances (Zq values).

Western-blot analysis

Total soluble proteins were separated using 10% SDS-polyacrylamide gel electrophoresis, blotted to PVDF membranes and analysed with anti-serum specific for HSP101 (Agrisera), HSP90.1, cytosolic HSP70 (Agrisera), Oleo2 (Shimada *et al.*, 2008) and Actin (Sigma).

qRT-PCR analysis

qRT-PCR was performed in a Eco real-time PCR machine (Illumina) using Kapa sybr fast one-step qRT-PCR kit (Kapabiosystems) following the manufacturer's instructions. *TUB5* (At1g20010) was chosen for normalization and RNA from 7-day-old Col-0 Arabidopsis plants grown at 22 °C was used as calibrator. Each experiment was conducted in three technical replicates with three biological replicates. Relative gene expression was determined using the Delta-delta cycle threshold method. Primer sequences are listed in Supporting Information Table S1.

Bioinformatic analysis

Unless cited, the proteins specifically described in the text as significantly changed were those fulfilling in both replicates of the experiment a $Zq \leq -2$ and a mean fold change ≥ 1.2 for the induced proteins or a $Zq \geq 2$ and a mean fold change ≤ 0.83 for the repressed proteins at a specific condition (A, CS or CD) compared to C.

GProX was used for clustering and Gene Ontology (GO) enrichment analysis (Ashburner *et al.*, 2000, Kumar & Futschik, 2007, Rigbolt *et al.*, 2011). Unsupervised clustering based on the fuzzy c-means algorithm (Futschik & Carlisle, 2005) was performed on standardized data (Rigbolt *et al.*, 2011). Six clusters were generated with the proteins significantly changed ($|Zq| \geq 2$ in both replicates). Enrichment analyses were carried out for the members of the clusters using as background the proteins not fulfilling the threshold above. Enrichment was done on GO biological process terms using a binomial test and adjusting *P*-values by the Benjamini and Hochberg correction (Benjamini and Hochberg, 1995). Venn diagrams and their GO classification were carried out using the Venny tool http://bioinfogp.cnb.csic.es/tools/venny/index.html and the 'Plant GeneSet Enrichment Analysis Toolkit' (PlantGSEA) http://structuralbiology.cau.edu.cn/PlantGSEA/, respectively.

RESULTS

Identification of an early time-point of the heat stress response where a clear commitment to plant survival or death is observed for acclimated and non-acclimated plants

As stated before, one of our goals was to uncover new proteins specifically involved in the commitment to plant survival or death under heat stress. Thus, it was important to establish the experimental conditions that determine these two final fates in response to heat. In this case, we took advantage of the well-known capability of Arabidopsis seedlings to acclimate to high temperatures (Larkindale *et al.*, 2005, Larkindale & Vierling, 2008). For our assays, we have selected a severe condition of 45 °C for 3 h. This challenge, when applied isolated, drives the plant to death as shown in Fig. 1b. However, the same severe regime can be tolerated and lead to plant survival after acclimation to 38 °C for 1 h followed by a recovery of 1 h at 22 °C (Fig. 1a).

Because we were interested in the eliciting response that will finally lead to both different fates, but none of the treatments resulted in an immediate macroscopic alteration of phenotype (see Fig. 1a, 5 h after the corresponding 45 °C treatment), we evaluated the *de novo* protein synthesis as an early marker of metabolism activity. One of the earliest responses to heat stress is a global inhibition of translation (Matsuura *et al.*, 2010, Ueda *et al.*, 2012, Yanguez *et al.*, 2013). This inhibition is clear during the 38 °C challenge but more acute when the temperature is shifted to 45 °C (Fig. 1c). This latter repression is observed independently of the plant fate, as the *de novo* synthesis of proteins is greatly reduced after 2 and 3 h at 45 °C independently of the occurrence of a prior priming event (Fig. 1c). However, after 5 h at 22 °C following the challenge, those acclimated plants that are committed to survival (CS) are able to resume protein synthesis, while those that have not been acclimated and are committed to death (CD) seem unable to do so (Fig. 1c). These results indicate that 5 h after the severe heat challenge the acclimated plants, in sharp contrast to the non-primed ones, restart their protein synthesis, being this one of the earliest symptoms of recovery from stress.

Once one early marker of the response associated to the ability of plants to successfully acquire thermotolerance was established, those time-points (CS and CD) were selected for further studies together with C and A (Fig. 1).

Selection of a proteome approach to analyse the plant heat stress response during the chosen recovery periods

In order to determine the most appropriate approach to study the acclimation process and to uncover the mechanisms specifically involved in determining plant survival or death, we decided to analyse the transcriptomic changes cored during the selected treatments and to compare them with the expected protein accumulation.

To do so, we selected three different well-known heat stress responsive genes such as *HSP90.1*, *HSP101* and *HSP70-5*, while *BAG7* [an Arabidopsis Bcl-2-associated athanogene that is involved in the unfolded protein response (Williams *et al.*, 2010)]

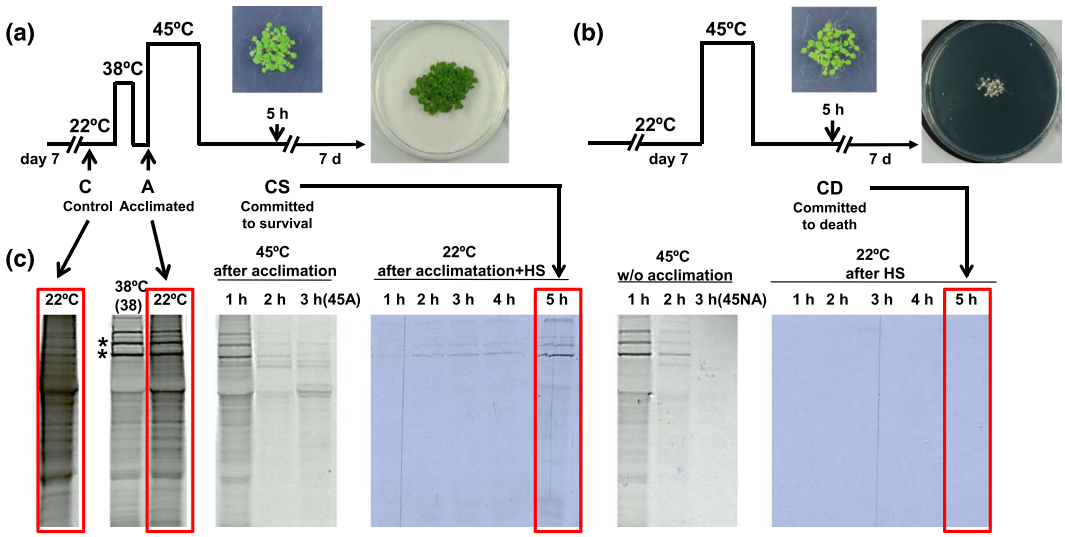

Figure 1. Identification of an early time-point of the heat stress response where a clear commitment to plant survival or death is observed for acclimated and non-acclimated plants. (a–b) 7-day-old Arabidopsis seedlings were subjected to the stress treatments represented in the figure that leads to plant survival (a) or death (b). Photographs of the plants taken after 5 h and 7 days from the exposure to the 45 °C treatments are shown above the 5 h time-point and at the right of each heat stress diagram, respectively. (c) Metabolic labelling of *de novo* synthesized proteins during the course of the heat shock (HS) treatments described in a and b. The seedlings were labelled for the last 1 h of the time-points indicated in the figure. The labelling corresponding to C (control), A (acclimated), CS (committed to plant survival) and CD (committed to plant death) are boxed. The arrows in a and b represent the time-points selected for further analysis. 38, 45A and 45NA labels are included as a reference labels are included as a reference for the time-points used in Fig. 2. The asterisks mark the positions of the newly synthetized HSP90 and HSP70 isoforms.

was used as control, because its mRNA levels do not significantly change during the heat stress response (Yanguez *et al.*, 2013). Measurements of the relative transcriptional changes were carried out by qPCR analysis during the experiment. For mRNA analyses, samples at C, immediately after the 38 °C treatment (38), at A, immediately after the 45 °C treatment for acclimated or not-acclimated plants (45A and 45NA, respectively) and at CS and CD (see Fig. 1 for a more clear location of these treatments) were collected.

As expected, a clear induction of the heat stress responsive genes is observed during the different heat stress treatments [38 °C and 45 °C, independently that the plants were (45A) or not acclimated (45NA)] (Fig. 2). However, at A (1 h of recovery after the treatment at 38 °C), there is a clear transcriptional reduction of the heat induced genes compared to the level accumulated at 38 °C. Studies in our lab using a L-azidohomoalanine for labelling nascent protein synthesis (by the Clik-it technology) demonstrated that the main labelled bands under the 38 °C and the 45 °C heat stress periods correspond to different members of the HSP70 and HSP90 family (data not shown). As it can be easily spotted HSP70 and HSP90 (marked by an asterisk in Fig. 1c) are highly translated during 38 °C, and this translation is maintained during A. This translational maintenance is expected to result in an accumulation of these proteins in A, despite their mRNA levels are reduced at this stage (at least for the highly induced *HSP70-5* and *HSP90.1* as shown in Fig. 2). This situation could also apply to other genes with a similar pattern of mRNA expression under heat recovery as *AtHSP101* (Fig. 2). Indeed, in *Brassica napus*, *HSP101* has been shown to have a peak of expression during a heat challenge with a sharp reduction when returned to the control conditions while the protein encoded is almost absent during the heat episode but highly accumulates during the recovery time (Dhaubhadel *et al.*, 1999).

The complete opposite situation, which may also lead to further and clear inconsistencies, applies for the recovery periods after the 45 °C treatments leading to CS and CD. During this periods and, specifically, during CD, a strong repression of translation could lead to the inhibition of the synthesis and accumulation of specific proteins (Fig. 1c) even in the presence of high amounts of mRNAs encoding them (Fig. 2).

This lack of correlation between the mRNA and the protein levels during the recovery periods strongly reinforces the need to perform a proteomics analysis as a more accurate assessment to understand the molecular response during plant acclimation and during the early responses to heat stress leading to survival or death.

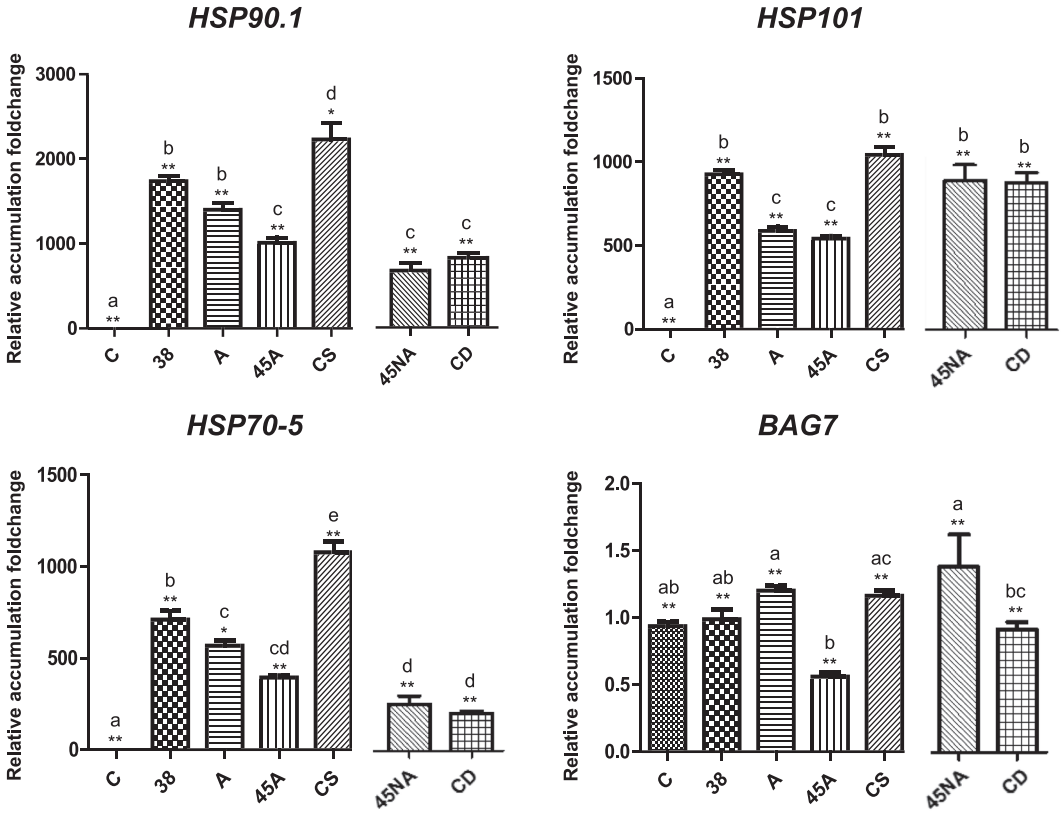

Figure 2. Analysis by qPCR of the transcriptional changes of different heat stress responsive genes along the heat stress treatments. Transcript levels of three different well-known heat stress responsive genes *(HSP90.1*, *HSP101* and *HSP70-5*) and one control gene not induced by heat (*BAG7*) are shown. Accumulation of the different gene transcripts from non-treated (C) and heat stressed plants (38, A, 45A, CS, 45NA and CD) are expressed as fold change values related to the control sample, which was arbitrarily assigned value 1 after normalization to *TUB5* used as internal control. 38, 45A and 45NA correspond to samples collected immediately after the heat stress treatment of 1 h at 38 °C (38) or 3 h at 45 °C with (45A) or without the acclimation treatment (45NA) (see Fig. 1c for further details). A, CS and CD correspond to the time-points described in Fig. 1a–b.

Analysis of the proteome during heat acclimation and at the early stages of the heat stress response that leads plants to survival or death

Based on the previous data, we decided to use a high-throughput multiplexed quantitative approach, iTRAQ (isobaric tags for relative and absolute quantitation), to identify proteins and their relative abundance changes to a control condition during A, CS and CD (Fig. 1).

A total of 5909 proteins were consistently identified in both replicates, out of which 3623 proteins were quantified with at least two peptides at 5% FDR. The average number of peptide identifications per protein was 10, which allowed a good accuracy of quantification of most identified proteins. Among the identified proteins, 221 showed a significant abundance change ($|Zq| \geq 2$ in both replicates) between any of the different treatments (A, CS and CD) and C. From these differentially expressed proteins, 114 showed a significant induction while 123 showed a significant repression.

These significantly changing proteins were subjected to unsupervised clustering, grouping them according to their similar behaviour in the response to the different treatments. As a result, six clearly distinct clusters were generated (Fig. 3a), defining the pattern of the responsive proteins. The cluster members were analysed for GO enrichment to find families and proteins involved in similar biological processes across the different treatments. In these clusters several GO categories appear significantly overrepresented (Fig. 3b). Most of them have been already reported as being related to the plant heat response but not necessarily in the context of thermotolerance acquisition, survival or death. One of the most interesting processes found to change in the heat acclimation experiment is translation, with proteins suffering a very acute increase in A compared to control, maintaining an over-accumulation in CS while being at the same level as control in CD (cluster 5). This includes several ribosomal proteins and translation factors. Within this cluster there are as well proteins related to various abiotic stress responses such as light intensity, salt, oxidative and metal stresses, among many others.

Similar abiotic stress responses appear overrepresented in cluster 3. However, this cluster is characterized by the vast majority of its proteins being heat responsive- and protein folding-related, with a high number of HSPs and chaperones. These proteins show an increased level in A with respect to control reaching the highest point in CS, while a much lower over-accumulation occurs in CD.

It is also noteworthy how proteins categorized as related to various biotic stress responses, overrepresented in cluster 1, do not show a clear change in A relative to control, going then up in CS but experimenting the highest accumulation in CD. These proteins are most of them also categorized as responsive to jasmonic acid, a signalling activated by a wide range of biotic and abiotic stresses (Turner *et al.*, 2002).

Another interesting pattern is the one defined by cluster 2, where proteins are under-accumulated compared to control in all the treatments in study, but in a slight level in A, going more acute in CS and showing the strongest under-accumulation at

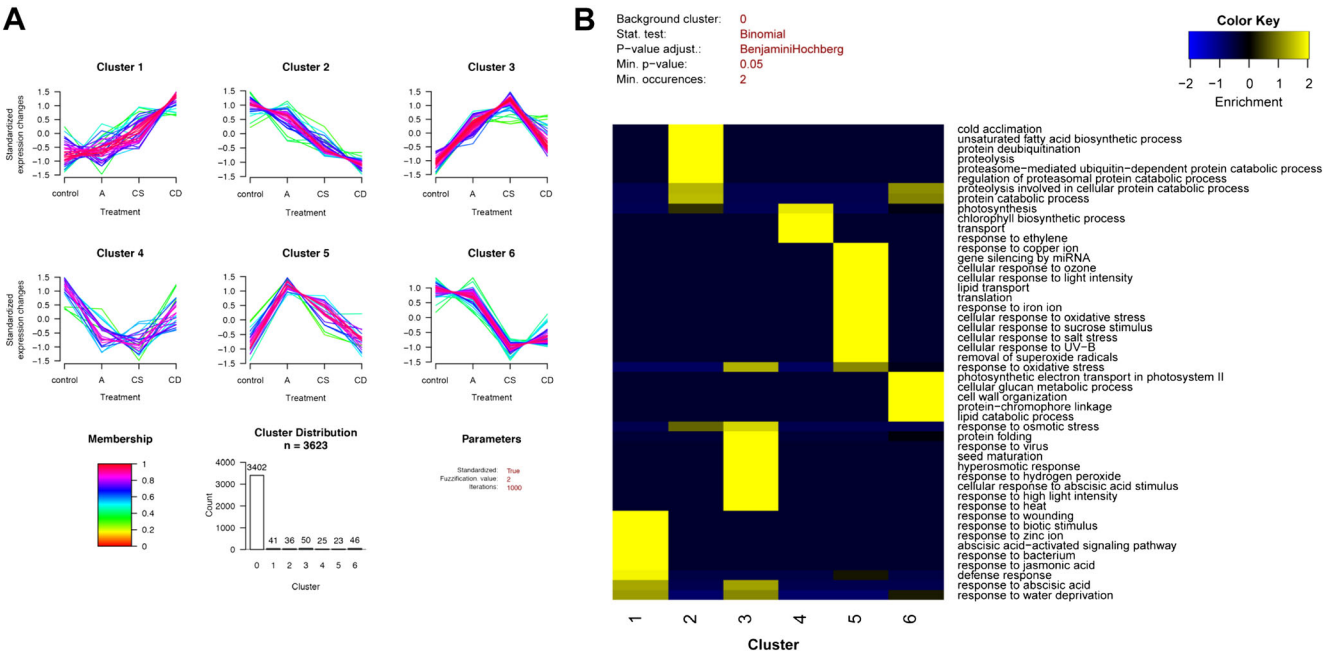

Figure 3. Clustering and GO enrichment of the identified proteins. (a) Significantly changing proteins ($|Zq| \geq 2$ in both replicates) were grouped into clusters based on their changing pattern in A, CS and CD relative to control. Unsupervised clustering based on the fuzzy c-means algorithm was performed on standardized data. Membership represents how well each protein belongs to its corresponding cluster according to its trend. Cluster distribution refers to the number of proteins belonging to each cluster, where cluster '0' is composed by the non-significantly changing proteins. (b) Cluster members were analysed for GO enrichment revealing pathways and processes changing in same trends. Standardized enrichment values are colour-coded and range from −2 (intense blue) to +2 (intense yellow), corresponding the value +2 to the highest enrichment.

CD. In this cluster, some processes related to proteolysis are enriched, including a number of aspartic proteases, cysteine and serine proteases and four TRAF-like family proteins.

Validation of the data by independent approaches

In order to validate these results, western-blot analyses were performed to assess the changes in the levels of five proteins with different heat stress expression profiles in the iTRAQ data. Most of them correspond to heat responsive proteins because of the lack of availability of antibodies raised against proteins involved in the other categories. As shown in Fig. 4, a clear accumulation of the HSP90.1 during A, CS and CD with a peak of expression at CS is observed by western-blot analysis. This pattern of expression correlates with the iTRAQ results that also determine that HSP90.1 is induced during the three heat stress treatments with a higher accumulation during the early stages leading to plant survival. Additional western blot analysis using antibodies raised against a mixture of all cytosolic HSP70 proteins show a basal accumulation of these proteins at control conditions and a higher induction during A and CS. This pattern of expression could be accounted, respectively, by the constitutive expression of Hsp70-1, Hsp70-2 and HSP70-3, and the heat-inducible expression of HSP70 and HSP70B. It has to be noted that in the western blot the higher accumulation of the HSP70 proteins is observed during A and CS, which correlated with the stages of higher quantification of the heat responsive HSP70 and HSP70B in the iTRAQ data. An additional peak of induction at A and CS is also observed for HSP101 by both approaches. Further correspondence is also observed between these two independent quantification methods for OLEO2. In both cases, the data demonstrate a clear accumulation of OLEO2 specifically at CS. The results described above demonstrate that in general, the western-blot results show a good correlation with the iTRAQ analysis, confirming the absence of biases imposed by the adopted methodology and validating the iTRAQ results.

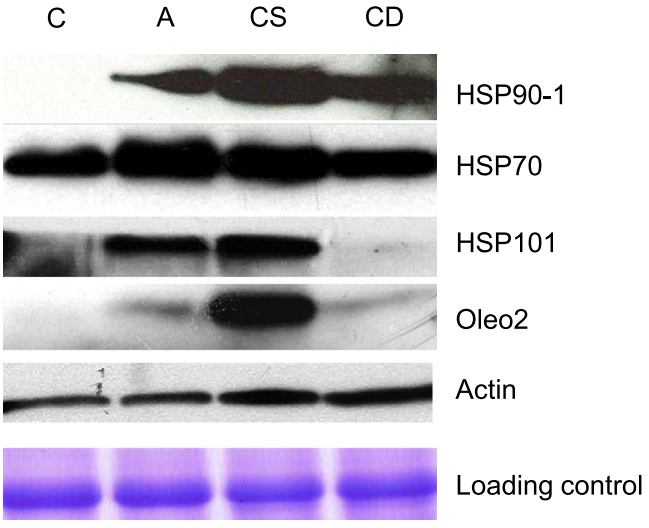

Figure 4. Validation of the iTRAQ data by western-blot analysis Accumulation of different proteins, HSP90.1, HSP70s, HSP101, Oleo2 and Actin was analysed by western-blot using specific antibodies. Protein extracts were obtained from C, A, CS and CD time-points. Last row shows the Coomasie blue stain of the gel as loading control.

Furthermore, the role in thermotolerance of some of proteins with a significant change in our iTRAQ assay was reported previously in different genetic studies, demonstrating the capacity of our analysis to identify important players of the heat stress response (Supporting Information Table S2).

The different heat stress regimes affect protein expression levels in different ways

In addition to the clusters, we carried out Venn diagram analysis in order to analyse the overlap among the different treatments and to uncover the specific proteins involved in each particular response. These Venn diagrams clearly demonstrate that the different heat stress responses to the different treatments impinge in common and distinct proteins (Fig. 5).

Specifically, there seems to be a general response along the three analysed treatments that includes the coordinate induction and repression of a small number of proteins. In addition, a common response in A and CS is observed that mainly affects the induced proteins. In parallel, a slightly higher degree of overlap is noted between CS and CD. This shared response is more accentuated for the repressed proteins. However, in sharp contrast to the responses described before, with the exception of the proteins involved in the general response, no overlap is observed between A and CD. Finally, this analysis clearly demonstrates that there is also a significant amount of proteins that are specifically induced or repressed at each of the different heat stress regimes.

All these data demonstrate that the heat stress players are only partially shared along the different assayed conditions and that the heat-triggered response is different depending on the capacity of the plant to cope with the stress.

Identification of proteins involved in the general heat stress response

As stated above, there is a general stress response that involves a significant induction and repression of 16 and 6 proteins, respectively (Supporting Information Table S3). This response is highly enriched in proteins involved in the gene GO category 'response to heat' (*P*-value 8.5e – 23, FDR 2.4e – 19) and includes the induction of 12 high and low molecular weight HSPs as HSP70, HSP70B, HSP101, HSP90.1 and HSP22.0, among others. HSPs are a major group of proteins involved in the maintenance of protein homeostasis under heat stress. These proteins have been proven to assist the folding of denatured proteins, the intracellular protein distribution, the protein degradation and the formation of complexes that mediate the transcriptional response to heat challenges (Morimoto, 2002, Wang *et al.*, 2004).

Along with these HSPs, four other proteins are also significantly induced: BAG6 [a calmodulin binding protein with a role in plant growth, development and pathogen defence (Kabbage & Dickman, 2008)], ADK1 (a protein similar to adenylate kinase), AnnAt1 [a Ca(2+) transport protein involved in

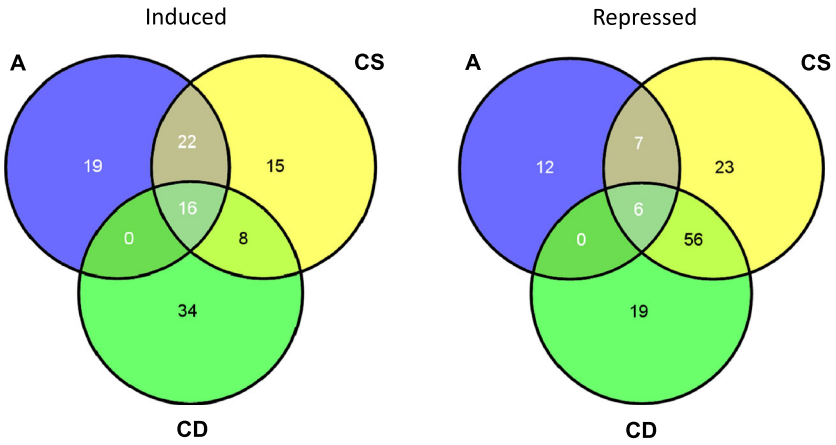

Figure 5. Venn diagrams of the differentially expressed proteins that were induced (Zq ≤ 2 in both replicates) or repressed (Zq ≥ 2 in both replicates) during A, CS and CD.

oxidative stress (Richards *et al.*, 2014)] and EGY3 [a close homolog of a membrane-associated and ATP-independent metalloprotease that is required for chloroplast development (Chen *et al.*, 2005)]. Despite their clear induction during the three different heat treatments, the involvement of these four proteins in the heat stress response has not been elucidated yet.

On the other hand, six proteins were coordinately repressed under A, CS and CD (Supporting Information Table S3). Two of them, CHL11 and PORB, are key proteins for chlorophyll biosynthesis (Jensen *et al.*, 1999, Frick *et al.*, 2003). In addition, GRP7 [a glycine-rich RNA-binding protein involved in the regulation of stomatal opening (Kim *et al.*, 2008)], a GDSL-like lipase and two unknown proteins belonging to the TRAF-like and HAD superfamily are also significantly repressed among the different treatments.

These results demonstrate that plants launch a general response to heat that is triggered independently of whether the heat stimulus does or does not constrain plant survival. This response affects only a small portion of the heat responsive proteome and largely involves the induction of a specific set of HSPs.

Analysis of proteins that change coordinately during A and CS and during CS and CD

During A and CS, a clear induction of specific proteins belonging to the HSP and cochaperone families is also noted (Supporting Information Table S4). This set of HSPs is different from the one induced during the heat general response and includes the HSP70T-2, HSP23-6, HSP21 and HSP18.2, among others. In addition, during this common response, there is a clear accumulation of proteins with reservoir activity such as PAP85 and the three members of the cruciferin family (CRU1, CRU2 and CRU3). In the same way, at CS and CD, there is also a significant accumulation of some specific proteins (Supporting Information Table S4). In this case, a significant induction of SUMO1, a small ubiquitin-like modifier protein involved in the SUMOylation of the heat shock transcription factor AtHsfA2 (Cohen-Peer *et al.*, 2010), is observed. Moreover, the glutathione S-transferase GSTF9 (Sappl *et al.*, 2009) and IPIAT1 [an isopentenyl diphosphate isomerase involved in isoprenoid biosynthesis (Okada *et al.*, 2008)] are also identified as CS and CD responsive proteins.

Among the proteins repressed during the different treatments, a high proportion of proteins related to photosynthesis stand out (Supporting Information Table S4). However, it is during the coordinated response to CS and CD where the highest enrichment in this category is detected. These repressed photosynthesis-related proteins include photosystem II reaction centre protein A (PSBA), the photosystem II subunit P-1 (PSBP-1), the chlorophyll synthase CHLG (Lin *et al.*, 2014) and different PsbP-related proteins as PPL1 (Ishihara *et al.*, 2007), PPD1 (Liu *et al.*, 2012) and PPD5 (Roose *et al.*, 2011), among others. Moreover, a significant coordinated repression of three different thylakoid lumenal proteins with unknown function (At1g12250, At3g63540 and At5g52970) is also monitored. Along with these photosynthesis-related proteins and in concordance with the clusters information (Fig. 3), during CS and CD a large number of repressed proteases can be identified (*P*-value 3.69e – 06; FDR 1.98e – 3, Supporting Information Table S4).

All together, these data suggest that there is a shared response during A and CS that mainly affects the induction of specific HSPs, cochaperones and storage proteins while the common response triggered during CS and CD mainly involves a tight repression of specific photosynthesis-related proteins and proteases.

Identification of specific markers of the different heat stress responses

In addition to the proteins that are coordinately induced along two different stress conditions, we also observed proteins that are specifically induced at a particular treatment (Supporting Information Table S5).

Thus, among the proteins highly induced only during the acclimation response a clear enrichment for proteins

responsive to oxidative stress is detected. This is the case of the (Cu–Zn) superoxide dismutase SOD2 and two members of the peroxidase family, At3g01190 and At5g64120. Both SODs and PERs are considered ROS scavenger enzymes with a role in the protection of cells against the oxidative damage. Finally, proteins related to ribosome composition as the 60S ribosomal protein L36a and the translation initiation of eIF3-4 are also specifically induced during this period.

During CS the significant induction of the haem oxygenase (HY1) and of the oleosin, OLEO2, is specifically noted. Both proteins are well-known regulators of the abiotic stress response. HY1 plays a major role in UV-C and salt acclimation signalling (Xie *et al.*, 2011, Xie *et al.*, 2012), while OLEO2 is a protein found to surround oil bodies and control their expansion and positioning during germination under freezing followed by imbibition at 4 °C (Shimada *et al.*, 2008).

Finally, the group of proteins induced specifically during CD is the most heterogeneous one with a high enrichment of proteins involved in secondary metabolism (*P*-value 1.58e – 07; FDR 2.19e – 4). Indeed, different proteins involved in serine, glycine and cysteine metabolic processes along with proteins involved in isoprenoid and carotenoid biosynthesis are observed in this group. In addition, the category 'defence response' is also represented (*P*-value 1.6e – 04 FDR 9.01e – 03). Indeed, key proteins involved in oxilipin biosynthesis as LOX2, a chloroplast lipoxygenase required for wound-induced jasmonic acid accumulation in Arabidopsis (Bell *et al.*, 1995), are increased specifically during CD. The induction of LOX2 is concomitant to the accumulation during this phase of jasmonic acid responsive proteins as JR1, JR2, the arginine amidohydrolase ARGAH2 (Brauc *et al.*, 2012) and the invertase ATBFRUCT1.

A repression of proteins related to photosynthesis is also observed at different specific stages. Among them, the antenna light harvesting complex LHCA2, AtOSA1 (a protein involved in the biogenesis of the plastid cytochrome b6f complex (Manara *et al.*, 2013)) and SVR7 (a pentatricopeptide repeat protein required for FtsH-mediated chloroplast biogenesis (Liu *et al.*, 2010)) are specifically repressed during CS or at CD, respectively. In addition, proteins involved in carbon allocation and adaptation to growth conditions can be spotted at these stages. In this sense, EXL2 and ICL, an isocytrate lyase with a role in providing the additional source of carbon required for lipid breakdown during seed survival and recovery after prolonged dark conditions (Eastmond *et al.*, 2000), are significantly repressed during CS and CD, respectively. In addition, proteins involved in pathogen defence as HRI2 (Qi *et al.*, 2011) and GTR1 (Nour-Eldin *et al.*, 2012) are shown to be repressed correspondingly during CD and CS.

The identification of specific proteins that are particularly induced or repressed in a certain heat stress treatment reinforces the idea that plants launch specialized programmes depending on the heat treatment and fate of the plant, impacting specifically on particular proteins to modulate specific processes.

Characterization of some of the proteins significantly changed in the iTRAQ data as new players of thermotolerance acquisition

As stated before, the role in thermotolerance of some of the proteins identified in this study was not directly assayed before. Therefore, we took a reverse genetic approach to analyse the thermotolerance phenotype of two of them, *JR1 and BAG6*. As shown in Supporting Information Tables S5 and S3, their coded proteins were significantly induced during CD and during the general heat stress response, respectively. A search in the Arabidopsis T-DNA insertion mutant database (http://signal.salk.edu/cgi-bin/tdnaexpress) retrieved the existence of a line, SALK_000461C, which has a T-DNA insertion in the first exon of the *JR1* coding sequence (Supporting Information Fig. S1). In addition, an insertional line within the first exon of AtBAG6 was also identified, SALK_004760 (Fig. 6). The localization of the T-DNAs was corroborated by further genotyping and sequencing (data not shown). qRT-PCR results confirmed that these lines show a reduced expression of *JR1* of *AtBAG6* (Supporting Information Figs S1 and 6, respectively), and therefore, they were selected to carry out thermotolerance assays. As shown in Fig. 7, a hypersensitivity to the basal heat stress treatment was repeatedly observed for the *jr1* mutant compared to the control plants. However, the opposite phenotype, although mild, was observed for the *AtBAG6* mutant line. As the function of these proteins in thermotolerance was not reported previously, these data demonstrate that JR1 and BAG6 have a key role in the basal heat response, confirming that the proteomic analysis described in this article is a valuable tool to identify new regulators of this process.

AtBAG6 interacts by yeast two hybrid with different calmodulin (CaM) isoforms, including the AtCaM3 (Kang *et al.*, 2006). This later protein, AtCaM3, has been proven to have a key role in the heat stress transduction pathway by modulating the transcriptional activation of *HSP18.2* and *HSP25.3* (Zhang *et al.*, 2009). To get a deeper insight into the molecular role of BAG6 and its relation to CaM3 activity, we carried out qRT-PCRs to study if the moderate increase tolerance of the *AtBAG6* knockdown lines was associated to a change in the expression of the *HSP18.2* and *HSP25.3.* The results shown in Fig. 6 clearly demonstrate that there is a clear induction of both transcripts in the described *bag6* mutant in comparison with Col-0 plants during the heat challenge. The same result is obtained with an independent *bag6* line, SALK-015968 (Fig. 6). These data demonstrate a role of AtBAG6 in the regulation of the heat induced small HSP (sHSP) transcriptional cascade and suggest an opposite role to CaM3 during the heat shock signal transduction in Arabidopsis.

DISCUSSION

Proteomics can significantly contribute to the understanding of the molecular response of plants to heat stress, as this response seems closely linked to global and specific changes in protein levels. However, the proteomics response is only expected to be partially coincident under different

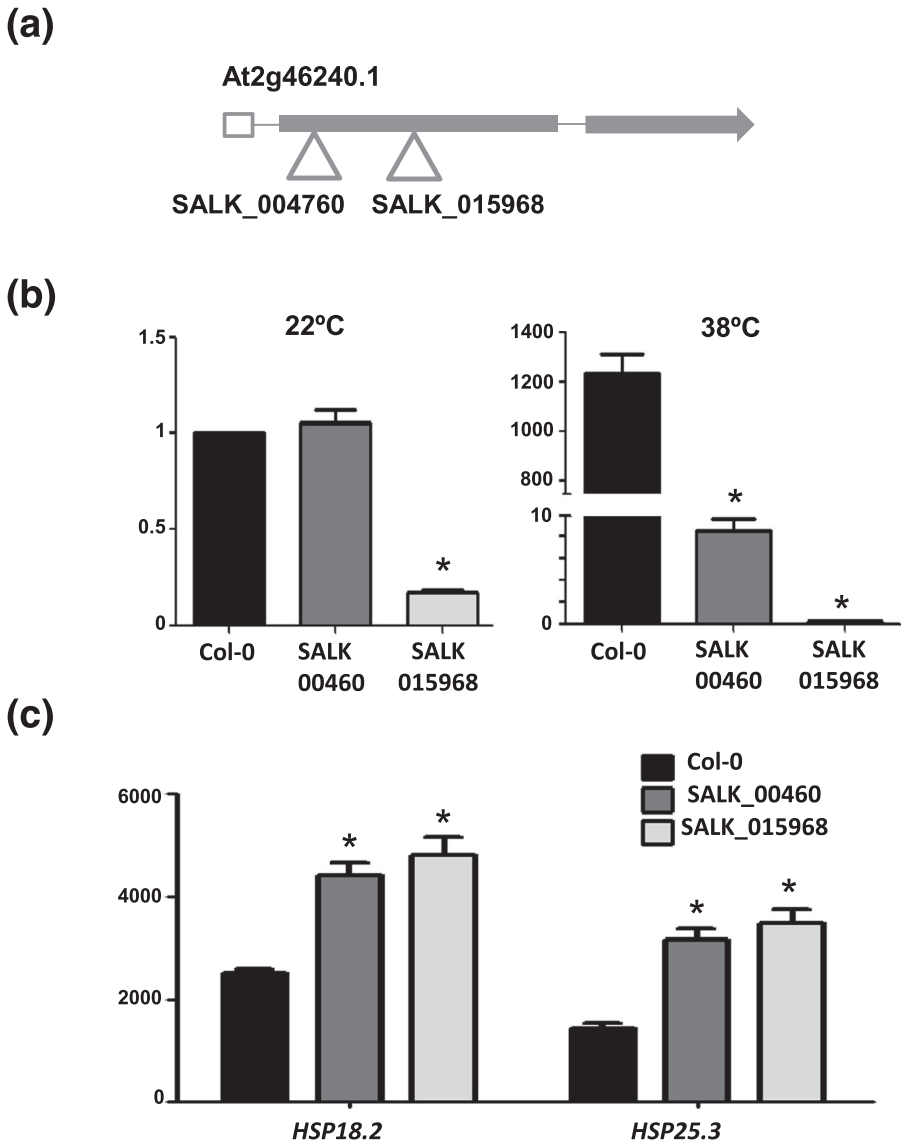

Figure 6. Analysis of the *bag6* T-DNA insertion mutants used for further analysis. (a) Genomic organization of *AtBAG6.* Exons are indicated as rectangles. The triangle marks the region of the T-DNA insertions. (b and c) qPCR analysis of the levels of *BAG6* (b) or *HSP18.2* and *HSP25.3* (c) in 7-day-old grown seedlings from control (Col-0) or from the T-DNA insertion mutants described in (a). For this analysis seedlings were grown at 22 °C (b, left panel) or challenged for 1 h at 38 °C (b, right panel and c). Accumulation of the *different* transcripts is expressed as fold change values related to the control sample (Col-0) at 22 °C, which was arbitrarily assigned to value 1 after normalization to *TUB5*. Asterisks mean that there are statistical differences (ANOVA, $P < 0.01$) with the respective controls.

thermotolerance-determinant conditions as the heat regime, the developmental stage and the acclimation status of the plant. As our approach considers the response to different temperatures (38 °C and 45 °C) and to the early events that lead to acclimation or commitment to cell survival or death, this analysis has allowed us to dissect the proteome response to different conditions and to assign the changes in the levels of different proteins to the diverse physiological processes under study (Fig. 8).

In this sense, our data demonstrate that only a small number of proteins respond coordinetly to the three heat stress regimes: A, CS and CD. In addition, our results also show a significant overlap for the induced proteins during A and CS. This overlap could be partially explained because A and CS are sequential treatments and, therefore, the proteins accumulated in A, if stable, could be also monitored in CS. Interestingly, a significant overlap is also detected for CS and CD. The fact that a clear overlap is observed between CS and CD but no overlap is detected between A and CD suggests that the heat stress response may be partially conditioned to the intensity of the heat insult (38 °C for A and 45 °C for CS and CD) that, in turn, determines the severity of the plant cell injuries. In this sense, it could be possible that the larger number of proteins with reduced levels at CS and CD compared to A could be observed due to the more drastic repression of protein synthesis during the 45 °C challenge versus the 38 °C treatment.

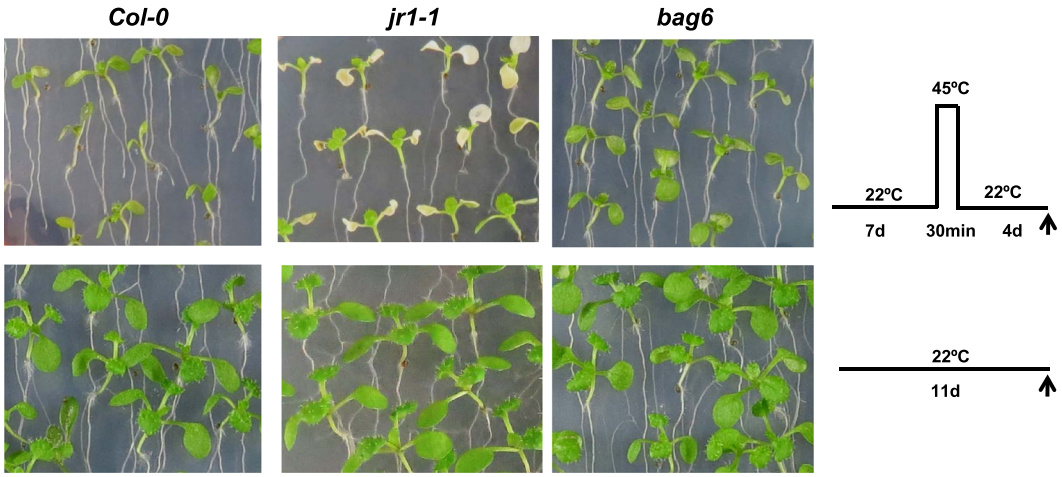

Figure 7. *jr1-1* and *bag6* mutant plants have a modified heat stress response. 7-day-old Col-0, *jr1-1* and *bag6* mutant plants were either challenged with a severe heat stress of 45 °C for 30 min (upper panel) or grown at control conditions (lower panel). Treatment regimes are shown at the right of each panel. Photographs were taken at the end of the treatment (4 days after the heat challenge or 11 days of growth at control conditions, respectively).

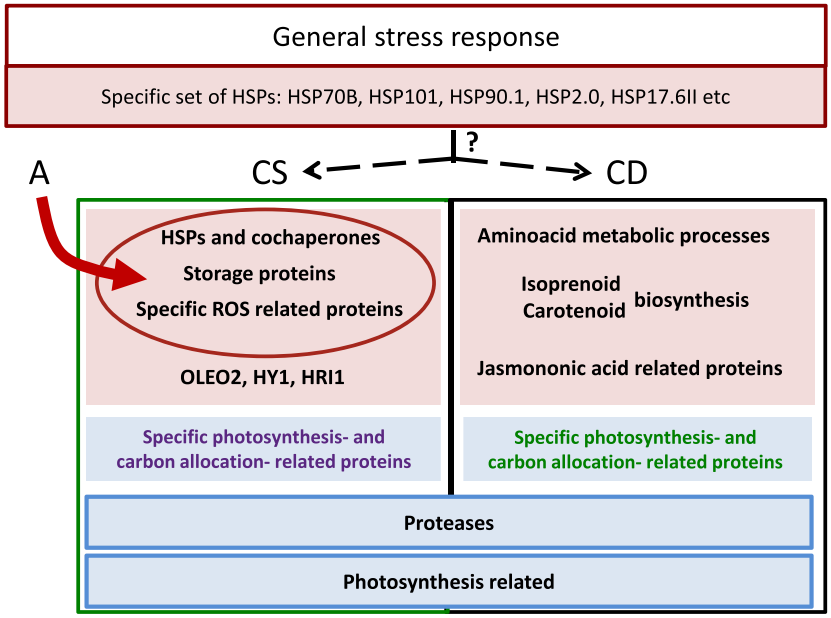

Figure 8. Proposed model highlighting the function of the proteins that could have a major role in the commitment of plants to heat induced survival or death. The different categories and the specific proteins that are induced or repressed in each condition are shown on a red or blue background, respectively. Those proteins that were induced during the acclimation stage and could contribute to the commitment to survival are shown in a circle. Some specific proteins induced during the general stress response are also included. Although these proteins are induced independently of the fate of the plants, it could be still possible that their proper accumulation could be determinant of the commitment to survival or death.

Finally, a large number of proteins are also changed specifically during a single treatment, which suggests also a proteome specialization during the different heat stress responses. These proteins could serve as potential markers for proper acclimation to high temperatures and as markers for the early heat responses leading to plan survival or death.

Previously published proteomics results assigned an essential role in the heat stress response to the HSPs, ROS scavenger enzymes, proteases and proteins involved in hormone signalling and production (Wahid *et al.*, 2007, Kosova *et al.*, 2011, Bokszczanin & Fragkostefanakis, 2013). However, as it has been established before, the heat stress response cannot be determined in general terms, and therefore, this kind of comprehensive and systemic analyses, as the one described here, could be pivotal to identify, among these functional categories, the specific proteins involved in the physiological responses to different heat stress treatments.

HSPs and HSFs

Within the proteins highly induced during A, CS and CD, a large number of HSPs could be identified and, among them, some regulators with a proven role in thermotolerance are included. However, as these proteins are induced along the three treatments independently of triggering plant survival or death, our results suggest that their induction themselves do not assure thermotolerance acquisition. This may imply either that its induction is necessary for the establishment of the response, but other players are needed to determine the final fate of the plant, or, alternatively, that a certain protein threshold could be key for the plant survival. Finally, although a large number of HSPs converge during the general heat stress response not all of them are coordinately expressed in the three heat stress treatments. Indeed, certain HSPs as HSP18.2 and HSP21, among others, seem to be specific of the A and CS response, reinforcing the idea of a proteome specialization during the different responses and stressing the need of this type of analysis.

HSFs are central regulators of the heat stress response (Nover & Scharf, 1997) and among them, probably the best studied factors are Hsf1As and HsfA2. Specifically, different data demonstrate that *hsfa1s* and *hsfa2* loss of function mutants show a decreased basal and acquired thermotolerance while overexpression of HsfA2 enhances heat tolerance under stress conditions (Ogawa *et al.*, 2007, Liu *et al.*, 2011). As transcriptional regulators, these factors seem to play an important role in controlling the expression of a large number of the heat responsive genes including HSPs and chaperones (Nishizawa *et al.*, 2006, Schramm *et al.*, 2006, Liu *et al.*, 2011). In our assays neither the Hsf1s nor the HsfA2, which is a target of Hsf1A itself, were confidently detected and quantified, probably because of their low expression levels even in the presence of the challenge. However and besides this fact, 18 HsfA2 target genes were identified as significantly induced proteins in our proteomics analysis (Supporting Information Table S6). These data suggest that these transcription factors, and specifically the HsfA2, could be active under our stress conditions. Finally, our data suggest that along the HsfA2 targets, one of the mechanisms that control HsfA2 activity, AtSUMO1 (Cohen-Peer *et al.*, 2010), is also significantly induced during CS and CD.

ROS-related proteins

One of the molecular responses to different kinds of environmental stresses, including heat, is the generation of reactive oxygen species (ROS) as singlet oxygen, superoxide radical, hydrogen peroxide and hydroxyl radicals (Bokszczanin & Fragkostefanakis, 2013, Wahid *et al.*, 2007). To counteract the ROS destructive effect, plants have evolved highly efficient ROS scavenger enzymes such as superoxide dismutases (SODs), catalases (CATs), peroxidases (PERs) and the enzymes involved in the ascorbate-glutathione cycle (APXs and GPXs, respectively). The activation of these ROS scavengers seems to be important for heat adaptation as an increase in the activity of some of these enzymes have been correlated to the acquisition of thermotolerance in wheat cultivars (Almeselmani *et al.*, 2006). In our assays, a large number of ROS scavenger enzymes have been identified and quantified in the different treatments. However, only significant variations of some SODs, GPXs and PERs have been observed. Strikingly, these proteins seem to be specifically induced during some treatments instead of being coordinately regulated. For example, the induction of SODC2, and of two peroxidases, At3g01190 and At5g64120, is specifically observed during acclimation, but not during CS and CD. However, GSTF9 has been identified among the proteins induced coordinately during CS and CD, while the dehydroascorbate reductase DHAR1 was only significantly induced during CD. As these enzymes scavenge different ROS species (superoxide radical and hydrogen peroxide) and use different hydrogen donors as gluthathione and dehydroascorbate, respectively, the specific induction of these proteins may reflect the accumulation at the different stages of specific reactive species or substrates.

In addition to the proper scavenger enzymes, many other ROS-related proteins seem to be regulated differentially during A, CS and CD. Clear examples are the significant induction during CD of FLU, which prevents the peroxidation of chloroplast membrane lipids (op den Camp *et al.*, 2003) or the induction during CS of HY1, an haem oxygenase involved in carotenoid and flavonoid biosynthesis (Xie *et al.*, 2012).

Proteases

Heat stress usually causes protein unfolding and misfolding. Chaperones play an important role to prevent these processes (Saibil & Ranson, 2002, Sharma *et al.*, 2009) but, when this mechanism is overwhelmed proteases degrade the unfolded and denatured proteins releasing aminoacids for recycling (Roberts *et al.*, 2012, Hansen & Hilgenfeld, 2013). In our data, a clear and coordinated induction of chaperones could be observed in all the different treatments. Moreover, a repression of a large number of proteases is monitored during CS and CD. These data are in agreement with the relative low amount of degraded proteins through the different treatments and may suggest that the selected time-points (even in the case of CD) correspond to quite early stages in the response to heat.

Jasmonate-related proteins and BAG6

In Arabidopsis, the possible role of some jasmonate-related proteins in thermotolerance has been demonstrated. For example, it has been described that the *coi1-1* mutant, which is defective in different JA-dependent responses, is thermosensitive to a heat stress of 38 °C for 16 h (Clarke *et al.*, 2009). In our case, we observe a clear accumulation of LOX2 and at least four proteins encoded by JA-inducible genes during CD, including JR1. The induction of the main enzyme involved in JA synthesis and of different JA responsive genes suggest an important role of JA metabolism in thermotolerance acquisition. JR1 is repressed at A but accumulates at CD compared to C, a protein pattern that is consistent with the proposed role of jasmonate-related proteins in programmed cell death (PCD). However, based on the phenotype of the *jr1* mutant, we may speculate that the induction of these jasmonate-related proteins during CD could be part of an extreme molecular mechanism

launched by plants in an attempt to be protected against the high levels of injuries caused by the heat in non-acclimated plants. Whether this extreme mechanism could be somehow related to PCD should await further characterization.

BAG6 is a calmodulin binding protein that was shown to interact with CaM3 (Kang *et al.*, 2006), and this latter protein was shown to act as transcriptional activation of *HSP18.2* and *HSP25.3* during the heat stress (Zhang *et al.*, 2009). In this article, we observe a clear accumulation of the AtBAG6 in the general response to heat. Furthermore, we demonstrate an up-regulation of *HSP18.2* and *HSP25.3* during the heat challenge in the *bag6* mutant lines that correlates with the enhance thermotolerance of the *bag6* mutant. These results suggest that BAG6 has an opposite role to CaM3 in the heat stress signal transduction, restricting the induction of some sHSPs during the heat challenge. If so, BAG6 may be involved in limiting the extension of the heat stress response, as it is the case of ROF2 (Meiri *et al.*, 2010) and SUMO1 (Cohen-Peer *et al.*, 2010). Finally, BAG6 has been also proposed to be a positive regulator of PCD in plants, and this role seems to be directly associated to its CaM binding domain (Kang *et al.*, 2006). This role could also explain the heat-tolerance phenotype of the mutant; however, whether its role in PCD is important during the heat stress response and, if so, how this could be associated to its possible function in the regulation of the transcriptional cascade should be studied in more detail.

Translation-related proteins

One of the main aspects of the heat stress response is the regulation of translation, and different proteomics analysis have identified differentially expressed translation factors and ribosomal proteins under heat stress conditions (Ahsan *et al.*, 2010, Kosova *et al.*, 2011, Zou *et al.*, 2011). In some cases, as it is the case of chloroplast ET-TU, the levels of this elongation factor have been positively correlated with the acquisition of thermotolerance in rice (Momcilovic & Ristic, 2007). In our assays the levels of AtIF3 and SVR3 are significantly increased and repressed during A and CD, respectively. The specific regulation of these chloroplast translational factors may play an important role by boosting the selective translation of specific mRNAs at these particular stages. In addition to these translation factors, two chloroplastic ribosomal proteins, PSRP3 and PRPL28 (Romani *et al.*, 2012, Tiller *et al.*, 2012), significantly increased their levels during CS, and another four 60S and one specific 40S ribosomal proteins are specifically expressed in our dataset. Recently, a stress specific change in the subunit composition in the ribosomes has been described during the response to phosphate- and iron-deficiency in Arabidopsis roots (Wang *et al.*, 2013), and, a similar observation was done previously during the hypoxic stress response of maize seedlings (Bailey-Serres & Freeling, 1990). The fact that we also observed significant changes in the accumulation of specific ribosomal proteins during the different heat stress treatments may suggest that the composition of the ribosomes could be also altered during the different heat stress responses and that this mechanism could be important for the adaptation to the diverse heat stress challenges. In addition, the main accumulation of translation-related proteins observed during A (cluster 5, Fig. 3) could reflect the key role of this process in the accumulation in CS of proteins directly involved in thermotolerance acquisition as HSPs and cochaperones, among others.

Photosynthesis and carbon allocation-related proteins

One of the main effects of our heat stress treatments, which highly correlates with the data obtained in other studies (Ahsan *et al.*, 2010, Li *et al.*, 2013, Rocco *et al.*, 2013), is the decrease in the levels of proteins involved in photosynthesis and in carbon allocation and adaptation to growth conditions. However, although the final result is the inhibition of photosynthesis, this response seems to be complex affecting different proteins during A, CS and CD. These data, along with the situation observed for other processes, highlight the specificity of the heat stress proteome response to the different treatments assayed.

Finally, it is important to mention that this analysis has focused on quantifying the relative abundance of the proteins during the different heat stress treatments compared to control conditions; however, the activity of some of these proteins could be highly modulated by posttranslational modifications that are mainly missed in our proteomics study. Exploring this higher level of regulation will contribute importantly to our current understanding of how plants adapt to this especially harmful condition.

ACKNOWLEDGMENTS

The research leading to these results has received funding from the European Research Council under the European Union's Seventh Framework Programme (FP/2007–2013)/ERC Grant Agreement 260468 to M. Mar Castellano and from the grants RTA2013-00027-00-00 and S2013/ABI-2734-CM. We thank Dr. A. Muñoz for critical reading of the manuscript, Dr. R. Toribio for his help in the annotation of gene descriptions and Dr. Hara-Nishimura and Dr. Hans-Peter Mock for kindly providing the OLEO2 and HSP90.1 antibodies, respectively. Plant Response is also thanked for its support in the project. The authors declare no conflict of interest.

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Received 30 July 2015; received in revised form 14 October 2015; accepted for publication 16 October 2015

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Analysis of the *jr1* T-DNA insertion mutant used for the thermotolerance assay.
Table S1. Primer sequences used for qRT-PCR analyses.
Table S2. Description of the reported thermotolerance phenotypes associated to gain and loss of function mutants of some genes whose proteins show a significant change in the iTRAQ analysis.
Table S3. Proteins significantly induced and repressed during the general heat stress response.
Table S4. Proteins coordinately changed during A and CS and CS and CD, respectively.
Table S5. Proteins specifically induced or repressed during A, CS or CD treatments.
Table S6. Proteins significantly changed in the iTRAQ data and whose mRNA levels are regulated by the HsfA2. The AGI number, the name of the protein according to TAIR description, the number of peptides identified for each protein in each independent experiment and the fold change of each protein in each condition compared to C (shown as average ± standard deviation) are shown.