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Cyclic AMP mediates heat stress response by the control of redox homeostasis and ubiquitin-proteasome system

Annalisa Paradiso[1] | Guido Domingo[2] | Emanuela Blanco[3] | Alessio Buscaglia[1,2] | Stefania Fortunato[1] | Milena Marsoni[2] | Pasquale Scarcia[4] | Sofia Caretto[5] | Candida Vannini[2] | Maria Concetta de Pinto[1]

[1]Department of Biology, University of Bari "Aldo Moro", Bari, Italy

[2]Department of Biotechnology and Life Science, University of Insubria, Varese, Italy

[3]Institute of Biosciences and Bioresources, National Research Council, Bari, Italy

[4]Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari "Aldo Moro", Bari, Italy

[5]Institute of Sciences of Food Production, CNR, Research Division Lecce, Lecce, Italy

Correspondence
Candida Vannini, Department of Biology, University of Bari "Aldo Moro", Via Orabona 4, Bari 70125, Italy.
Email: candida.vanninini@uninsubria.it

Maria Concetta de Pinto, Department of Biotechnology and Life Science, University of Insubria, via J.H. Dunant 3, Varese I-21100, Italy.
Email: mariaconcetta.depinto@uniba.it

Present address
Alessio Buscaglia, Lonza, Monteggio, Switzerland

Abstract

Heat stress (HS), causing impairment in several physiological processes, is one of the most damaging environmental cues for plants. To counteract the harmful effects of high temperatures, plants activate complex signalling networks, indicated as HS response (HSR). Expression of heat shock proteins (HSPs) and adjustment of redox homeostasis are crucial events of HSR, required for thermotolerance. By pharmacological approaches, the involvement of cAMP in triggering plant HSR has been recently proposed. In this study, to investigate the role of cAMP in HSR signalling, tobacco BY-2 cells overexpressing the 'cAMP-sponge', a genetic tool that reduces intracellular cAMP levels, have been used. in vivo cAMP dampening increased HS susceptibility in a HSPs-independent way. The failure in cAMP elevation during HS caused a high accumulation of reactive oxygen species, due to increased levels of respiratory burst oxidase homolog D, decreased activities of catalase and ascorbate peroxidase, as well as down-accumulation of proteins involved in the control of redox homeostasis. In addition, cAMP deficiency impaired proteasome activity and prevented the accumulation of many proteins of ubiquitin-proteasome system (UPS). By a large-scale proteomic approach together with in silico analyses, these UPS proteins were identified in a specific cAMP-dependent network of HSR.

KEYWORDS

cyclic AMP, heat shock proteins, heat stress response, proteomics, reactive oxygen species, redox homeostasis; tobacco BY-2 cells, ubiquitin-proteasome system

1 | INTRODUCTION

The constant rise in temperature represents one of the most damaging environmental stress for plants, that can lead to a significant reduction in crop yield (Lesk, Rowhani, & Ramankutty, 2016). A moderate but persistent heat stress (HS) causes a strong reduction in cell growth, due to the inhibition of both cell division and cell expansion (Centomani et al., 2015; Potters, Pasternak, Guisez, Palme, & Jansen, 2007; Sgobba, Paradiso, Dipierro, De Gara, & de Pinto, 2015). If temperatures exceed 40°C, severe cellular injury may occur, resulting in rapid cell death (Echevarria-Zomeno et al., 2016; Locato, Gadaleta, De Gara, & de Pinto, 2008; Petrov, Hille, Mueller-Roeber, & Gechev, 2015; Vacca et al., 2004). At a molecular level, HS alters membrane fluidity, induces denaturation and aggregation of proteins and increases the production of reactive oxygen species (ROS), causing an impairment in major physiological processes (Bokszczanin & Fragkostefanakis, 2013; Hasanuzzaman, Nahar, Alam, Roychowdhury, & Fujita, 2013; Kotak et al., 2007; Li, Gao, Ren, & Tang, 2018). In order to

Annalisa Paradiso and Guido Domingo should be considered joint first author.

counteract the harmful effects of high temperature and maximize the chance of survival, plants activate the heat stress response (HSR) that is aimed at restoring cellular metabolic balance. HSR comprises complex signalling networks that lead to changes in gene expression and protein synthesis, as well as in post-translational modification (Bokszczanin & Fragkostefanakis, 2013; Li et al., 2018; Mittler, Finka, & Goloubinoff, 2012; Ohama, Sato, Shinozaki, & Yamaguchi-Shinozaki, 2017).

The network of heat shock factors (HSFs)—heat shock proteins (HSPs) represents one of the most relevant defence mechanisms of HSR (Ohama et al., 2017). The expression of HSPs, preventing misfolding, aggregation and denaturation of proteins, is a major reprogramming event required for the acquisition of thermotolerance (Jacob, Hirt, & Bendahmane, 2017; Sable & Agarwal, 2018).

Calcium ions (Ca^{2+}) and ROS have been proposed as key second messengers involved in the activation of HSR (Larkindale, Hall, Knight, & Vierling, 2005; Liu, Sun, & Zhou, 2005; Saidi et al., 2009; Volkov, Panchuk, Mullineaux, & Schoffl, 2006). The plasma membrane fluidization, due to HS, causes a Ca^{2+} influx in the cytosol, which is controlled by membrane-associated cyclic nucleotide gated calcium channels (CNGCs; Finka, Cuendet, Maathuis, Saidi, & Goloubinoff, 2012; Finka & Goloubinoff, 2014; Los & Murata, 2004; Saidi et al., 2009). A tight connection linking HS, Ca^{2+} influx and ROS production is given by the respiratory burst oxidase homolog D (RBOHD), a Ca^{2+}-dependent NADPH oxidase located in the plasma membrane, playing diverse roles in plant response to stress. Elevation of Ca^{2+} levels in response to HS activates this NADPH oxidase which produces ROS, causing an imbalance in cellular redox homeostasis (Miller et al., 2009; Qu, Yan, & Zhang, 2017; Suzuki et al., 2011). ROS, being able to react with lipids, proteins, carbohydrates and nucleic acids, can lead to significant cell damages. However, the temporary oxidative imbalance can activate signalling pathways allowing cells to acclimate to environmental stress (Foyer & Noctor, 2005; Jaspers & Kangasjarvi, 2010; Suzuki, Koussevitzky, Mittler, & Miller, 2012). In order to function as second messengers, and not as toxic molecules, ROS must be finely regulated (Geigenberger & Fernie, 2014; Suzuki & Mittler, 2006). Plants possess an adaptable and plastic antioxidant machinery encompassing enzymatic and non-enzymatic components. The hydrophilic redox couples ascorbate (ASC)/dehydroascorbate (DHA) and glutathione (GSH)/glutathione disulphides (GSSG), connected by a reaction network, namely ASC-GSH cycle, play a crucial role in preserving redox homeostasis and in contributing to redox signalling (Foyer & Noctor, 2011, 2013). The imbalance among ROS-producing and ROS-scavenging systems gives information on cellular environment, acting as a redox signal able to induce adaptive responses (Paciolla, Paradiso, & de Pinto, 2016). Thus, ROS-scavenging systems, regulating ROS levels, play a critical role in the acquisition of thermotolerance, which is strictly related to plant redox homeostatic capacity (de Pinto, Locato, Paradiso, & De Gara, 2015; Katano, Honda, & Suzuki, 2018).

3′,5′-Cyclic Adenosine—Monophosphate (cAMP) has been widely recognized as a second messenger in plants, where it plays important roles in regulating responses to different biotic and abiotic stress (Alqurashi, Gehring, & Marondedze, 2016; Blanco, Fortunato, Viggiano, & de Pinto, 2020; Gancedo, 2013; Gao et al., 2012; Gehring & Turek, 2017; Ma et al., 2009; Sabetta et al., 2019). A role for cAMP has also been proposed in HSR; in Arabidopsis, the increase in intracellular cAMP levels, due to a mild heat shock, activating CNGC6, caused a cytosolic Ca^{2+} influx (Gao et al., 2012). In addition, the treatment of Arabidopsis plants with exogenous cAMP analogues induced the expression of some HSPs (Gao et al., 2012) and caused an enrichment in proteins involved in temperature stress response (Alqurashi et al., 2016; Thomas, Marondedze, Ederli, Pasqualini, & Gehring, 2013). However, these data were obtained with pharmacological approaches which do not consider the importance of physiological concentrations and endogenous fluctuations of cAMP. To overcome these problems, tobacco Bright Yellow-2 (BY-2) cells and *Arabidopsis thaliana* plants overexpressing the chimeric protein 'cAMP-sponge' have been recently produced (Sabetta et al., 2016; Sabetta et al., 2019). 'cAMP-sponge' is a non-invasive genetic tool, composed by the two-high affinity cAMP-binding domains of the human PKA Iβ regulatory subunit, that allows in vivo cAMP depletion (Lefkimmiatis, Moyer, Curci, & Hofer, 2009). Both Arabidopsis and tobacco BY-2 transgenic lines (cAS lines) compared to wild type (WT) showed around half of free cAMP (Sabetta et al., 2016; Sabetta et al., 2019). In tobacco BY-2 cells cAMP dampening inhibited cell growth, delaying cell cycle progression and cell division, and enhanced defence systems, indicating that these cells sensed cAMP deficiency as a stress condition (Sabetta et al., 2016). On the other hand, probably due to compensatory mechanisms, cAS Arabidopsis plants did not show phenotypic changes at resting conditions, although had reduced resistance to an avirulent strain of *Pseudomonas syringae* (Sabetta et al., 2019). Tobacco BY-2 cells represent an optimal tool for functional genomics when the phenotypic changes, due to the expression of a specific gene, can be hidden by in planta compensatory mechanisms (Bhat & Thompson, 2004). Furthermore, BY-2 cells have already been used as a model system in the study of physiological response to HS (Centomani et al., 2015; Konigshofer, Tromballa, & Loppert, 2008; Sgobba et al., 2015).

With the aim to identify specific cAMP-dependent components of HSR, by means of in vivo cAMP dampening, in this study the behaviour of WT and cAS lines of tobacco BY-2 cells, grown at 27°C (control) or 35°C (HS), was evaluated and compared. After 3 and 5 days of culture at the two temperatures, cell growth, HSPs expression, as well as redox homeostasis of the two genotypes were analysed. In order to identify specific cAMP-dependent HS-responsive proteins involved in heat tolerance, a large-scale comparative proteomic analysis of the two genotypes grown for 3 days in control or HS conditions was performed. A two-way ANOVA analysis allowed to recognize proteomic changes exclusively due to genotype or temperature or influenced by genotype–temperature interaction. The proteomic approach together with in silico analyses permitted to identify the activation of ubiquitin-proteasome system (UPS) as a cAMP-dependent response of HSR.

2 | MATERIALS AND METHODS

2.1 | Cell cultures, heat treatment and cell growth parameters

Wild type tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) cell suspensions were routinely propagated and cultured according to Nagata, Nemoto, and Hasezawa (1992). Tobacco BY-2 line over-expressing the 'cAMP-sponge', namely cAS line, previously obtained (Sabetta et al., 2016), was routinely propagated in liquid selective medium (50 μg/ml kanamycin). For the experiments, cAS cells were cultured in non-selective medium. Two millilitres of both WT and cAS stationary cell suspensions (7 days) were diluted in 100 ml of fresh culture medium in 250-ml flasks and grown at 27°C (control) or 35°C (HS) for 5 days. Where indicated, cAS cells were pre-treated with 20 μM cAMP. After 3 and 5 days of culture, aliquots of cell suspensions were collected for the determination of mitotic index and cell viability. Alternatively, cells were collected by vacuum filtration on Whatman 3MM paper, frozen in liquid nitrogen and stored at −80°C until the analyses. In total, five independent biological replicates were performed.

Cell growth was expressed as cell fresh weight (mg) in 1 ml of cell suspension. Mitotic index was measured by DAPI staining according to Centomani et al. (2015). Cell viability was determined by trypan blue staining as described in de Pinto, Francis, and De Gara (1999).

2.2 | cAMP and ROS determination

Free cAMP was measured according to Sabetta et al. (2016). Briefly, cells (500 mg) were ground in liquid nitrogen and homogenized in two volumes of phosphate buffered saline [PBS: 0.8% (w/v) NaCl, 0.028% (w/v) KCl, 0.144% (w/v) Na_2HPO_4, 0.024% (w/v) KH_2PO_4, pH 7.4]. The supernatants obtained after centrifugation at 12,000*g* for 15 min, were transferred into 10 kDa Amicon® Ultra-4 Centrifugal Filter Units (Merck Millipore, Germany) and centrifuged at 4,000*g* for 30 min at 4°C. The eluted samples were utilized to measure free cAMP by the cAMP-Glo™ assay kit (Promega, Madison, WI) according to Promega's instructions. This assay is based on the principle that cAMP stimulates protein kinase A activity, decreasing available ATP and leading to decreased light production in a coupled luciferase reaction. The luminescence was read by a microplate luminometer (Victor3 Multilabel Plate Readers; PerkinElmer, Massachusetts, MA), and correlated to the cAMP concentrations by using a cAMP standard curve in the range of 0–15 nM.

Extracellular H_2O_2 was determined in the culture medium, after the removal of cells by centrifugation (10,000*g*, 60 s, 25°C), according to de Pinto, Paradiso, Leonetti, and De Gara (2006). Briefly, 500 μl of culture medium was added to 500 μl of assay reagent (500 μM ammonium ferrous sulphate, 50 mM H_2SO_4, 200 μM xylenol orange and 200 mM sorbitol) and after 45 min the absorbance of the Fe^{3+}-xylenol orange complex at 560 nm was detected. The specificity for H_2O_2 was tested by adding catalase in the reaction mixture.

Intracellular ROS were monitored by detecting the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) into the highly fluorescent compound 2′,7′-dichlorodihydrofluorescein (DCF) according to Zhao, Fujita, and Sakai (2005). Fluorescence intensity was measured by a microplate reader (Victor3 Multilabel Plate Readers; PerkinElmer) at 495 nm excitation and 530 nm emission wavelengths.

2.3 | Analysis of enzymatic and non-enzymatic antioxidants

Ascorbate and glutathione contents and redox states were determined according to Sgobba et al. (2015).

For enzymatic assays, cells were ground in liquid nitrogen and homogenized at 4°C in a 1:3 (w/v) ratio with extraction buffer (50 mM Tris–HCl pH 7.5, 0.05% cysteine, 0.1% bovine serum albumin). Homogenates were centrifuged at 20,000*g* for 15 min and the supernatants used for spectrophotometric analyses. Proteins were determined according to Bradford (1976), using bovine serum albumin as a standard. Catalase (CAT, EC 1.11.1.6) activity assay was performed by following H_2O_2 dismutation at 240 nm in a reaction mixture consisting of 0.1 M phosphate buffer, pH 7.0, 50 μg protein and 18 mM H_2O_2 (ϵ = 39.6 M^{-1} cm^{-1}). Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to Beauchamp and Fridovich (1971), measuring NBT reduction in the light in absence and in presence of 15 μg proteins. The reaction mixture consisted of 100 mM potassium-phosphate buffer pH 7.8, 0.1 mM EDTA, 13 mM methionine, 75 μM NBT. The reaction was started by adding 2.5 μM riboflavin in the mixture. After illumination for 15 min, absorbance was measured at 530 nm. One unit of SOD was defined as the amount of enzyme that inhibited the rate of NBT reduction by 50%. Ascorbate peroxidase (APX, EC 1.11.1.11), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2) activities were measured according to de Pinto, Tommasi, and De Gara (2000).

2.4 | Activities of proteases and proteasome

Activity of total proteases was determined spectrophotometrically according to Distefano, Palma, Gomez, and Rio (1997). In brief, cells were ground in liquid nitrogen and homogenized at 4°C in a 1:3 (w:v) ratio with extraction buffer (100 mM potassium-phosphate buffer pH 7.8, containing 1 mM EDTA). Supernatants were collected after centrifugation at 18,000*g* for 15 min at 4°C. Aliquots of protein extracts (200 μg) were incubated at 37°C with 0.15% (w/v) azocasein in 0.1 M Tris–HCl, pH 8.5. After 4 hr, the reaction was stopped with 12% TCA on ice and the samples centrifuged at 10,000*g* for 10 min. Absorbance of samples at 340 nm was read before and after incubation. Enzymatic activity was calculated considering that one unit corresponds to the amount of enzyme needed to cause a 0.01 increase in absorbance at 340 nm per 2 hr.

Proteasome activity was determined as described in Ustun et al. (2016), with minor modifications. Cells (0.1 g) were ground in liquid nitrogen and homogenized in a 1:3 (w:v) ratio with extraction buffer (50 mM Hepes-KOH, pH 7.2, 2 mM DTT, 2 mM ATP and 250 mM sucrose). Supernatants were collected after centrifugation at 20,000*g* for 15 min at 4°C. Ten microlitres of samples, with 1 mg/ml protein concentration, were mixed with 220 μl of assay buffer (100 mM Hepes-KOH, pH 7.8, 5 mM $MgCl_2$, 10 mM KCl and 2 mM ATP); after 15 min of incubation at 30°C, the reaction was started by addition of the fluorigenic substrate Suc-LLYY-NH-AMC (Calbiochem) and the release of amino-methyl-coumarin (360 nm ex/460 nm em) was monitored between 0 and 120 min, by Infinite M200 Pro (Tecan, Austria).

2.5 | Quantitative real time PCR

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA concentration was determined by measuring the absorbance at 260 nm with NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized from 1 μg of total RNA, using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Primers for quantitative real-time PCR (qRT-PCR) were designed on cAMP-sponge construct and on *Nicotiana tabacum* Actin, HSP18, HSP26 and HSP101 gene sequences (Table S1). qRT-PCR assays were performed on the Applied Biosystems StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) using the iTaq Universal SYBR Green SuperMix (Biorad, California). Actin was used as housekeeping gene. Reaction mixtures (10 μl) included the following components: 1X master mix, 0.2 μM of each primer and 1 μl cDNA. Cycle conditions were 95°C for 20 s and then 40 cycles of 95°C for 15 s and 60°C for 30 s. Separation of real-time PCR products on 2% (w/v) agarose gels revealed single bands of the expected size whose identities were confirmed by direct sequencing. Relative quantification was performed according to the comparative Ct (threshold cycle) method ($2^{(-\Delta\Delta Ct)}$; Bustin, 2000; Livak & Schmittgen, 2001). In all experiments, appropriate negative controls, containing no template, were used. Analysis was performed on three independent biological replicates per cell suspension sample, and three technical replicates for each sample, and reactions were repeated twice to verify reproducibility.

2.6 | Workflow of proteomic and downstream bioinformatic analyses

Proteins were extracted following SDS/phenol method with minor modifications (Wu, Xiong, Wang, Scali, & Cresti, 2014). Cells (1 g) were ground with liquid nitrogen and then homogenized in extraction buffer (0.15 M TRIS–HCl, pH 8.8, SDS 1%, 1 mM EDTA, 0.1 M DTT, 1 mM PMSF, 0.1 mg/ml Pefabloc, 1 mM Na_3VO_4, 1 mM NaF). After centrifugation (15,000*g* for 10 min), the supernatant was collected and mixed with an equal volume of phenol at room temperature (RT) for 30 min. After centrifugation (15,000*g* for 5 min at RT) phenol phase was collected and proteins were precipitated (overnight at −20°C) with five volumes of 0.1 M ammonium acetate in methanol. After centrifugation, the protein pellet was washed one time with 0.1 M ammonium acetate in methanol and one time with 80% (v/v). After centrifugation (15,000*g* for 5 min at 4°C), the pellet was air dried, resuspended in SDS Lysis Buffer (100 mM Tris–HCl, pH 7.5, 4% SDS) and quantified with 2D Quant Kit (GE Healthcare).

Proteins were digested with trypsin via the Filter Aided Sample Preparation (FASP) as described by Vannini et al. (2019). Peptides were desalted using SPE (Phenomenex Strata C18-E). All the procedures were carried out in positive pressure, except the loading and elution of the sample which exploited the gravity force. The column was conditioned with 3 ml 0.1% TFA in methanol and equilibrated with 2 ml Equilibration Buffer (0.1% TFA in H_2O). The sample loading was maximized flowing 1 ml of Equilibration Buffer. The final elution was performed by loading 1 ml of Elution Buffer (0.1% TFA in 70:30 ACN:H_2O).

The peptides were analysed by LC MS/MS as described by Alqurashi et al. (2018). After LC separation peptides were sprayed into the mass spectrometer and eluting ions were measured in an Orbitrap mass analyzer, set at a resolution of 35,000 and scanned between m/z 380 and 1,500. Data dependent scans (Top 20) were employed to automatically isolate and generate fragment ions by higher energy collisional dissociation (HCD); Normalized collision energy (NCE): 25% in the HCD collision cell and measurement of the resulting fragment ions was performed in the Orbitrap analyser, set at a resolution of 17,500. Peptide ions with charge states of 2^+ and above were selected for fragmentation. Raw data were searched against the *Nicotiana tabacum* Uniprot protein database (version 2019-01, 76,141 entries) with MaxQuant program (v.1.5.3.3). For the quantitative analysis, the 'ProteinGroups' output file from MaxQuant were processed as described by Vannini et al. (2019). Briefly, after replacing of missing value, inconsistent identifications were filtered out: only protein groups detected in at least three of the four biological replicates (75%) in almost one analytical group (WT27, WT35, cAS27, cAS35) were considered for statistical analysis. LFQ intensities were Log_2 transformed, centred by subtracting the median within each replicate, and subjected to principal component analysis (PCA) by the Perseus software. Log2 transformed and centred LFQ intensities were used for ANOVA based multiple sample test followed by post-hoc test (FDR cut-off of 0.01 based on the Tukey's test) in order to discover differentially abundant proteins (DAPs) in WT35 versus WT27, cAS35 versus cAS27, cAS27 versus WT2 and cAS35 versus WT35. In each comparison proteins imputed 50% (imputation value = 2) in almost one analytical group or ≥75% (imputation value 3 or 4) in both were filtered out. On this dataset a two-way statistical analysis (*p*-value <.01) was performed. Hierarchical clustering analysis was carried out using the Perseus software and default parameters.

In order to use bioinformatic tools available only for *A. thaliana* a local BLAST of *Nicotiana tabacum* proteins using the *A. thaliana* blastset TAIR10 (version 2012-05-07), was performed. Functional classification was obtained with the MapMan 3.5.1R2 software using the

Arabidopsis thaliana ISOFORM_TAIR10_2012 protein database as background.

The enrichment analysis was achieved using the AgriGO Singular Enrichment Analysis (SEA) compare tool (http://bioinfo.cau.edu.cn/agriGO/analysis.php?method=compare), with *A. thaliana* TAIR10_2017 protein database as background, default parameters and a false discovery rate threshold of 0.01 (Du, Zhou, Ling, Zhang, & Su, 2010) and ShinyGO v0.61 (http://bioinformatics.sdstate.edu/go/) using a *p*-value cut-off of .05.

2.7 | Upstream motif enrichment analysis

For the enrichment analysis of TGGGC motif of the Proteasome-Related cis-Element (PRCE), the upstream regions (1.5 Kb) of selected genes were extracted from the *A. thaliana* orthologues proteins, using RSAT retrieve sequences tool (http://rsat.eead.csic.es/plants/retrieve-seq_form.cgi) and default parameters (Nguyen et al., 2018; van Helden, Andre, & Collado-Vides, 2000). In order to remove repetitive DNA elements, the FASTA sequences of the promoter regions obtained from RSAT retrieve sequences analysis were submitted to the RepeatMasker software (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker), selecting *A. thaliana* as DNA source (Tempel, 2012). Motifs were predicted using MEME tool (http://meme-suite.org/tools/meme; Bailey et al., 2009). MEME parameters were set as follow: number of searched motifs was set to 10, site distribution was set to any number of repetitions, background was set to 0 order model of sequences, minimum and maximum motifs widths were 5 and 12, respectively.

3 | RESULTS

3.1 | Cyclic AMP preserved cell viability under HS in a HSP_S-independent way

In order to elucidate the role of cAMP in plant HSR, WT and three cAS lines (A, B, C) of tobacco BY-2 cells, previously characterized for the presence of cAMP-sponge and reduced levels of free cAMP (Sabetta et al., 2016), were grown for 3 and 5 days at control temperature (27°C) or at 35°C, which represents a moderate long-term HS for BY-2 cells (Sgobba et al., 2015). Coherently with the data previously reported (Sabetta et al., 2016), after 5 days of culture at control temperature, cAS lines compared to WT showed an inhibition of cell growth. A substantial decline in growth occurred in both genotypes subjected to HS, although the fresh weight was significantly lower in the three cAS lines than in WT (Figure 1a). Although cAS lines showed a lower mitotic index (MI) than WT in control conditions, the exposure of BY-2 cells to HS for 3 days caused a clear and similar decrease of mitotic index (MI) in both the genotypes (Figure 1b). On the other hand, after 3 and 5 days of HS, cell death was significantly higher in cAS lines than in WT (Figure 1c). The pre-treatment of the three cAS lines with exogenous cAMP was able to rescue cell growth and to reduce cell death occurring under HS, which reached values comparable to WT (Figure 1d). As the three transgenic lines had such a similar response to HS, the A line, hereafter simply referred as cAS, was chosen for further characterization of HSR.

After verifying that HS did not alter the expression of cAMP-sponge in cAS cells (Figure S1), the levels of free cAMP were determined in both WT and cAS lines grown in control and HS conditions (Figure 2a). As expected, at 27°C the free cAMP content in cAS cells was significantly lower than in WT, confirming the ability of cAMP-sponge to sequester this second messenger (Sabetta et al., 2016). The growth for 3 days at 35°C caused an increase of cAMP levels in WT cells, which did not occur in cAS cells: indeed, at the same time-point cAMP levels in cAS cells subjected to HS remained low and comparable with the control. Probably due to cAMP-sponge saturation, after 5 days of HS, an increase in cAMP occurred in cAS cells, though reaching values comparable to that of WT cells grown at 27°C (Figure 2a). The higher cell death observed in cAS line subjected to HS seemed to be not due to a failure in HSPs expression (Figure 2b–d). In both WT and cAS cells, 3 and 5 days of HS induced a significant increase in the transcription of HSP101 (Figure 2b). Similarly, under HS the expression of the two low molecular weight HSPs, HSP18 and HSP26, increased in both WT and cAS lines (Figure 2c,d). The induction of HSP18 expression under HS was transient in WT cells while in cAS cells remained higher than control after 5 days of HS (Figure 2d).

3.2 | Involvement of cAMP in the control of redox homeostasis under HS

To verify whether cAMP deficiency could cause changes in cellular redox environment, the presence of ROS was checked in the two cell lines, under control and HS conditions. After 3 and 5 days of HS a significant rise in extracellular H_2O_2 occurred in both genotypes (Figure 3a). On the other hand, under HS the increase in intracellular ROS, measured by the DFC-DA probe, was significantly higher in cAS than in WT cells (Figure 3b). The activity of SOD, which is involved in the removal of superoxide anion, under control conditions was higher in cAS than in WT cells. HS caused a significant increase in SOD activity, which reached comparable values in the two cell lines (Figure 3c). Unlike SOD, under HS the activity of APX, the enzyme involved in the removal of hydrogen peroxide at the expense of ASC, significantly increased only in WT cells (Figure 3d). CAT activity, which was comparable between WT and cAS cells at 27°C, after HS only increased in WT cells (Figure 3e).

To verify whether the increase in intracellular ROS caused an oxidation in cAS cell environment, the content and redox state of ASC and GSH were determined. In control conditions, total ascorbate content was significantly higher in cAS than in WT cells. After 5 days of HS, a significant increase in ascorbate content was observed in WT cells. Conversely, in cAS cells total ascorbate content after HS was comparable to that found in cells grown in control conditions (Figure 4a). Furthermore, only in cAS cells, HS led to a significant decrease in the redox state (ASC/ASC + DHA) of this metabolite

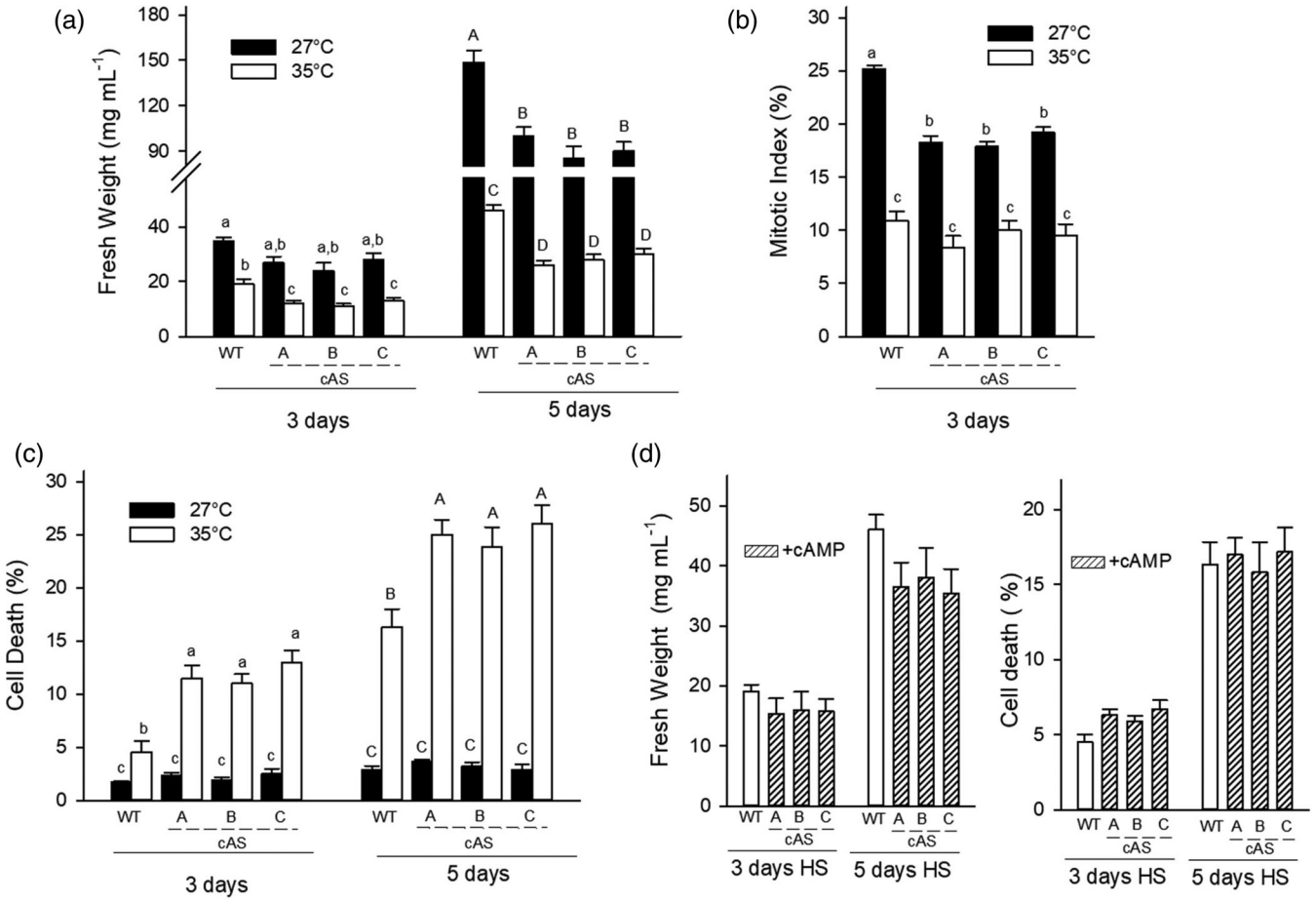


FIGURE 1 cAMP dampening negatively affects thermotolerance in tobacco BY-2 cells. (a) Fresh weight of WT and three cAS (A, B, C) lines grown for 3 and 5 days in control (27°C) and heat stress (HS, 35°C) conditions. (c) Mitotic index of WT and cAS lines grown for 3 days in control and HS conditions. (d) HS-dependent cell death in WT and cAS lines. (e) Fresh weight and cell death of cAS cells treated with exogenous cAMP (20 μM) before HS. The values are the means ± *SE*s of five independent experiments. Different letters indicate significant differences obtained by one-way ANOVA test ($p < .05$)

(Figure 4b), which was rescued by the pre-treatment with exogenous cAMP (Figure 4b). Although the activity of DHAR increased during HS in both cell lines, it remained lower in cAS cells than in WT ones (Figure 4c). Changes in GSH content and redox state were like those observed for ASC. Indeed, after 5 days of culture at 27°C total glutathione content was significantly higher in cAS than in WT cells (Figure 4d), whereas 5 days of HS led to an increase in glutathione only in WT cells (Figure 4d). On the other hand, GSH oxidation under HS occurred only in cAS cells, as shown by the decrease in the redox state of this metabolite. The addition of exogenous cAMP before HS was able to rescue glutathione redox state in cAS cells (Figure 4e). After 3 and 5 days of HS, GR activity increased in WT cells and did not change in cAS ones (Figure 4f).

3.3 | Specific genotype- and temperature-dependent changes in proteomic profiles

The proteomic analysis of WT and cAS lines, grown for 3 days at 27 or 35°C, followed by one-way ANOVA comparison test (FDR < 0.01), permitted to identify 1,201 DAPs in the four analytical groups (Dataset S1). After a post-hoc Tukey's test 497 DAPs in WT35 versus WT27 comparison, 435 in cAS27 versus WT 27 comparison, 537 DAPs in cAS35 versus WT35 comparison and 492 in cAS35 versus cAS27 one, were identified (Dataset S1). PCA showed that biological replicates were plotted very closely in the PCA space, indicating a good correlation between the replicates, due to the high homogeneity of BY-2 cells. The heat-stressed samples (WT35 vs. WT27 and cAS35 vs. cAS27) were separated in PC1 and this accounted for 38.1% of the total variation; cAS and WT samples were separated in PC2, which accounted for 34.7% of the total variation, indicating that both HS and the presence of transgene (cAMP-sponge) affected more than 30% of tobacco cells proteome (Figure S2).

In order to discriminate among the effects on proteomic changes, due to genotype or temperature or the interaction between these two factors, DAPs were subjected to two-way ANOVA analysis that permitted to obtain three separate protein lists: genotype-dependent, temperature-dependent and genotype-temperature interaction-dependent (Dataset S2).

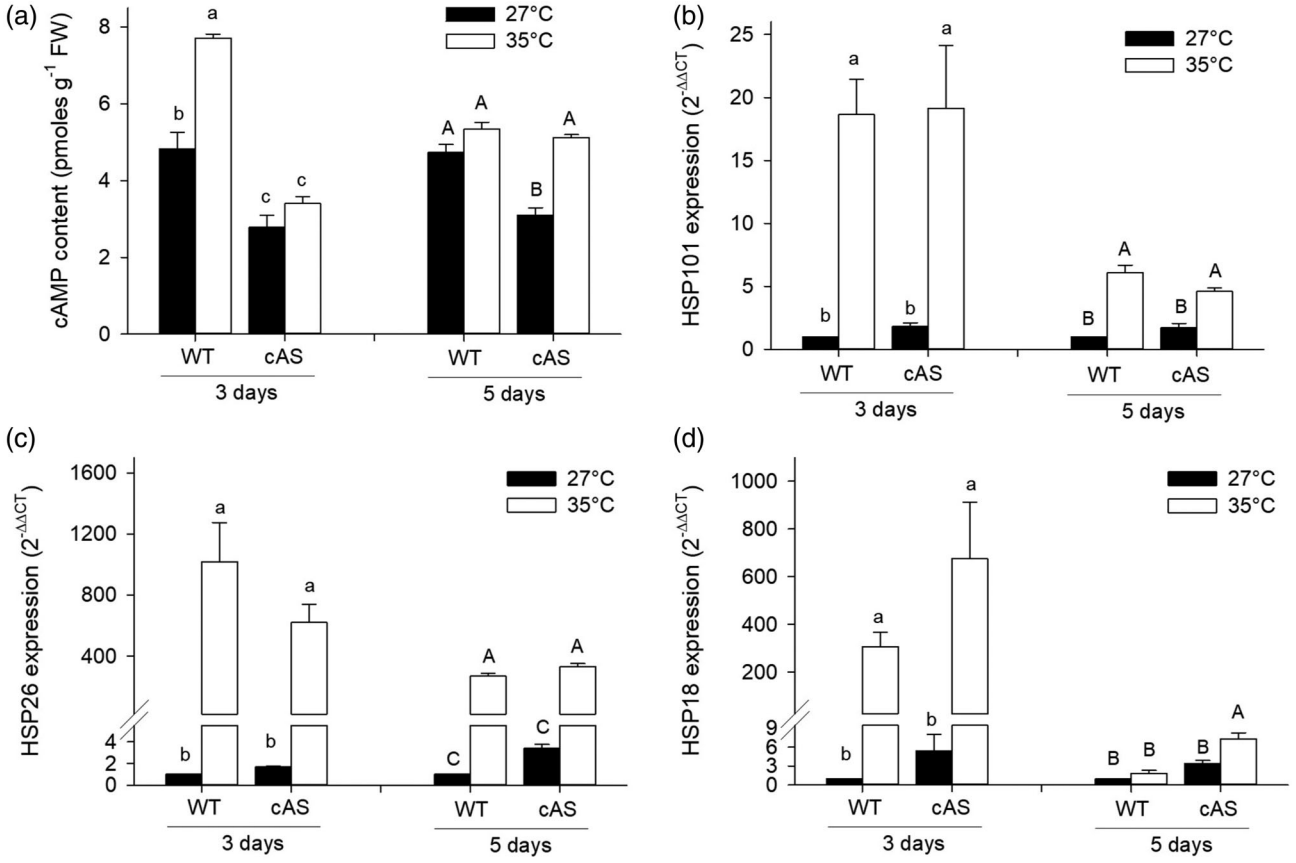


FIGURE 2 cAMP accumulation under heat stress is not needed for the induction of heat shock proteins (HSPs). (a) Free cAMP content of WT and cAS cells grown for 3 and 5 days in control (27°C) and heat stress (HS, 35°C) conditions. Relative expression of (b) HSP101, (c) HSP26 and (d) HSP18 of WT and cAS cells grown for 3 and 5 days in control (27°C) and HS (35°C) conditions. The expression level of HSPs was normalized to that of Actin. At each time point, gene expression was relativized to the WT (27°C). The values are the means ± *SE*s from three independent experiments, with three technical replicates for each experiment. Different letters indicate significant differences obtained by one-way ANOVA test ($p < .05$)

Singularly, both genotype and temperature affected proteome with 412 and 441 DAPs, respectively (Dataset S2). These proteins were analysed and graphically represented by MapMan cellular functions overview. Both genotype and temperature negatively affected 'protein synthesis' and 'RNA processing' (Figure 5a). 'Stress', 'redox', 'signalling' and 'protein degradation', which were among the more enriched categories in both genotype- and temperature-dependent lists, were represented by heat maps (Figure 5b). In the 'stress' category, among the genotype-dependent DAPs, RBOHD (A0A1S3YBQ2) was found to be up-accumulated in cAS cells in comparison with WT. On the other hand, 23 HSPs were positively regulated by temperature in both genotypes. Most proteins of the 'redox' category, among which two APX1 (A0A1S3YK10, Q40589), were accumulated in response to HS in both WT and cAS cells. On the other hand, among the genotype-dependent proteins of the 'redox' category, both up- and down-regulated proteins were found in cAS cells. In the list of redox proteins negatively influenced by the low cAMP levels, the chloroplastic APX6 (A0A1S3ZZS2) was found. In addition, in the same list, the GR2 (P80461) and the DHAR2 (A0A1S4CJW2), which conversely were positively regulated by HS, were also found. The low level of cAMP also negatively influenced proteins of 'signalling' category and in particular those belonging to 14-3-3 and G-proteins signalling. In the signalling category also three kinases were down-regulated in cAS cells, among which the phosphatidylinositol 4-kinase (PI4K, A0A1S4CNE1), which, conversely, was up-regulated in a temperature-dependent manner. A temperature-dependent accumulation of 16 proteins belonging to 'protein-degradation' category was observed in both the genotypes. Conversely, a down-regulation of 10 genotype-dependent proteins of 'protein-degradation' category occurred in cAS cells.

3.4 | Proteomic changes dependent on genotype–temperature interaction

Genotype–temperature interaction affected 503 proteins (Dataset S2). The positively regulated proteins of WT and cAS cells under HS, belonging to this list of genotype–temperature interaction, were analysed with AgriGO SEA COMPARE (Figure S3). Although response to stress was enriched in both genotypes, 'response to abiotic

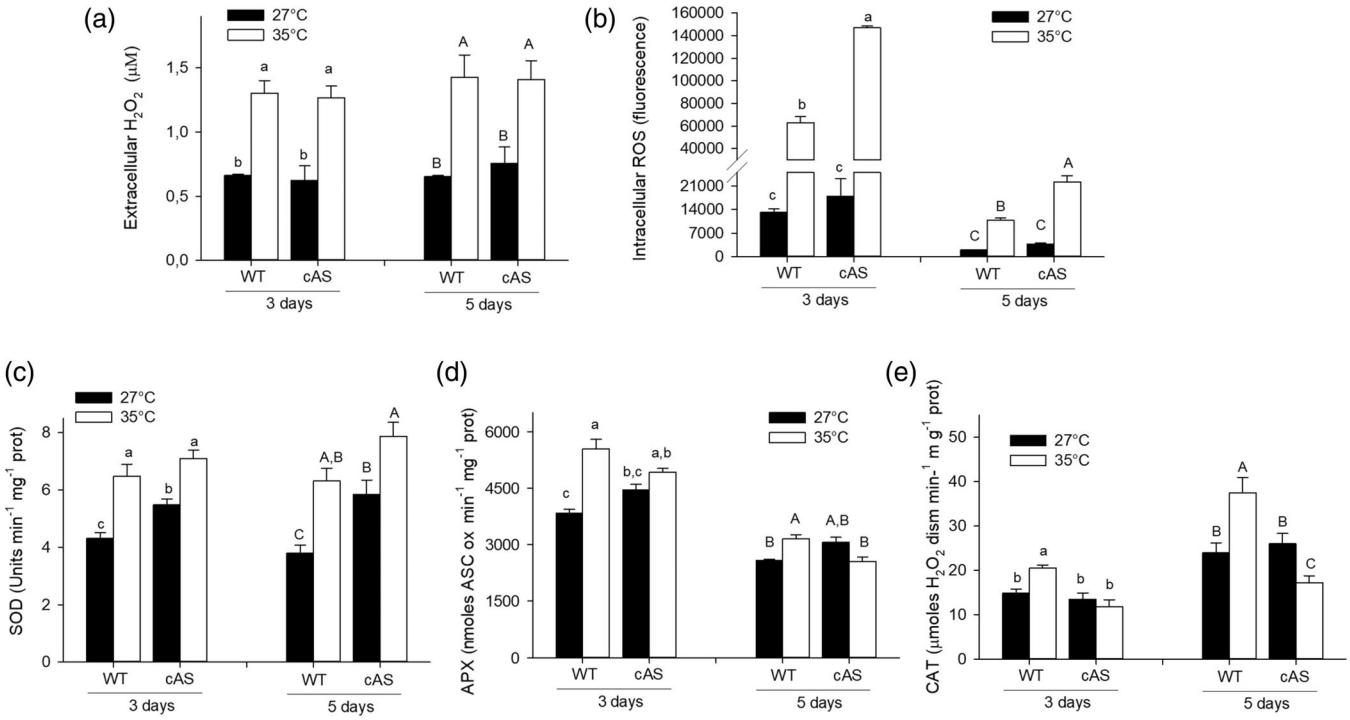


FIGURE 3 Accumulation of reactive oxygen species (ROS) and changes in the activities of ROS-removal enzymes in WT and cAS cells exposed to heat stress (HS). (a) Extracellular H_2O_2 content, (b) intracellular ROS levels and activities of (c) superoxide dismutase (SOD), (d) ascorbate peroxidase (APX) and (e) catalase (CAT) in WT and cAS cells grown for 3 and 5 days in control (27°C) and HS (35°C) conditions. The values are the means ± *SE*s of five independent experiments. Different letters indicate significant differences obtained by one-way ANOVA test ($p < .05$)

stimulus', as well as 'response to heat' appeared more enriched in cAS cells. Moreover, an enrichment of proteins of 'response to chemical stimulus' and 'cellular localization' was observed exclusively among the up-regulated proteins in cAS line. On the other hand, categories of 'proteolysis' and 'ubiquitin-dependent protein catabolic process' were enriched only in WT cells.

To have more information on specific protein changes in the two genotypes in response to HS, DAPs of temperature–genotype interaction list were clustered (Figure 6a; Dataset S3). The 89 proteins accumulated in response to HS exclusively in cAS cells (Cluster 1), provided information about adaptive responses elicited by cells with cAMP deficiency (Figure 6b; Dataset S3). ShinyGO allowed to identify 'response to chemical', 'response to abiotic stimulus', 'response to heat' and 'protein folding' as the more enriched protein categories of the adaptative response of cAS cells to HS. In particular, among these proteins, four small HSPs (A0A1S4BGR4, A0A1S4BYT1, A0A1S3X239, A0A1S4A8D1), one HSP90 (A0A1S3XHJ8), the chloroplast-targeted Hsp101 homologue ClpB3 (A0A1S4CAS7), the Hsp70-Hsp90 organizing protein 3-like (A0A1S4CII4) and two proteins of DNAJ heat shock family (A0A1S3XKW9, A0A1S3ZS67) were found.

Cluster 2 and Cluster 3, which were, respectively, composed by proteins up-regulated in WT cells (43 proteins) and down-regulated in cAS cells (33 proteins) after HS, were together indicated as 'cAMP-dependent HS-responsive proteins' (Figure 6c; Dataset S3). The more enriched categories of the cAMP-dependent HS-responsive proteins were 'cofactor and coenzyme metabolic processes' and 'proteasomal protein catabolic process'. In this list of proteins, it is worth mentioning an isoform of catalase (L0SQ20), the nudix hydrolase homolog 2 (A0A1S4CY46) and the glucose-6-phosphate dehydrogenase 6 (O65855), which are involved in the control of redox homeostasis. Moreover, the non-specific phospholipase C1 (A0A1S3YVC3), the BAG (Bcl-2–associated athanogene) family molecular chaperone regulator 7-like (A0A1S4DEU7) and the Hsp70 nucleotide exchange factor Fes1 (A0A1S4DEU7), which are involved in thermotolerance acquisition, also belong to the list of cAMP-dependent HS-responsive proteins.

3.5 | Involvement of cAMP in the control of protein degradation under HS

To verify whether cAMP deficiency could be responsible for a failure in protein degradation in response to high temperatures, activities of total proteases and 26S proteasome were determined in WT and cAS cells grown at 27 and 35°C. Total protease activity under control conditions did not show significant differences between WT and cAS cells, both at 3 and 5 days. On the other hand, HS caused an increase of protease activity only in WT cells (Figure 7a). In cAS cells, proteasome activity was impaired already under control conditions,

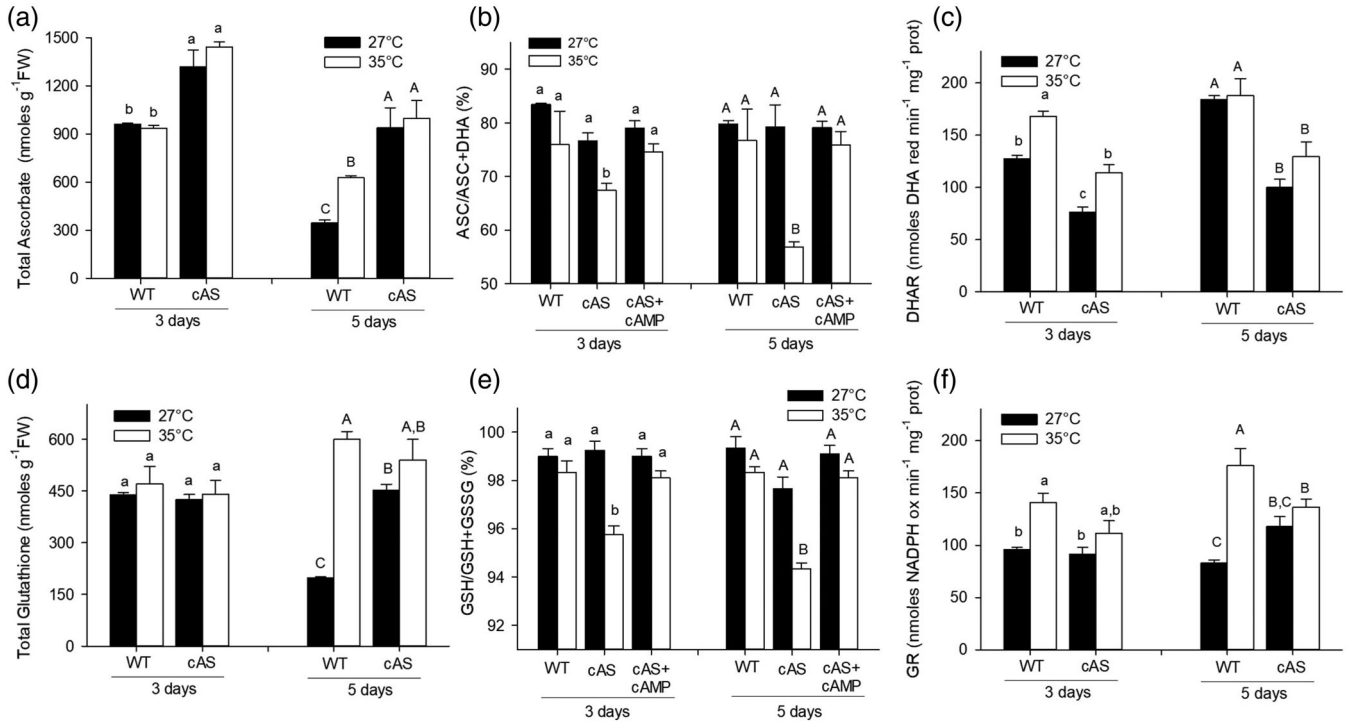


FIGURE 4 cAMP deficiency causes oxidation of ascorbate (ASC) and glutathione (GSH) during heat stress (HS). (a) Total content (ASC + dehydroascorbate-DHA) and (b) redox state (ASC/ASC + DHA) of ascorbate and (c) activity of DHA reductase (DHAR) in WT and cAS cells grown for 3 and 5 days at 27 and 35°C. (d) Total content (GSH + glutathione disulphide -GSSG) and (e) redox state (GSH/GSH + GSSG) of glutathione and (f) activity of glutathione reductase (GR) in WT and cAS cells grown for 3 and 5 days at 27 and 35°C. The values are the means ± *SE*s of five independent experiments. Different letters indicate significant differences obtained by one-way ANOVA test ($p < .05$)

being significantly lower than that observed in WT cells. Moreover, HS caused an increase of proteasome activity only in WT cells (Figure 7b). A heat map and graphical representation by MapMan proteasome overview of the 15 proteins belonging to UPS, found in the genotype–temperature interaction list, were reported in Figure 7c. Proteins belonging to 20S core particle and to non-ATPase regulatory particles (RPN), as well as proteins of ubiquitin family failed to be accumulated in cAS cells under HS. Using the motif-based sequence analysis tools MEME we found that 9 of the 15 genes encoding these proteins were enriched in their promoters with the TGGGC core sequence of the Proteasome-Related cis-Element (PRCE; Figure 7c), which is recognized by NAC78 and NAC 53 transcription factors (Gladman, Marshall, Lee, & Vierstra, 2016; Nguyen et al., 2013).

Interestingly, NAC78 has been found to be up-regulated in both shoots and roots of Arabidopsis plants exposed for 3 hr to HS at 38°C (Figure S4; Kilian et al., 2007; Winter et al., 2007), indicating a possible involvement of this transcription factor in the HSR. Thus, the TGGGC sequence was searched in the promoters of genes coding for 'cAMP-dependent HS-responsive proteins' (Cluster 2 and Cluster 3). MEME analysis showed that 32 of the 76 genes encoding these proteins contained one or more TGGGC sequence (Table S2). Among these proteins, those belonging to UPS, as well as the mitochondrial GrpE protein homolog 2 (A0A1S3X0Q4), the HOP2 protein (A0A1S4D331) and the serine/threonine-protein kinase STY46 (A0A1S3Z8L7) were found. These proteins could be part of a specific cAMP-dependent HSR network activated by NAC78.

4 | DISCUSSION

Many pieces of evidence, collected in the last decades, highlight that cAMP is involved in the signalling pathways of environmental stress response (Blanco et al., 2020). An increase in cAMP occurs in plant response to pathogens or elicitors, as well as in response to wounding and heat (Gao et al., 2012; Jiang, Fan, & Wu, 2005; Ma et al., 2009; Sabetta et al., 2019; Swieiawska et al., 2014; Zhao, Guo, Fujita, & Sakai, 2004). Although a complete knowledge of cAMP signal transduction in stress response is still lacking, it has been proposed that this molecule might act mainly through the regulation of CNGCs, leading to the alteration of ion fluxes (Gehring & Turek, 2017; Jha, Sharma, & Pandey, 2016). In Arabidopsis, a mild heat shock caused an increase of intracellular cAMP levels, which in turn, stimulating CNGC6, triggered a cytosolic Ca^{2+} influx; consistently, mutations in CNGC6 impaired thermotolerance (Gao et al., 2012).

Here we report that an increase in free cAMP, occurring in response to long-term HS, is also an important component of HSR in tobacco BY-2 cells. The failure in the HS-dependent cAMP elevation makes these cells less tolerant to high temperature. Although both cAMP dampening and HS decreased mitotic index (Centomani

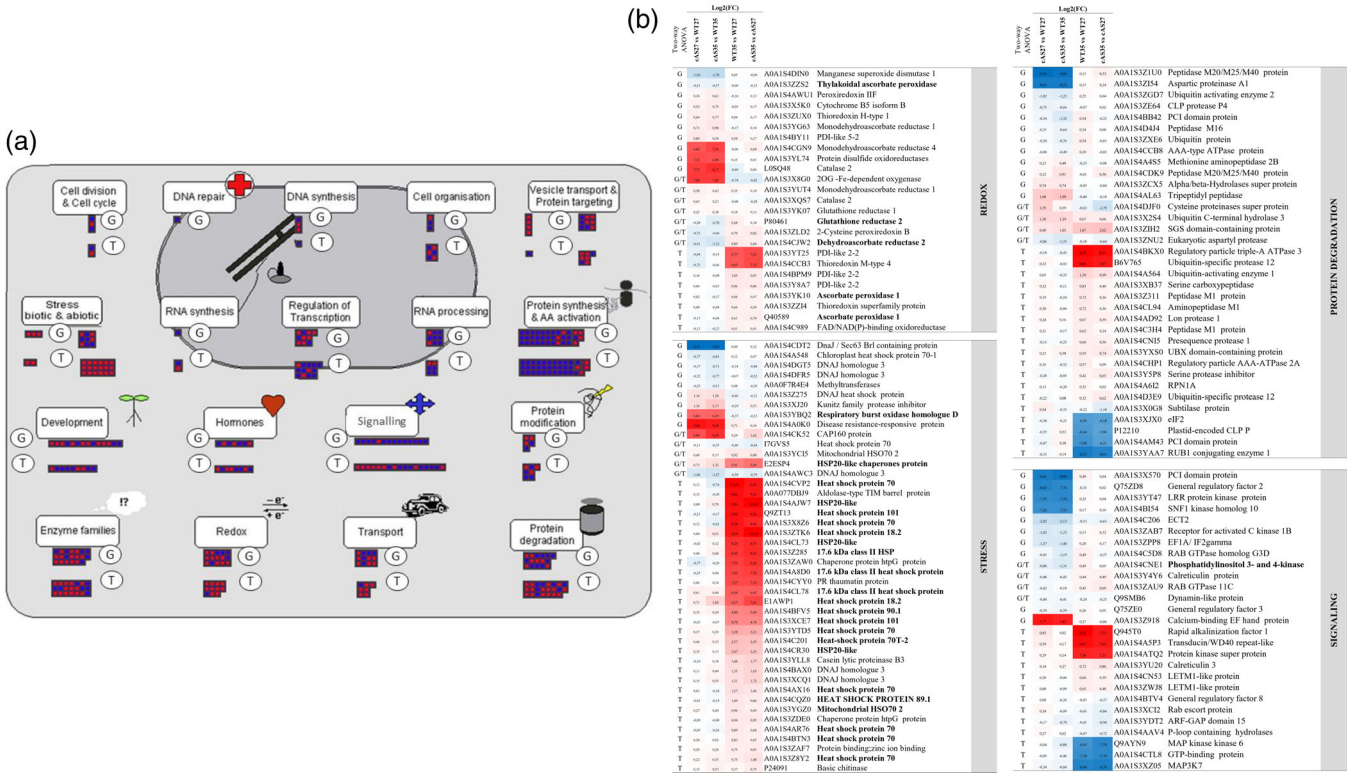


FIGURE 5 Genotype- and temperature-dependent changes in the proteome of tobacco BY-2 cells. (a) Representation of genotype- (G) or temperature-(T) dependent proteins assigned to different categories by MapMan general cellular responses function. In the figure, each square represents a protein. Blue and red boxes show down- and up-regulated proteins, respectively. (b) Heat maps of differently accumulated proteins belonging to 'redox', 'stress', 'protein degradation' and 'signalling' categories in cAS27 versus WT27, cAS 35 versus WT35, WT35 versus WT27 and cAS35 versus cAS27 comparisons [Colour figure can be viewed at wileyonlinelibrary.com]

et al., 2015; Sabetta et al., 2016), the lower cell growth observed in cAS cells after HS is not due to a further slow-down of cell division, but to a rise in cell death. Interestingly, the treatment of cAS cells with exogenous cAMP permitted the recovery of cell viability, which is consistent with the increased thermotolerance of Arabidopsis plants treated with a cAMP analogue (Gao et al., 2012).

Exposure to high temperatures induces ROS increase, which is required for the activation of HSF-dependent pathways and for the expression of defence genes (de Pinto et al., 2015; Saidi, Finka, & Goloubinoff, 2011; Suzuki et al., 2011). Our data confirm that HS increases the extracellular and intracellular content of ROS in tobacco BY-2 cells (Konigshofer et al., 2008; Sgobba et al., 2015). However, cAMP deficiency resulted in a greater accumulation of intracellular ROS, which is consistent with the genotype-dependent accumulation of RBOHD in cAS cells. The positive regulation of NADPH oxidase in cAMP-deficient cells resembles what is known to occur in animal systems. Decreased levels of cAMP in vascular smooth muscle cells of spontaneously hypertensive rats (SHR) are responsible of enhanced oxidative stress due to the higher expression of Nox1/Nox2/Nox4 and p47phox proteins and enhancement of NADPH oxidase activity. The treatment with cAMP-elevating agents of SHR lowered NADPH oxidase levels and reduced oxidative stress (Gusan & Anand-Srivastava, 2013; Saha, Li, & Anand-Srivastava, 2008).

Thermotolerance in plants is generally associated with an increase in antioxidant enzymes (Chou, Chao, & Kao, 2012; de Pinto et al., 2015; Mittal, Madhyastha, & Grover, 2012; Sgobba et al., 2015). HS enhanced the activity of the ROS-removal enzymes SOD, CAT and APX in tobacco BY-2 cells, thus indicating the ability of these cells to eliminate ROS excess, thereby reducing their oxidative potential (Sgobba et al., 2015). In cAMP-deficient cells, the failure in the enhancement of APX and CAT activities after HS, corroborated by the genotype-dependent down-regulation of the chloroplastic APX6 (A0A1S3ZZS2) and the failed up-regulation of the cAMP-dependent HS-responsive isoform of CAT2 (L0SQ20), may be co-responsible for the high accumulation of intracellular ROS. The high ROS accumulation in cAS cells subjected to HS, leads to an alteration in cellular redox environment, as shown by the oxidation of ASC and GSH, which can be rescued by the addition of exogenous cAMP. Interestingly, glutathione oxidation has been indicated as a suitable sensor of increased H_2O_2 (Tuzet, Rahantaniaina, & Noctor, 2019). In cAMP-deficient cells, the lower activity of the recycling enzymes DHAR and GR, probably due to the genotype-dependent down-accumulation of GR2 (P80461) and DHAR2 (A0A1S4CJW2), can contribute to the higher oxidation of the cellular environment. Moreover, nudix hydrolase homolog2, which encodes an ADP-ribose pyrophosphatase involved in the maintenance of NAD levels (Ogawa et al., 2009), and

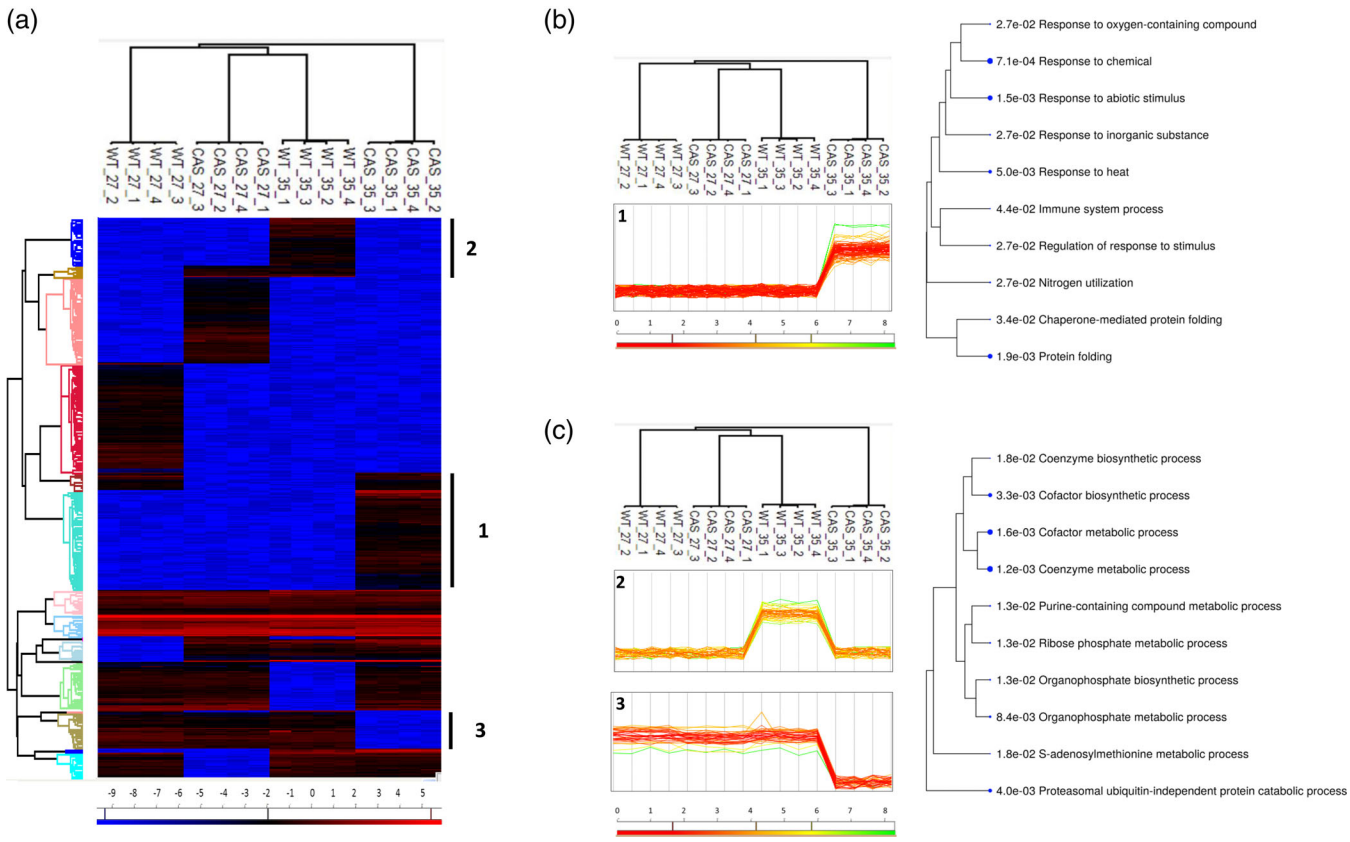
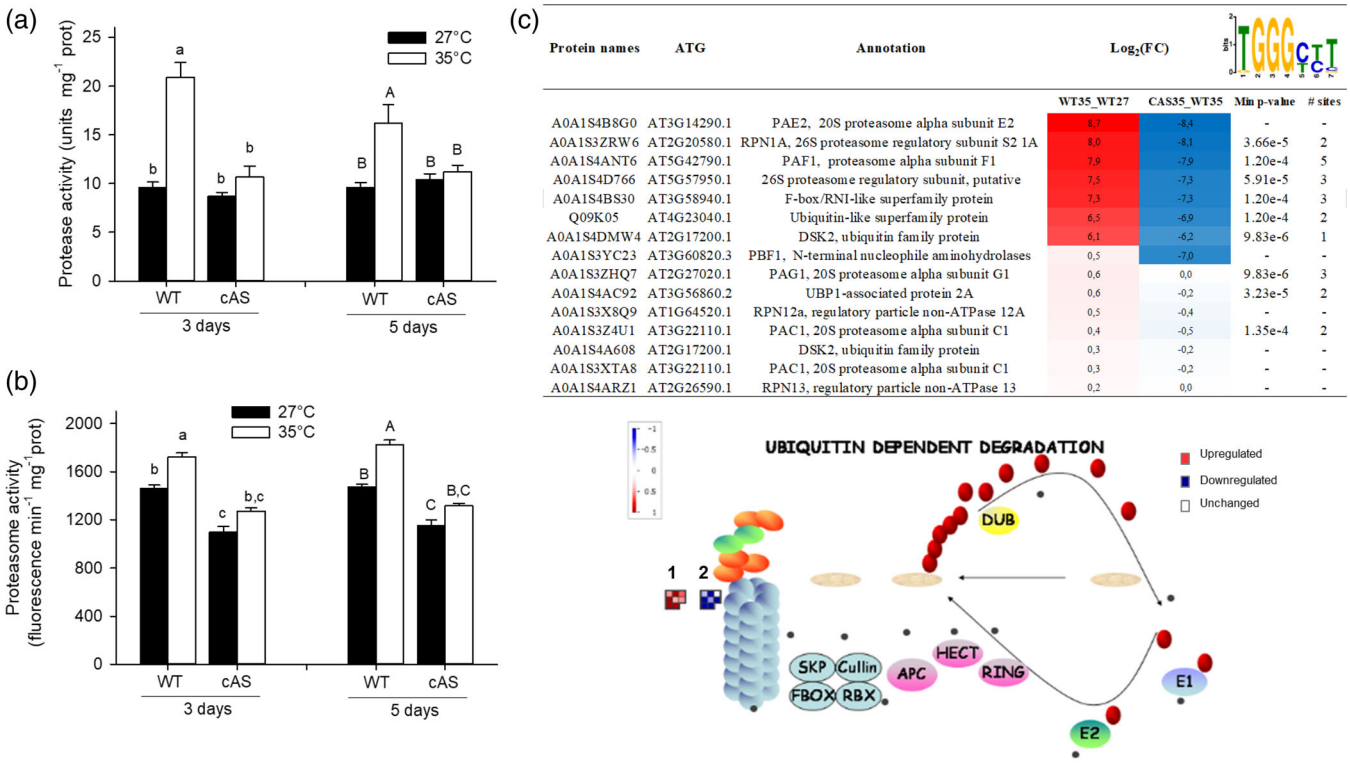


FIGURE 6 Proteomic changes dependent on genotype–temperature interaction in tobacco BY-2 cells. (a) Hierarchical clustering of the 503 differently accumulated proteins dependent on genotype–temperature interaction. Expression profile and ShinyGO enrichment analysis of (b) Cluster 1, composed by 89 proteins accumulated in response to HS only in cAS cells and (c) Clusters 2 and 3, composed by proteins up-regulated only in WT cells (43 proteins) and down-regulated only in cAS cells (33 proteins), respectively [Colour figure can be viewed at wileyonlinelibrary.com]

the cytosolic glucose-6-phosphate dehydrogenase 6, which provides NADPH for the antioxidant systems (Esposito, 2016), were also found among the cAMP-dependent HS-responsive proteins that are lacking in cAS cells. Remarkably, NADPH availability, impacting on monodehydroascorbate reduction, might regulate glutathione redox state (Tuzet et al., 2019). Thus, the higher oxidation in the cellular environment of cAMP-deficient cells could be in part responsible for the higher cell death observed in response to HS.

High temperatures per se can trigger protein denaturation (Pinto, Morange, & Bensaude, 1991) and the refolding of aggregated proteins, mediated by HSPs, is one of the key plant mechanisms for thermotolerance achievement (Baniwal et al., 2004; Hasanuzzaman et al., 2013). However, HS-dependent cAMP elevation is not needed for the induction of HSPs in tobacco BY-2 cells. Indeed, the expression of HSP18, HSP26 and HSP101 were similar in cAS and WT cells. In addition, the accumulation of the majority of HSPs occurred in a temperature-dependent way in both the genotypes. These data are apparently in conflict with the work of Gao et al. (2012), reporting that cAMP, by the activation of CNGC6 and the promotion of Ca^{2+} influx, is responsible of HSPs expression. However, it should be noted that the increase in cytosolic Ca^{2+} may also be due to depletion of intracellular reserves (Gong, van der Luit, Knight, & Trewavas, 1998; Liu et al., 2006; Saidi et al., 2009; Zheng et al., 2012). Consistently, Arabidopsis *cngc6* mutants showed a higher HSPs expression than WT plants, suggesting that, a stimulation of Ca^{2+} release from intracellular reserves can compensate for the loss of CNGC6 (Gao et al., 2012).

The proteomic analysis showed that among the proteins depending on genotype–temperature interactions, the category 'response to heat' was more enriched in cAS cells. However, despite the higher accumulation of some HSPs and folding proteins, cAS cells were more sensitive to HS. Notably, cAMP-deficient cells failed to accumulate many proteins of UPS, suggesting that the avoidance of proteotoxic stress could be a critical step of HSR. The selective protein degradation mediated by UPS has been proposed as an important plant strategy to deal with environmental stress (Aristizabal, Rivas, Cassab, & Lledias, 2019; Smalle & Vierstra, 2004; Stone, 2014; Xu & Xue, 2019; Zhou, Chang, & Qiu, 2010). Plant survival to proteotoxic stress, due to protein aggregation at high temperatures, requires that disaggregation and refolding of proteins must be coordinated with proteolysis (McLoughlin, Kim, Marshall, Vierstra, & Vierling, 2019). Consistently, deficiency in the expression of proteasome subunits, as well as in E3 ligases increases plant susceptibility to HS (Li et al., 2015; Liu et al., 2016; Wang, Kurepa, & Smalle, 2009),



Protein names	ATG	Annotation	Log2(FC) WT35_WT27	Log2(FC) CAS35_WT35	Min p-value	# sites
A0A1S4B8G0	AT3G14290.1	PAE2, 20S proteasome alpha subunit E2	8,7	-8,4	-	-
A0A1S3ZRW6	AT2G20580.1	RPN1A, 26S proteasome regulatory subunit S2 1A	8,0	-8,1	3.66e-5	2
A0A1S4ANT6	AT5G42790.1	PAF1, proteasome alpha subunit F1	7,9	-7,9	1.20e-4	5
A0A1S4D766	AT5G57950.1	26S proteasome regulatory subunit, putative	7,5	-7,3	5.91e-5	3
A0A1S4BS30	AT3G58940.1	F-box/RNI-like superfamily protein	7,3	-7,3	1.20e-4	3
Q09K05	AT4G23040.1	Ubiquitin-like superfamily protein	6,5	-6,9	1.20e-4	2
A0A1S4DMW4	AT2G17200.1	DSK2, ubiquitin family protein	6,1	-6,2	9.83e-6	1
A0A1S3YC23	AT3G60820.3	PBF1, N-terminal nucleophile aminohydrolases	0,5	-7,0	-	-
A0A1S3ZHQ7	AT2G27020.1	PAG1, 20S proteasome alpha subunit G1	0,6	0,0	9.83e-6	3
A0A1S4AC92	AT3G56860.2	UBP1-associated protein 2A	0,6	-0,2	3.23e-5	2
A0A1S3X8Q9	AT1G64520.1	RPN12a, regulatory particle non-ATPase 12A	0,5	-0,4	-	-
A0A1S3Z4U1	AT3G22110.1	PAC1, 20S proteasome alpha subunit C1	0,4	-0,5	1.35e-4	2
A0A1S4A608	AT2G17200.1	DSK2, ubiquitin family protein	0,3	-0,2	-	-
A0A1S3XTA8	AT3G22110.1	PAC1, 20S proteasome alpha subunit C1	0,3	-0,2	-	-
A0A1S4ARZ1	AT2G26590.1	RPN13, regulatory particle non-ATPase 13	0,2	0,0	-	-

FIGURE 7 cAMP involvement in the control of protein degradation during heat stress (HS). Activities of (a) proteases and (b) proteasome in WT and cAS cells grown for 3 and 5 days in control (27°C) and HS (35°C) conditions. The values are the means ± *SE*s of four independent experiments. Different letters indicate significant differences obtained by one-way ANOVA test ($p < .05$). (c) Heat map and MapMan representation of proteins related to the ubiquitin-proteasome system. In the figure, each square represents a protein, with the red ones showing the positively accumulated proteins and the blue ones the down-accumulated; (1) and (2) are referred to WT35 versus WT27 and cAS35 versus WT35 comparisons, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

whereas the overexpression of ubiquitin or ubiquitin E3 ligases enhances thermotolerance (Lim et al., 2013; Liu et al., 2014, 2016; Tian et al., 2014).

The accumulation of some HSPs only in cAS cells exposed to HS might reflect an attempt of these cells to increase the protein folding capacity, in order to cope with the absence of protein degradation. The higher HSPs accumulation can also be due to proteasome inhibition that extends the half-life of HSPs, through the decrease of their degradation (Imai, Yashiroda, Maruya, Yahara, & Tanaka, 2003; Stangl et al., 2002). Consistently, in Arabidopsis mutants of RPN1a (26S regulatory particle non-ATPase 1a), sHSP-interacting proteins are more abundant than in wild type, implying that these proteins are substrates of proteasomes (McLoughlin et al., 2019).

However, cAMP-deficient cells failed to accumulate some HS-responsive proteins, involved in the acquisition of thermotolerance, such as non-specific phospholipase C1 (Krckova et al., 2015), BAG7 (Williams, Kabbage, Britt, & Dickman, 2010) and the Hsp70 nucleotide exchange factor Fes1 (Gowda, Kandasamy, Froehlich, Dohmen, & Andreasson, 2013; Zhang et al., 2010). BAG7 is a cochaperone localized in the endoplasmic reticulum that acts as an essential factor for the correct preservation of the unfolded protein response; in Arabidopsis BAG7 knockouts are sensitive to HS (Williams et al., 2010). Fes1 has been reported to be induced by high temperatures and Arabidopsis Fes1 mutants show a heat-sensitive phenotype (Zhang et al., 2010). Interestingly, Fes1 is involved in the promotion of ubiquitin-dependent proteasomal degradation of misfolded proteins (Gowda et al., 2013).

The deficiency in UPS components in cAS cells was accompanied by a decrease in proteasome activity. Remarkably, in rat cerebral cortical neurons TAU pathology, triggered by the accumulation of ubiquitinated proteins, was mitigated by cAMP analogues that stimulate proteasome activity (Metcalfe, Huang, & Figueiredo-Pereira,- 2012). Likewise, cAMP rise in rat spinal cord neurons enhanced 26S proteasome activity, elevating the expression of different UPS components, including the proteasome subunits Rpt5 and Rpt6, the E3 ligase CHIP and the ubiquitin gene ubB (Myeku, Wang, & Figueiredo-Pereira, 2012). Thus, it is possible that similarly to what happens in animals, cAMP regulates the expression of some UPS components in BY-2 cells.

Interestingly, nine genes coding for the cAMP-dependent proteins of the UPS and accumulated in response to HS, show in the promoters at least one TGGGC sequence of the PRCE. The TGGGC sequence has been identified in many genes of the transcriptional network activated by proteotoxic stress, comprising genes encoding components of UPS, as well as chaperone proteins and proteins involved in cellular detoxification (Gladman et al., 2016). This sequence is

recognized by NAC78 and NAC53, two transcriptional factors involved in the activation of proteasome stress regulon (Gladman et al., 2016; Nguyen et al., 2013; Yabuta et al., 2011).

NAC78 has been reported to increase in both shoots and roots of Arabidopsis plants in response to a moderate HS (Kilian et al., 2007; Winter et al., 2007). Thus, the 32 genes encoding 'cAMP-dependent HS-responsive proteins', having the TGGGC sequence in their promoters, can represent a specific HSR network activated by this transcription factor. Among these proteins, those belonging to 20S core protease and 19S regulatory particle of the 26S proteasome, as well as proteins involved in protein ubiquitylation have been identified. The mitochondrial GrpE protein homolog 2, which has been reported to be essential for conferring thermotolerance to long-term exposure at moderately high temperature (Hu, Lin, Chi, & Charng, 2012; Mayer & Bukau, 2005), belongs to this HSR network. The HOP2 protein, also belonging to this cAMP-dependent HSR network, plays a significant role in protein quality control, affecting plant capability to acclimate to high temperatures for long periods (Fernandez-Bautista et al., 2018). Finally, the serine/threonine-protein kinase STY46, which is involved in the regulatory network of chloroplast protein import and has been reported to be induced by HS also in maize and spinach leaves (Hu et al., 2015; Zhao et al., 2018), has been identified among the 'cAMP-dependent HS-responsive proteins'.

Recently, phosphorylation of NAC78, by the PI4Kγ5 has been proposed as a crucial event for the cleavage and normal function of this transcription factor (Tang, Zhao, Tan, & Xue, 2016) and interestingly, a PI4K has been identified among the genotype-dependent down-accumulated proteins in cAS cells. Hence, our data suggest that cAMP could be involved in the induction of a specific HSR network, which comprises components of UPS, through the activation of NAC78 transcription factor.

However, it is possible that in addition to changes in protein abundance, cAMP signalling in HSR could be transduced by phosphorylation. In support of this hypothesis, many literature data suggest that in animals, 26S proteasome activity is regulated by cAMP-mediated phosphorylation (Lokireddy, Kukushkin, & Goldberg, 2015; Myeku et al., 2016; Myeku & Duff, 2018; Zhang et al., 2007).

In conclusion, our data offer a new view of the multi-faceted HSR in plants, identifying at least in tobacco BY-2 cells, cAMP as a key signal for the control of redox homeostasis and UPS functioning.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ORCID

Milena Marsoni https://orcid.org/0000-0003-0613-1581
Maria Concetta de Pinto https://orcid.org/0000-0002-2937-1935

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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