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Dual coordination of the SUMOylation and phosphorylation pathways during the response to heat stress in *Solanum tuberosum*

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ABSTRACT

Most plant species are adapted to a relatively narrow range of ambient temperatures. Temperatures that are above the preferred range elicit a variety of responses at tissue, cellular and sub-cellular level. One of the earliest responses is post-translational modification by SUMOylation and phosphorylation; modulating heat stress (HS)-induced gene expression, metabolism adaptation and metabolite synthesis to improve plant tolerance to the heat stress.

In this study we used a combination of three-dimensional gel electrophoresis fractionation, Western-blot analysis, difference in gel electrophoresis (DIGE) technology and mass spectrometry to identify those proteins that first showed changes in SUMOylation and phosphorylation in potato leaves exposed to sublethal HS. We found that these early response proteins are mostly involved in photosynthesis, or defence mechanisms, or energy metabolism. The relationship between the fractional change in SUMOylated proteoforms and in serine-phosphorylated proteoforms was not constant, but varied between proteins. The physiological significance of these differences needs further investigation.

1. Introduction

High temperature is one of the most important factors affecting crop yield and quality, especially in the context of climate change. Even short-term and moderate increases in temperature above the optimum growth temperature (heat stress, HS) cause adverse alterations in plant growth. It is now known that there are a number of adaptive mechanisms which act to minimize these deleterious effects of above-optimal temperatures (Kotak et al., 2007). One of the earliest responses to be triggered by increase in temperature is an increase in the abundance of the so-called heat shock proteins (HSP). HSPs act as molecular chaperones, helping particular proteins to maintain their native state. Other early events in plant responses to HS include (Hemme et al., 2014) the arrest of the cell cycle, the production of small molecules known to be active in stress protection, and the reprioritization of photosynthetic activity toward the de novo synthesis of saturated fatty acids (which help increasing membrane fluidity). Adaptive responses to HS also involve various post-translational modifications (PTM) of protein molecules. There is now a substantial and detailed literature on the effects of HS on PTMs such as protein phosphorylation (Gallie et al., 1997; Rokka et al., 2000; Chen et al., 2011; Evrard et al., 2013). HS has also been shown to enhance the SUMOylation of particular proteins (Miller and Vierstra, 2011); the reversible covalent attachment to a given protein of a «Small Ubiquitin-like Modifier protein» (SUMO). SUMOs are 10–12 kDa molecules. They are encoded by a small family of genes whose members vary in number from 1 (*Sorghum bicolor*) to 8 (*Arabidopsis thaliana*); the pathway itself is considered to be evolutionarily conserved.

The process is an ATP-dependent enzymatic cascade involving the SUMO Activating Enzyme E1 (SAE, composed of 2 subunits, SAE1 and SAE2) and the SUMO Conjugating Enzyme E2 (SCE) to attach the SUMO molecule to a specific Lys residue of its target. This Lys is, in most cases, located in a specific consensus sequence ΨKXD/E (Ψ, hydrophobic amino acid; K, lysine; X, any amino acid; D, aspartic acid; E, glutamic acid). However, the SUMOylation of a Lys located outside this consensus sequence has also been observed in up to 45% of all SUMOylated proteins identified so far. A third enzyme, the SUMO-ligase E3 facilitates the transfer of SUMO molecules to substrate acceptor

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Lys residues; including the cases when SUMOylation occurs at Lys residues outside of the consensus motif (Novatchkova et al., 2004; Saracco et al., 2007). The best characterized SUMO ligase of *Arabidopsis* is SIZ1 (Park et al., 2010). In *Arabidopsis thaliana*, hundreds of SUMO conjugates have been identified, they are found in most cell compartments and have been shown to be implicated in a variety of metabolic pathways (Elrouby and Coupland, 2010; Miller et al., 2010; Park et al., 2011; Elrouby et al., 2013). SUMOylation has many very different biological consequences, modulating for instance protein localization, stability, activity and interactions with other proteins (Matunis et al., 1996; Meulmeester and Melchior, 2008; Chaikam and Karlson, 2010). The phenomenon of crosstalk between SUMOylation and phosphorylation has recently gained experimental support (Hietakangas et al., 2006; Anckar and Sistonen, 2007; Stehmeier and Muller, 2009; van den Burg and Takken, 2010; Yang and Sharrocks, 2010). In mammals the "heat shock transcription factor" HSF1, was shown to be activated by SUMOylation in a phosphorylation-*dependent manner* (Hietakangas et al., 2006); and a similar situation may exist in plants as the activity of the *Arabidopsis thaliana* heat shock transcription factor HSFA2 was shown to be regulated by phosphorylation (Evrard et al., 2013) and by SUMOylation (Cohen-Peer et al., 2010).

Potato (*Solanum tuberosum*), is not only one of the most important food crops in the world, it also displays high sensitivity to high temperature stress (Levy and Veilleux, 2007). However the mechanisms involved in the response of *Solanum tuberosum* to HS remain largely unexplored. In this study we have examined at the proteome level the rapid changes in protein SUMOylation and serine phosphorylation, which are induced by HS in potato leaves. The identification of SUMOylated and phosphorylated proteins was performed by combining three-dimensional gel electrophoresis fractionation, Western analysis using antibodies to SUMO1/2 or phosphoserine, and mass spectrometry (Colignon et al., 2013). Our results confirm the effectiveness of using a gel-based proteomics strategy to identify and quantify endogenous proteins with multiple PTMs under given physiological conditions. They suggest that HS responses may be modulated by a coordinated interplay between the SUMOylation and the phosphorylation of particular protein molecules.

2. Materials and methods

2.1. Plant material and heat stress treatment

Solanum tuberosum L. cv. Désirée plants were multiplied in vitro on Murashige and Skoog medium (Murashige and Skoog, 1962) and grown for 10 days. Plantlets were transplanted and grown in a controlled environment chamber with a 16 h day/night photoperiod (light intensity = 270 µmol $m^{-2} s^{-1}$) at 21 °C/18 °C. Three-week-old plants were used for the experiments. Heat stress was applied in the light by transferring plants to a controlled environment chamber at 40 °C. Three entire leaves were collected after 0, 1, 2 and 4 h of HS and immediately frozen in liquid nitrogen until use.

2.2. Quantitative RT-PCR analysis

Total RNA was extracted from control and heat treated leaves by the TRIzol method (Life Technologies) as per the manufacturer's instructions. First strand cDNAs were synthesized from 1.5 µg total RNA using random hexamers and RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) as per the manufacturer's instructions. Quantitative reverse transcription PCR (qRT-PCR) was performed using the CFX96 system (Bio-Rad). The method of designing PCR primers for each potato SUMO enzyme, was based on the alignment of corresponding coding genes from several different plant species so as to locate conserved motifs among the sequences. The gene-specific potato primers for quantification of the SUMOylation machinery transcripts that were used in the qRT-PCR analysis were designed by Primer3 software (http:/frodo.wi.mit.edu) (Table 1). A set of potato 18S rRNA primers was also designed for use as reference gene for normalization. The qPCR reactions were performed in a final volume of 25 µL containing 2x Maxima™ SYBR Green qPCR Master Mix (Life Technologies), 300 nM of each direct and reverse gene specific primer, and 400 ng of cDNA template in a CFX96 thermocycler (Bio-Rad). The thermal cycling conditions were as described by Colignon et al. (2017a). Two biological replicates and two technical replicates of each treatment combination were analyzed. To quantify the transcripts accumulation, we used the comparative Ct (threshold cycle value) method of relative quantification ($2^{-\Delta\Delta Ct}$) as described by Livak and Schmittgen (2001). Tukey's range test was used to test the significance of the treatment effects. All statistical analyses were performed using the SYSTAT 8.0 package.

Table 1
Sequences of primers (5′ – > 3′) used in qRT-PCR analysis.

SAE1b-D	TGT-CCA-TCT-TCG-ATG-CTG-AT
SAE1b-R	AAT-ATC-TCA-CCG-CAG-GAG-TCA
SUMO1-D	TAA-GGG-TCA-GGA-TGG-GAA-TG
SUMO1-R	CAG-GAG-TTT-GCT-CTG-CCC-TA
SAE2-D	TGC-AAG-AGC-TGG-TGT-CCA-CC
SAE2-R	CGG-TTA-CTG-GTG-GCA-CAA-TTC
SIZ-D	CGG-TTG-GTG-ACC-GAA-GAT
SIZ-R	CAG-CAT-CGA-AGA-TGG-ACA-GA
SCE1-D	ACA-CTT-TGG-CGG-TTT-ACT-CG
SCE1-R	TTG-CTA-AGC-CGG-AGA-CAC-TT
18S-D	GTG-ACG-GGT-GAC-GGA-GAA-TT
18S-R	GAC-ACT-AAT-GCG-CCC-GGT-AT

2.3. Protein extraction

Total protein was extracted from leaves using the procedure of Islam et al. (2004) and solubilized in the following buffer: 8 M urea, 2 M thiourea, 4% w/v CHAPS, 2% v/v IPG buffer and 40 mM DTT. The protein concentration was measured by the Bradford's method (Bradford, 1976).

2.4. Gel-based fractionation

1D PAGE was performed on 7.5% or 10% polyacrylamide gels using 30 µg of protein extracts. Gels were run using the discontinuous buffer system of Laemmli (Laemmli, 1970).

2D PAGE. The DIGE saturation-labeling procedure was used. For 2D gels dedicated to Western blot analysis, 200 µg of proteins were labeled using the CyDye5 saturation dye for preparative PAGE (GE Healthcare); for 2D gels dedicated to MS/MS analysis, a total of 300 µg of proteins were labeled with the CyDye 5 for *preparative PAGE* (Colignon et al., 2013).

The labeled proteins were separated on IEF strips (pH 4.5–7, 24 cm, GE Healthcare,) according to the manufacturer's instructions. The strips were equilibrated in 10 mm DTT and 125 mm IAA, and mounted on top of a 10% polyacrylamide -gel. The latter were run overnight using the discontinuous buffer system of Laemmli (Laemmli, 1970).

3D PAGE. The 2D spots were picked from 2D gels, (Ettan Spot Picker robot, Amersham GE Healthcare) and submitted to a third electrophoretic fractionation using either the NuPAGE® Bis-Tris (MES buffer) or the NuPAGE Tris-acetate discontinuous buffer system (Colignon et al., 2013). These gels were run as recommended by the manufacturer (Life Technologies). The PageRuler Plus Prestained Protein Ladder (Thermo Scientific, Waltham, MA, USA) was used as size standard.

2.5. Western-blot analysis

Following electrophoretic fractionation, the gels destined for Western-blot analysis were electroblotted onto either Hybond ECL (1D gels) or PVDF-LF (2D- and 3D gels) blotting membranes as per the

manufacturer's instructions (Amersham GE Healthcare).

For the detection of SUMO1 conjugates or phosphoserine, the polyclonal *Arabidopsis* SUMO1 antibody (1/1000 dilution, Agrisera) or anti-phosphoserine polypeptides (1/2000 dilution, Life Technologies) antibody were used. The anti-AtSUMO1 antibody is predicted to detect SUMO1, but also SUMO2 in *A. thaliana* and in *Solanum lycopersicum*. Therefore nodistinction was made between these two isoforms and the abbreviation "StSUMO1/2" has been used.

2.6. Enzymatic digestion, MS/MS analysis and bioinformatics analysis

3D protein spots were analysed using nano-liquid chromatography (UltiMate 3000, ThermoFisher) tandem mass spectrometry (maXis, Bruker, Bremen Germany). Proteins were in-gel digested with trypsin (Promega, The Netherlands). The digests were separated by reverse-phase LC using a 75-µm × 150-mm reverse phase Thermo column (Acclaim Pep- Map 100 C18). Mobile phase A was composed of 5% acetonitrile and 95% of 0.1% formic acid in water. Mobile phase B was 0.1% formic acid in acetonitrile. One microliter of the digest was injected and the organic content of the mobile phase was increased linearly from 5 to 40% phase B over a period of 40 min, and from 40% to 100% B in 5 min. The column effluent was connected to an ESI nanosprayer (Bruker). In survey scan, MS spectra were acquired for 0.5 s in the range between 50 and 2200 *m/z*. The most intensely represented peptide ions, 2+ and 3+, were sequenced. The CID energy was automatically set as a function of the *m/z* ratio and charge state of the precursor ion. Compass HyStar 3.2 software (Bruker) piloted the maXis and Thermo systems. Mascot 2.2 was used as search engine (Matrix Science, London, UK). The maximum number of missed cleavages per peptide was set at 1. Methionine oxidation was set as variable modification and carbamidomethylation was fixed as constant. All MS/MS samples were analyzed using the Mascot (version 2.2) search engine. Peptide identifications were accepted if they could be established with a minimal individual identity score greater than 30 (Silvestre et al., 2010). For protein identification we did start with the potato database "Uniprot", which has about 53k entries for *Solanum tuberosum*; but more than 90% of the entries in that database are for "uncharacterized protein". We therefore switched to the Viridiplantae database and used protein identification by sequence homology (a score equal or greater than 60) to search the 11,500 k entries.

3. Results

3.1. Comparison of temporal changes in protein SUMOylation and serine phosphorylation in potato leaves under heat stress

Following earlier work on a number of plant species which showed that HS brings about an increase in both protein phosphorylation and protein SUMOylation (Chen et al., 2011; Miller and Vierstra, 2011), we have analyzed the time course and the magnitude of these post transcriptional modifications in leaves of the potato. Total potato leaf protein extracts were resolved by 1D-SDS/PAGE, electro-blotted onto PVDF-LF membranes, and then probed with antibodies to AtSUMO1/2 and to phosphoserine. The corresponding Western-blots are presented in Fig. 1A and B.

As observed previously for HS in *Arabidopsis thaliana* (Miller and Vierstra, 2011), we found that HS in potato resulted in a transient increase in the global level of SUMOylation, which reached a peak after 1 h of HS (Fig. 1A). The Western blotting analysis suggested that this early accumulation of StSUMO conjugates occurred at the expense of the pool of free StSUMO1/2 which was largely consumed after 1 h HS and which returned to about the control level some hours later.

The time course of the response of phosphoserine proteins was somewhat different, reaching a maximum after 2 h of HS (Fig. 1B).

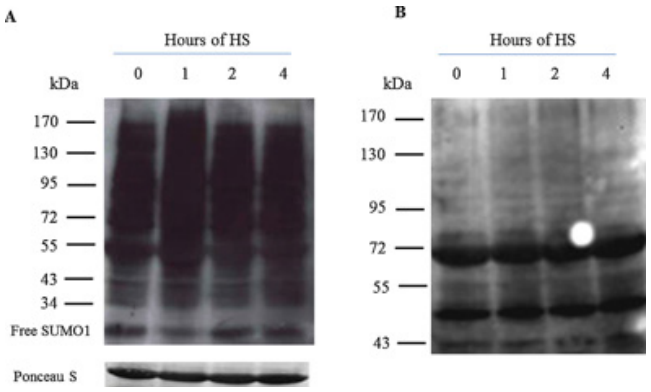

Fig. 1. Results of Western blot analysis of StSUMO1/2 conjugates (A) and protein serine phosphorylation (B) in potato leaves exposed to sub-lethal HS for 0–4 h. Total protein extracts from leaf samples withdrawn at the indicated times were subjected to SDS-PAGE and duplicated Western blots were probed with either anti-SUMO or anti-phosphoserine polypeptides antibodies. The free SUMO was more easily observed on the over exposed image of the western blot. The images are representative of three independent experiments. Ponceau S staining was used as loading control.

3.2. Comparative time course expression profiling of StSUMO enzymes by qRT-PCR in potato leaves under heat stress

The 1D approach has some limitations, Western blotting is semi-quantitative and the resolution of the 1D separation is limited. To overcome these limitations a 2D/3D approach was used, as described later. But first, we wanted to have a better insight in the potato SUMOylation machinery. To this end the relative expression levels of StSUMO1/2-conjugating enzymes were investigated in control and in potato leaves exposed to HS. We found (Fig. 2) that the expression levels of StSUMO1/2 enzymes and of StSUMO1/2 for exposure times > 1 h increased in response to HS. Surprisingly we could not find any indication for the presence of *SAE1b* homolog gene *SAE1a* in potato cv. Désirée. These results suggest the interesting possibility that regulation of SUMO conjugates abundance during HS may be initially (1 h exposure time) controlled by the pool of free StSUMO1/2and then by SUMO-conjugating enzymes.

3.3. Mapping SUMO1/2 conjugates and phosphoserine proteins on 2D and 3D-gels

For the 2D mapping of SUMO1/2 conjugates and phosphoserine proteins, we used total protein extracts from leaves HS-treated for 1 h (these have a higher content of StSUMO1/2 modified proteins than leaves treated for a longer period of time). The StSUMO1/2 conjugates and phosphoserine proteins were visualized by combining a preparative 2D-PAGE with a 2D-Western blotting analysis (Colignon et al., 2017b). Because of the low abundance of the StSUMO1/2 protein conjugates, the protein samples were labeled with the highly sensitive fluorescent dye, CyDIGE fluor Cy3, which was specifically developed for two-dimensional difference gel electrophoresis (2D-DIGE) of scarce proteins. Approximately 2200 spots were detected in the 2D-gel by the DeCyder software (Fig. 3A). Proteins from the duplicated gels were then electroblotted onto PVDF-LF membranes and probed successively with antibodies to AtSUMO1/2 and to phosphoserine. Immuno-reactive spots were localized on gels by software-aided superposition of the gel and blot images (Fig. 3B) with the result that 69 spots of the original set showed a positive immuno-detection with antibodies to AtSUMO1/2, 60 showed a positive detection with antibodies to phosphoserine and 43 a positive detection with both antibodies). Some of the original set of spots revealed by the antibodies to SUMO1/2 or to phosphoserine could not be matched to the corresponding ones in the gel. This may be due at least in part to the higher sensitivity of the immuno-detection antibody

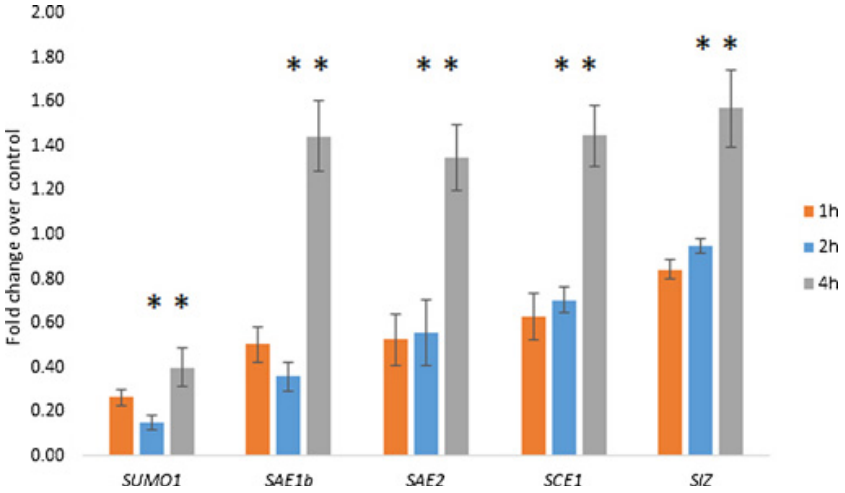

Fig. 2. Comparative expression of genes involved in the SUMOylation pathway in heat stressed potato leaves. Leaves were exposed to HS (0, 1, 2, and 4 h) corresponding to the maximal accumulation of SUMO protein conjugates. The accumulations of *SUMO1*, *SAE1b*, *SAE2*, *SCE*, and *SIZ* transcripts were assessed by qRT-PCR. Histograms represent the fold increase in level of expression over control leaves (0 h). Error bars indicate Standard Deviation of the mean (N = 2). Significance of the difference in the expression between treated and control samples, as determined by Tukey's test is indicated thus (*, $p < 0.05$).

procedure.

In total 86 protein spots were excised from 2-DE gels for MS/MS analysis. Since individual spots often contained several proteins (Table S1), they were submitted to an additional electrophoretic fractionation so as to resolve them into their individual components (resulting in 3D spots). These 3D spots were transferred onto PVDF-LF and reprobed with both antibodies so as to enable an unambiguous identification of StSUMO1/2 conjugate and phosphoserine modified protein. This 3D-step revealed 107 3D-spots. 87 of the 107 were post-translationally modified, 27 correspond to StSUMO1/2 targets, 17 are phosphoserine proteins and 43 are proteins that are both SUMOylated and phosphorylated (see Table S1). This is illustrated in Figs. 3C for 2 D gel spots N° 2, 40 and 45. It shows that for spot 2, protein A is SUMOylated; that spot 40 corresponds to a single protein both SUMOylated and phosphorylated; and that for spot 45, only protein B is modified i.e. positive for both StSUMO1/2 and phosphoserine.

3.4. Effects of one hour HS on the abundance of SUMO1/2 conjugates and phosphoserine proteins analyzed by the 3D-approach

Once the mapping step procedure had been established we investigated the changes brought about in the StSUMO1/2 and phosphoserine proteomes of *Solanum tuberosum* leaves after 1 h HS stress, using the quantitative DIGE strategy. Two 2D-gels were run, each containing one extract (control or 1 h HS), labelled with Cy3 and the internal standard labelled with Cy5. An internal standard comprised of a mixture of equal amounts of the control and the heat stressed sample was labelled with the Cy5. The inclusion of this internal standard allows the normalization of spot volumes so as to avoid gel running effects. Equivalent 2D-spots of interest (i.e. spots that displayed HS-associated in the 2D gel pair) were excised and re-electrophoresed in gels using alternative buffers systems (3C). Then fluorescence images were used for quantification. A typical result is illustrated for three spots in Fig. 4A. 2D-spot n°2, which had previously been shown to be positive for the AtSUMO1/2 antibody was resolved by this new step into 2 spots 'A` and 'B`. After 1 h HS, only the abundance of spot A (Heat shock protein 90-StSUMO1/2 conjugate) increased, while the abundance of spot B remained constant. Following 1 h of HS the abundance of 2D-spot n°40, previously shown to contain a single protein which is both SUMOylated and phosphorylated, increased (Fig. 4B). As regards spot 45 which contains 2 unidentified proteins: the abundance of protein A, negative for both SUMO1/2 and phosphoserine, decreased after HS, while the abundance of protein B displaying both PTMs, increased (Fig. 4B).

This quantification step on the 3D-gels, enabled us to calculate (with ratios of minimum 1.5) the changes in the relative abundance of the SUMO and/or phosphoserine proteins (up- or down regulation) in response to HS. The results were of 20 StSUMO1/2 conjugates (17 up-

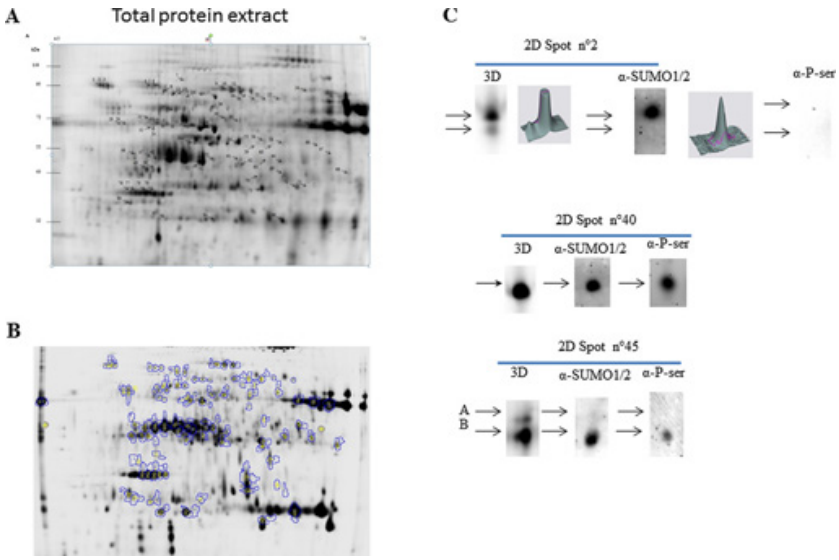

Fig. 3. High-resolution 2D-DIGE proteome analysis of *Solanum tuberosum* leaves following exposure to 1 h HS.
(A) Representative 2D-DIGE gel image of potato leaves exposed to 1 h sub-lethal HS. Following labelling with Cy3, proteins were separated by 2D electrophoresis using in the first dimension, isoelectric focusing (pH range 4.5–7.0), in the second dimension, 10% acrylamide 1D SDS PAGE in the Laemmli buffer system. Spots are indicated by a number. For the image clarity, only spots with protein identification are shown. Further information about protein identifications can be found in the Supplementary Table S1.
(B) 2D Western blot analysis of PVDF membrane probed successively with antibodies to AtSUMO1/2 and to phosphoserine. The fluorescent secondary antibody was coupled to Cy5. Note that some of the original set of spots revealed by the antibodies to AtSUMO1/2 or to phosphoserine could not be matched to the corresponding ones in the gel.
(C) Representative gel image of 3D SDS-PAGE fractionation and subsequent Western blot analysis for spots N° 2, 40 and 45. The spots were excised from the 2D gel and re-electrophoresed on NuPage Tris acetate 7% gel. Also shown is fluorescence volume of spot N°2 after 3D SDS PAGE and Western blot analysis which was performed as in Figure B.

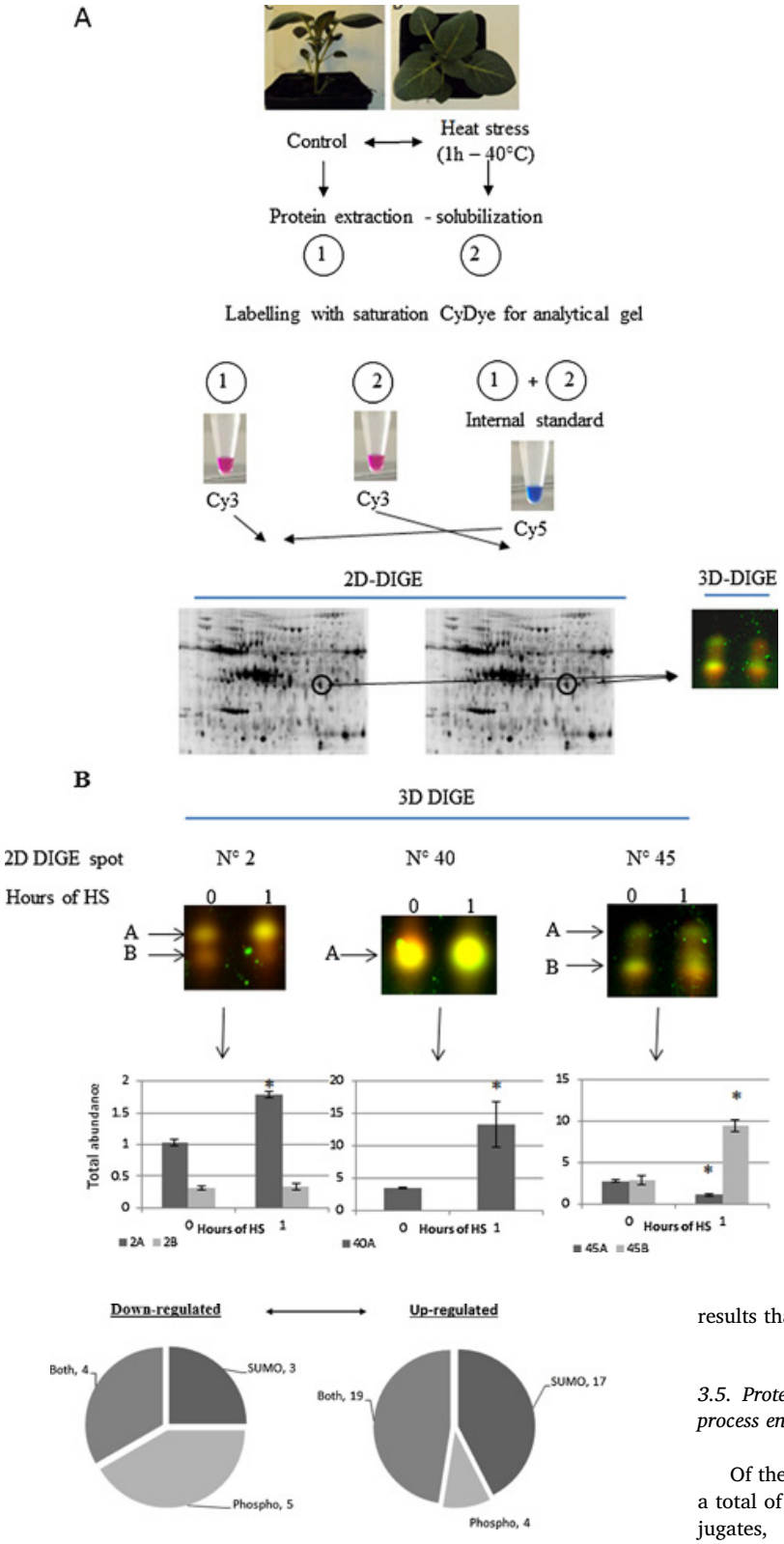

Fig. 4. Flow chart depicting the analytical steps in the quantitative analysis of SUMO-and phosphoserine proteomes using 3D-DIGE.
(A) Extracted proteins were labeled with the saturation CyDyes Cy3 or Cy5 for analytical gels and separated with, in the first dimension, IEF on an immobilized pH 4.5–7.0 gradient; and in the second, SDS-PAGE using the Laemmli buffer system. Each gel contained one sample from the experiment (either control or HS treated) together with an internal standard formed from equal amounts of every sample. For the separation in the third dimension, spots of interest were further resolved on NuPage gel using either Tris Acetate 7% or Bis-Tris Glycine 4–12% MES buffer (see Fig. 4B).
(B) The result of three dimensional difference gel electrophoresis (DIGE) analysis using the CyDye DIGE Fluor saturation dyes Cy2 and Cy3 to investigate spots excised from the 2 D-DIGE gels. Spots were 3D fractionated on NuPage gels, in either Tris-Acetate 7% (Spot 2) or Bis-Tris-Glycine 4–12% MES buffer (Spots 40 and 45). Spots were visualized using a Typhoon 9400 imager. The spot fluorescence volume of each sample protein was normalized against the spot fluorescence volume of the corresponding internal standard, and then treatment effects were calculated as the ratio of that normalized value to the fluorescence of control spots. The figure shows the results for three example spots and statistically significant difference ($p < 0.05$) is marked with asterisk (*). The additional fractionation resolved Spots 2 and 45 into two elementary spots. Spot 40 was not resolved any further.

Fig. 5. Relative proportions of SUMOylated ("SUMO"), phosphoserine modified ("Phospho") and SUMOylated and phosphoserine proteins ("Both") PTMs, between the twelve leaf proteins that were found to be down-regulated, and the forty leaf proteins that were up-regulated, in response to heat shock.

regulated and 3 down-regulated), 9 phosphoserine proteins (4 up-regulated and 5 down-regulated) and 23 proteins bearing both modifications (19 up- and 4 down-regulated) (Fig. 5). It is clear from these results that most of the SUMOylated proteins are up-regulated.

3.5. Protein identification by MS/MS and Gene Ontology (GO) biological process enrichment analysis

Of the 107 3D-spots which were processed for the MS/MS analysis, a total of 79 proteins were identified, comprising 25 StSUMO1/2 conjugates, 12 phosphoproteins and 33 proteins modified by both SUMOylation and phosphorylation (Table S1). A further nine proteins were identified but were not detected as post-translationally modified. A total of 46 non-redundant proteins were identified.

Analysis of the biological processes that are associated with the identified StSUMO1/2 conjugates indicates that cellular and metabolic processes are the most represented. A Gene Ontology (GO) biological process enrichment analysis was executed using the Blast2Go bioinformatics tool (https://www.blast2go.com), but no statistically significant result was found. As regards the functionality of the identified

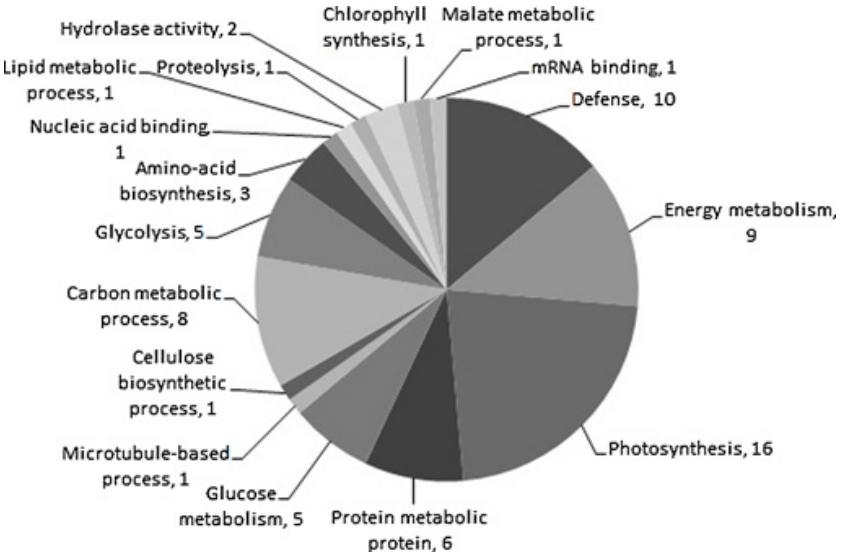

Fig. 6. Functional classification of phosphoserine and StSUMO1/2 modified proteins.
A total of 72 phosphoserine and/ or StSUMO1/2 modified proteins were categorized by Gene Ontology (GO) term analysis. This yielded 17 functional groups. Each number indicates the number of proteins assigned to that category.

HS-associated proteins, those proteins related to photosynthesis and photosynthesis associated processes were the most represented (Fig. 6). An enrichment of chaperone proteins such as the HSPs was also observed. The specific SUMO motif (Ren et al., 2009) was detected in 52% of the identified StSUMO1/2 conjugates, and further manual inspection failed to identify the inverted SUMO motif (Matic et al., 2010) in any of our samples. While the NetPhos 2.0 server did identified potential serine phosphorylation sites in each identified protein, we were unable to detect any sign of PDSM (Phosphorylation-Dependent SUMO Modification) sequence, which has been proposed to mediate a cross talk between the SUMOylation and phosphorylation pathways (Hietakangas et al., 2006).

4. Discussion

The first objective of this work was to apply a top-down methodology using newly developed proteomic technologies (3D electrophoresis) to investigate early events in the response of potato leaves to heat stress. The advantage of such “top-down” proteomics, as compared to bottom-up proteomics is that top-down proteomics characterizes intact proteins and therefore offers distinct advantages for PTMs and for the determination of mutant and alternatively spliced isoforms (Naryzhny, 2018). However top-down proteomic analysis of complex samples currently has a problem with sample fractionation. The successful implementation a top-down proteomics strategy therefore required reduced sample complexity. This was achieved by combining off-line orthogonal fractionation methods before to LC–MS/MS analysis (Padula et al., 2017). Recently Cesnik et al. (2018) have “provided a striking view” of salt-stress response in yeast by identifying and quantifying abundance changes of proteoform families (defined as the set of proteoforms that derive from a particular gene). Proteoforms identification was based on accurate mass difference measurements between experimental and theoretical proteoforms without protein fragmentation. We used a different approach, combining separation of proteins by high-resolution 3D-SDS PAGE with LC–MS/MS. We were able to take advantage of recent technical advances in DIGE technology for enhanced accuracy and reproducibility. By using of 3D electrophoresis with subsequent 3D Western-blot we have been able to detect and to quantitate HS- associated changes in the StSUMO1/2 and in the phosphoserine native proteoforms from a single tissue lysate at a very early stage (after 60 min). This methodology should have wider application in proteome analyses in particular for PTMs such as SUMOylation and Ubiquitylation which add amino acid chains of variable length to their protein targets which hampers protein identification using search engines.

The analytical and preparative procedures have been developed for an optimal resolution of the SUMO 1/2 and phosphoserine proteomes. They comprise three steps, illustrated in the flow charts (Figs. 3 and 4).

In the first step, the resolution step, total leaf extracts were labeled with the highly sensitive *CyDye DIGE fluor saturation Cy3 dye and fractionated by 2D-SDS PAGE and the StSUMO1/2- and phosphoserine-modified proteins were then visualized by fluorescent Western blotting.* 3D-SDS PAGE fractionation step followed in order was added to circumvent co-migration interferences and so as to locate the modified proteins unequivocally. In the second step, the quantification step, a 3D-DIGE analysis was performed to selectively quantify the HS-induced changes in abundance of StSUMO 1/2 and phosphoserine proteins. In the third step, the identification step, spots that had been noted as being of interest in step 1 were subjected to LC–MS/MS for identification of the proteins, after an additional 3D separation to improve the accuracy of determination. As regards sensitivity, the comparison of the 2D procedures in step 1 (SDS PAGE Western blot) confirms the greater sensitivity of fluorescent immuno-detection. As regards problems still to be resolved, spot picking for LC MS/MS analyses was not possible for some spots which appeared in the Western blot image but could not be matched in the gel. A possible strategy for resolving this problem might be to use a sub-proteome approach. Meanwhile our existing approach has enabled the simultaneous resolution at the leaf proteome level of two prominent and often inter-dependent PTMs, SUMOylation and serine phosphorylation, in response to heat-induced stress.

Our 1D-SDS PAGE analysis confirmed the earlier finding of Miller and Vierstra (2011) that the global increase in the SUMO1/2 conjugates occurs at the expense of the pool of free SUMO1/2. This suggests that this pool is functional and readily available for conjugation to protein targets. It thus appears that the SUMOylation pathway may play a central role in responses of some leaf cells to heat stress. The mechanisms by which the changes in the SUMOylation patterns are invoked under HS, are as yet unclear and remain to be explored in detail. While it is clear that the increased expression levels of the SUMO conjugating enzymes contribute to the SUMOylation increase, it remains to be explained why such an increase was not observed at short (1 h) exposure times. Because the SUMO-specific proteases have been

shown to be very sensitive to HS (Pinto et al., 2012) it is suggested that their inactivation could also contribute to the HS-associated global increase in SUMOylation.

Our 3D analysis revealed HS associated alterations in the leaf StSUMO1/2 proteome profiles, alterations which are more complex than those that have been suggested by previous 1D analyses. We found, for example, increases in the abundance of some StSUMO1/2 conjugates and decreases in the abundance of certain others. This may reflect differences in the sensitivity of specific SUMO proteases to HS or these changes in abundance of certain StSUMO1/2 conjugates could possibly be a consequence of changes in polySUMOylation. In support of the latter hypothesis is the observation that during HS the *Arabidopsis thaliana* mutants for the *PIAL1* and *PIAL2* genes (Protein Inhibitor of Activated Stat Like) accumulated SUMO protein conjugates. This was shown to be due to the suppression of polySUMO chain formation activity of PIAL1 and PIAL2 ligases (Tomanov et al., 2014).

A further, issue only recently emerged is the phenomenon of crosstalk between PTMs; that is the concerted action of PTMs (both agonistic and antagonistic interactions) in the regulation of protein function. The first example of PTMs crosstalk was characterized among histone H3 pSer10 and H3 acetylation at lysine 14 (Cheung et al., 2000; Lo et al., 2000). The resulting fine tuning of individual cellular functions, has now been demonstrated for various non histone proteins in plant, yeast and in human model systems (Yao et al., 2011; Cloutier and Coulombe, 2013; Venne et al., 2014). Cross talk between multiple PTMs may converge on the same target influencing the downstream cascade of regulatory events and, by consequence, determining the eventual phenotypic trait (Guerra et al., 2015). Global mass spectrometry (MS) investigations have uncovered the great variety of PTMs that exist; so it is possible that many traits may be regulated in this way. However because of the sub stoichiometric abundance of PTM modified proteins, these approaches require a specific enrichment of the peptides carrying the PTM of interest, which in most cases has precluded the detection of potential interactions with other PTMs that may be present on the same protein.

In the work reported here a significant fraction of the modified proteins displaying changes in abundance in response to HS appeared as StSUMO1/2 modified proteins only. While we cannot exclude the possibility that these proteins are phosphorylated on Tyr (Yao et al., 2011) most of the identified proteins were both SUMO1/2- and phosphoserine- modified. It is relevant to note that intensive cross talk between phosphorylation and SUMOylation has been shown to regulate the transcriptional response to HS (Hietakangas et al., 2003). It is therefore possible that a similar situation may prevail in plants; for example the *Arabidopsis* heat shock factor activity was found to be modulated by SUMOylation (Cohen-Peer et al., 2010) and phosphorylation (Evrard et al., 2013).

Alternatively phosphorylation and SUMOylation could independently regulate different functions of the modified protein (Huberts and van der Klei, 2010).

It has been suggested (Hietakangas et al., 2003; Stehmeier and Muller, 2009) that the interplay between the SUMOylation and phosphorylation pathways confers the fine regulation of a large array of biological functions; which together result in the acclimation response to HS. In the work reported here, the 3D-separation procedure enabled the identification of several SUMOylated and/or phosphorylated proteoforms which were shown to be members of the main classes of protein chaperones; including HSP90, HSP70, chaperonin and luminal binding protein, all of which have a role in proteome maintenance such as, *de novo* protein folding, the refolding of stress-denatured proteins, oligomeric assembly, protein trafficking and assistance in proteolytic degradation. In addition there is experimental evidence (Prodromou, 2016) which suggests that Hsp70 and Hsp90 serve as modulators of the heat shock transcription factor Hsf1. This functional diversity is made possible by a dynamic association with various co-chaperones and supported by PTMs such as acetylation, S-nitrosylation, ubiquitination, phosphorylation and SUMOylation. The SUMOylation of HSP90 has been demonstrated in yeast (on K178) and human (on K191) cells. In yeast and human cells SUMOylation of one of the protomers of the HSP90 dimer initiates the recruitment of the activator of HSP90 ATPase, Aha1 (Mollapour et al., 2014).

Because HSPs including HSP90 are thought to belong to a highly conserved family of proteins, we performed a genome-wide survey of the HSP90 gene family in potato. The objective was to test whether the SUMOylation motif is conserved in the potato Hsp90 s. Following a search for Hsp90 sequences in the *Solanum tuberosum* genome, the subsequent phylogenetic analysis using MEGA 6 package (Tamura et al., 2013) revealed that as observed in *Arabidopsis thaliana (*Krishna and Gloor, 2001), *S. tuberosum* HSP90 gene family includes 7 members, four of which constitute the cytoplasmic subfamily (Fig. S1), containing the highly conserved C-terminal pentapeptide MEEVD (Fig. S2a).

Our protein sequence analysis revealed the presence of a lysine residue (K179) in the N-domain in the four putative cytosolic *Arabidopsis* and potato HSP90s. The residue is within the SUMO motif LKED and it is at the same position observed in yeast and human HSP90 s (Fig. S2b). These data suggests that plants, fungi and mammals share an evolutionarily conserved SUMOylation-based component of HSP90 activity, namely the activation of the HSP90 ATPase activity by the SUMOylation dependent recruitment of endogenous activators such as Aha1.

The work reported here has shown that HS results in changes in the degree of SUMOylation and phosphorylation of a large number of endogenous phospho- and StSUMO1/2 conjugated proteins. Most of these are known to play a role in metabolic pathways associated with chloroplasts. This underscores the importance of this organelle in the response of the parent plant to HS (McDonald et al., 2011). In the current view, RuBisCo inactivation is the primary determinant of the HS induced depression of leaf photosynthesis (Hozein et al., 2010). Under these conditions photorespiration and alternative photosynthetic electron transport pathways serve as excess energy sinks to protect the chloroplasts from photoinhibitory damage (Kim and Portis, 2005; Li et al., 2016). It is known that RuBisCo activase (RCA) continuously activates RuBisCo by removing 2-carboxy-D-arabitinol 1-phosphate from its catalytic site. However RCA displays acute sensitivity to HS (Salvucci et al., 2001) and this may account for the reduced photosynthetic performance under HS (Hozain et al., 2010). We hypothesize that the increase in the degree of RCA SUMOylation observed here (Table S1, spots 54 and 55) may help to prevent HS-induced inactivation of RCA, hence improving photosynthetic performances under HS. It has been shown that the HS–associated decrease in photosynthetic electron transport is a result of injury to the PSII induced oxidative water splitting reactions (Sinsawat et al., 2004). However, our measurements of chlorophyll fluorescence (data not shown here) indicate that under our experimental conditions the photosystem II reaction center was still fully active. We therefore suggest that the increase in SUMOylation of OEC 33 (Oxygen Evolving Complex) and CA (Carbonic Anhydrase) proteins, both of which are involved in the PSII induced oxidative water splitting (Villarejo et al., 2002), has the overall result of stabilizing the labile water-splitting complex of PSII under mild HS.

5. Conclusion

In summary, our 2D/3D gel based proteomic analysis has shown that HS results in early changes in the degree of protein phosphorylation and of SUMOylation of a large set of individual molecules. We suggest that these early responses contribute to the cell homeostasis under HS by acting on cell metabolism at *multiple levels. In particular our findings suggest that SUMOylation has a major role* in the reprioritization of photosynthetic activity by acting on both the light and dark reactions in a way which suggests an interplay with protein phosphorylation. However elucidation of the detailed mechanisms that may be involved in such an interplay between SUMOylation and phosphorylation awaits

further experimental investigation.

Author contributions

All listed authors brought significant, direct and intellectual contribution for manuscript completion, and approved it for publication.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2019.02.024.

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