A Microsomal Proteomics View of H$_2$O$_2$- and ABA-Dependent Responses

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Abstract: The plant hormone abscisic acid (ABA) modulates a number of plant developmental processes and responses to stress. In planta, ABA has been shown to induce reactive oxygen species (ROS) production through the action of plasma membrane-associated nicotinamide adenine dinucleotide phosphate (NADPH)-oxidases. Although quantitative proteomics studies have been performed to identify ABA- or hydrogen peroxide (H$_2$O$_2$)-dependent proteins, little is known about the ABA- and H$_2$O$_2$-dependent microsomal proteome changes. Here, we examined the effect of 50 µM of either H$_2$O$_2$ or ABA on the Arabidopsis microsomal proteome using tandem mass spectrometry and identified 86 specifically H$_2$O$_2$-dependent, and 52 specifically ABA-dependent proteins that are differentially expressed. We observed differential accumulation of proteins involved in the tricarboxylic acid (TCA) cycle notably in response to H$_2$O$_2$. Of these, aconitase 3 responded to both H$_2$O$_2$ and ABA. Additionally, over 30 proteins linked to RNA biology responded significantly to both treatments. Gene ontology categories such as ‘response to stress’ and ‘transport’ were enriched, suggesting that H$_2$O$_2$ or ABA directly and/or indirectly cause complex and partly overlapping cellular responses. Data are available via ProteomeXchange with identifier PXD006513.

Keywords: hydrogen peroxide (H$_2$O$_2$); abscisic acid (ABA); microsomal proteomics; quantitative proteomics; mass spectrometry

1. Introduction

Plants, much like animals, are susceptible to oxidative damage by reactive oxygen species (ROS). The production of ROS, and in particular hydrogen peroxide (H$_2$O$_2$), increases during exposure to abiotic stress [1–3] and pathogen infection [4]. In pathogen infections, suppression of ascorbate peroxidase and catalase during the hypersensitive response enhances pathogen-induced programmed cell death [4]. The mitochondrial electron transport chain also synthesises a significant amount of ROS, mainly in the form of superoxide [5] that can cause oxidative stress [6,7]. Previous studies have shown that the oxidative stress imposed by H$_2$O$_2$ is a potent inhibitor of tricarboxylic acid (TCA) cycle enzymes such as citrate synthase [8], aconitase, and succinyl CoA ligase [9–11]. However, at low concentrations, H$_2$O$_2$ functions as a stress signal [12,13].
It has been demonstrated that ROS production is required for abscisic acid (ABA) signal transduction in guard cells [14,15]. In the guard cells, ABA is perceived by PYRABACTIN resistance (PYR)/PYR- Like 1/Regulatory components of ABA receptors (RCAR) [16,17], which in turn induce the production of ROS including H$_2$O$_2$, by nicotinamide adenine dinucleotide phosphate (NADPH) -oxidase [18,19]. ABA also induces stomatal closure but interestingly, treating the guard cells of ABA-insensitive mutants, abi1-1, with ABA did not induce ROS production but activation of hyperpolarised-activated Ca$^{2+}$ ($I_{Ca}$) channels and the induction of stomatal closure by H$_2$O$_2$, suggesting that abi1-1 disrupts ABA signalling between ABA reception and ROS production [20].

Besides, ABA regulates many plant developmental processes and induces increased tolerance to different stresses such as drought, salinity and low temperature [21]. ABA-induced ROS in the mitochondria of root tip cells operates as retrograde signal that regulate meristem activity in Arabidopsis [22]. In maize (Zea mays) leaves, water stress-induced ABA accumulation has been detected to elicit an increased production of H$_2$O$_2$ [23,24]. Also, in rice (Oryza sativa) seedlings, exogenous ABA has been observed to increase H$_2$O$_2$ content in leaves grown under potassium sufficient conditions [25].

Despite the considerable body of information on role of ABA in stress responses, the post-translational molecular targets at the microsomal level and particularly their relation with H$_2$O$_2$ is yet to be properly understood. Therefore, we set to find out whether H$_2$O$_2$ and ABA can induce a common response at the microsomal proteome level. To do this, downstream microsomal protein changes were investigated using label-free quantitative mass spectrometry analysis. Further, to find out whether ABA induces H$_2$O$_2$ production or vice versa, we tested for H$_2$O$_2$ production upon ABA treatment of Arabidopsis thaliana ecotype Columbia-0 cell suspension cultures or ABA production upon H$_2$O$_2$ treatment. In this study we showed that H$_2$O$_2$ and ABA have common and independent responses. The former is however, not surprising, as ABA has previously been shown to induce production of H$_2$O$_2$, thus the common response suggests an H$_2$O$_2$-dependent response, which can also be elicited via ABA.

2. Materials and Methods

2.1. Treatments of Arabidopsis Cell Suspension Culture

Cells derived from Arabidopsis thaliana (ecotype Columbia-0) roots were grown in Gamborg’s B5 basal salt mixture (Sigma-Aldrich, St. Louis, MO, USA), as described in [26,27]. At Day 7 post-subculturing, three biological replicate flasks containing cells were treated with 50 µM ABA or 50 µM H$_2$O$_2$ for 0 min (mock treatment), 5 min and 20 min. Cells were then harvested by draining off the media using a Stericup® filter unit (Millipore, Billerica, MA, USA), immediately flash frozen in liquid nitrogen, and stored at $-140{\degree}$C until further use.

2.2. Microsomal Protein Isolation

Approximately 1 g of cells was ground to a fine powder in liquid nitrogen and subjected to microsomal isolation, as described in [28]. The powder was incubated in a sucrrose buffer (50 mM Tris(hydroxymethyl)aminomethane (pH 8.0), 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), 0.25 M sucrrose and 1× protease inhibitor cocktail tablet (Sigma-Aldrich, St. Louis, MO, USA)) and centrifuged at 8000 × g for 15 min. The supernatant was subjected to ultracentrifugation using an Optima™ L-100K ultracentrifuge (Beckman Coulter, Brea, CA, USA) at 100,000 × g for 1 h. The supernatant, representing the cytosolic fraction, was pipetted out. The pellet, the microsomal fraction, at the bottom of the tube, was washed once in sucrrose buffer and centrifuged at 100,000 × g for 1 h. The microsomal fraction corresponding to the final pellet was suspended in sucrrose buffer, aliquoted into separate tubes and either used immediately or stored at $-80{\degree}$C.
2.3. Trypsin Digestion and Protein Identification by Tandem Mass Spectrometry

Approximately 0.2 mg of total microsomal protein extract was reduced with 5 mM DTT for 2 h at 37 °C and cooled. The sample was then alkylated with 14 mM iodoacetamide for 30 min at room temperature in the dark. Unreacted iodoacetamide was quenched by increasing DTT concentration to 10 mM and incubated for 15 min at room temperature in the dark. Proteins were incubated at 50:1 ratio with sequencing-grade modified trypsin (Promega, Madison, WI, USA) overnight at 37 °C with gentle agitation. Protein digestion was stopped by acidification of the mixture to pH 2.0 with trichloroacetic acid. Peptides were desalted using Sep-Pak Vac tC18 100 mg cartridges (Waters, Milford, MA), as described previously [27]. After desalting, peptides were re-suspended in 5% (v/v) acetonitrile and 0.1% (v/v) formic acid and analysed by the LTQ-Orbitrap Velos mass spectrometer (Thermo-Scientific, Bremen, Germany) coupled with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark) for nano-liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses as described in [29]. Five microlitres of the peptide mixtures was injected onto a 50 mm long × 0.3 mm Magic C18AQ (Michrom) column. A spray voltage of 1500 V was applied and the MS scan range used was m/z 350 to 1600. The top 10 precursor ions were selected in the MS scan by the Orbitrap with a resolution r = 60,000 for fragmentation in the linear ion trap using collision-induced dissociation. Normalised collision-induced dissociation was set at 35.0 and spectra were submitted to a local MASCOT (Matrix Science, London, UK) server and searched against Arabidopsis in the Arabidopsis Information Resource (TAIR, Release 10), with a precursor mass tolerance of 10 ppm, a fragment ion mass tolerance of 0.6 Da, and strict trypsin specificity allowing up to one missed cleavage. Carbamidomethyl modification on cysteine residues was selected as fixed modification, and oxidation of methionine residues as variable modification. Identified proteins were further validated with Scaffold version 4.0.4 (Proteome Software, Portland, OR, USA). Identified proteins were considered positive with a molecular weight search (MOWSE) score ≥32, number of peptides ≥2, a 95% protein and peptide probability, and a false discovery rate (FDR) ≤1%. Label-free quantitative analysis was performed using spectral counts with Scaffold software. Proteins were deemed responsive to treatment when present in at least two replicates with a fold change (FC) greater or equal to 1 ± 21, verified by Student t-test (p-value ≤ 0.05) in comparison to the mock-treated samples. All proteomics methods are extensively detailed elsewhere [26,29–31].

2.4. Intracellular ROS Assay and ABA Measurements

Cellular ROS levels were measured using OxiSelect™ intracellular ROS assay kit green fluorescence (Cambridge Bioscience, Cambridge, UK) after 20 min of 50 µM ABA treatment. The procedure used was according to the manufacturer’s protocol. ABA contents in samples treated with 50 µM H2O2 for 5 and 20 min were measured using Phytodetek® immunoassay kit for ABA (Agdia, Elkhart, IN, USA) according to the manufacturer’s instructions.

2.5. Bioinformatic Analyses

The gene ontology (GO) analysis toolkit in the database AgriGO [32] was used for the detection of enriched cellular components, biological processes and molecular functions.

3. Results

To gain insight on the microsomal cellular responses to H2O2 and ABA, a comparative proteomic analysis was undertaken. Microsomal proteins were isolated from Arabidopsis cell suspension cultures pre-treated with 50 µM of either H2O2 or ABA for 5 min or 20 min, digested with trypsin and analysed by MS/MS. Data was processed with MASCOT and Scaffold for identification and label-free quantitation. A total of 906 proteins from the enriched samples were identified (Table S1). Of these, 86 proteins were significantly (p ≤ 0.05) responsive to H2O2 treatment (Table 1) and 52 proteins to ABA.
A total of 21 responsive proteins were common to both ABA and H$_2$O$_2$ treatments and majority of the proteins displayed similar response signatures (Table 3).

### Table 1. Proteins responsive to H$_2$O$_2$ treatment.

| Accession Number | Protein Name | ANOVA (p-Value) | FC 5 min | FC 20 min |
|------------------|--------------|-----------------|----------|-----------|
| **1. Metabolism** |              |                 |          |           |
| AT1G17745        | D-3-phosphoglycerate dehydrogenase 2 | 0.0089 | 0.1 | 0.1 |
| AT5G26780        | Serine hydroxymethyltransferase 2 | 0.036 | 2.1 | 13 |
| AT3G61440        | Cysteine synthase C1 | 0.00074 | ns | 5 |
| AT4G14880        | O-acetylserine (thiol) lyase isozyme A1 | 0.015 | 0.1 | 7.9 |
| AT5G23300        | Pyrimidine d | 0.013 | 0.2 | ns |
| AT2G9820         | Adenosine kinase 1 | 0.0042 | 0.1 | 14.0 |
| AT5G17770        | NADH-cytochrome B5 reductase 1 | 0.017 | 0.4 | 2.5 |
| AT1G74790        | Catalytics | 0.045 | 5.7 | 4.6 |
| AT4G23850        | AMP-dep. synthetase and ligase protein | 0.017 | 0.5 | ns |
| AT1G65290        | Mitochondrial acyl carrier protein 2 | 0.044 | 2.9 | ns |
| AT3G47930        | L-galactono-1,4-lactone dehydrogenase | 0.019 | 0.2 | ns |
| **2. Energy** |              |                 |          |           |
| AT1G79550        | Phosphoglycerate kinase | 0.0069 | 0.2 | 2.0 |
| AT1G24180        | Pyruvate dehydrogenase E1 comp. α-2 | 0.014 | 0.1 | 0.2 |
| AT3G48800        | Aldehyde dehydrogenase 2B4 | 0.022 | 4.3 | 7.1 |
| AT3G60750        | Transketolase | 0.0087 | 0.5 | 4.5 |
| AT2G05710        | Aconitase 3 | 0.002 | 2.5 | 12.0 |
| AT2G20420        | ATP citrate lyase | 0.022 | ns | 5.0 |
| AT5G08300        | Succinyl-CoA ligase, alpha subunit | 0.006 | 2.4 | 2.5 |
| AT4G44330        | Citrate synthase family protein | 0.026 | ns | 5.9 |
| AT1G65930        | Cyt. NADP+-dep. isocitrate dehydrogenase | 0.033 | 0.1 | 2.9 |
| AT1G19120        | Ubiquinol-cytochrome C reductase hinge | 0.016 | 19 | 20 |
| AT3G03100        | NADH-ubiquinone oxidoreductase,17.2kDa | 0.0029 | 0.5 | ns |
| AT5G13430        | Ubiquinol-cytochrome C reductase, Fe-S | 0.02 | 0.2 | ns |
| AT4G14610        | Cytochrome P450, 72A, polypeptide 7 | 0.0026 | 0.1 | 5.4 |
| **3. Cell growth/division** | | | | |
| AT5G52240        | Membrane steroid binding protein 1 | 0.02 | 0.1 | 7.5 |
| AT1G10930        | DNA helicase (RECQl4A) | 0.0036 | 4.6 | 0.1 |
| AT3G44310        | Nitrilase 1 | 0.023 | 2.9 | ns |
| **4. Transcription** | | | | |
| AT1G14850        | Nucleoprotein 155 | 0.00094 | ns | 0.3 |
| **5. Protein synthesis** | | | | |
| AT1G01100        | 60S acidic ribosomal protein | 0.022 | ns | 0.4 |
| AT3G48930        | Nucleic acid-binding, OB-fold-like protein | 0.024 | 2.1 | ns |
| AT2G10930        | Nucleic acid-binding, OB-fold-like protein | 0.027 | ns | 0.3 |
| AT2G09200        | Ribosomal protein L10 | 0.012 | 2.9 | ns |
| AT3G04400        | Ribosomal protein L14p/L23e | 0.0095 | 3.8 | 3.0 |
| AT1G23290        | Ribosomal protein L18/L15 | 0.017 | ns | 0.5 |
| AT4G02220        | Ribosomal protein L19e | 0.0089 | ns | 0.3 |
| AT4G44120        | Ribosomal protein L30/L7 | 0.027 | ns | 0.5 |
| AT5G56710        | Ribosomal protein L31e | 0.013 | 4.6 | ns |
| AT3G45030        | Ribosomal protein S10p/S20e | 0.03 | ns | 2.2 |
| AT5G02960        | Ribosomal protein S12/S23 | 0.016 | 2.3 | ns |
| AT3G60245        | Zinc-binding ribosomal protein | 0.036 | 2.4 | ns |
| **6. Protein destination and storage** | | | | |
| AT1G14980        | Chaperonin 10 | 0.022 | ns | 2.9 |
| AT2G20500        | Chaperonin 60 family protein | 0.045 | 2.2 | 0.4 |
| AT3G20960        | Chaperonin 60 family protein | 0.041 | 2.1 | ns |
| AT5G56030        | Heat shock protein 81-2 | 0.012 | 0.4 | ns |
| AT3G25140        | Tetraicopeptide repeat-containing protein | 0.04 | 5.3 | ns |
| **7. Transporters** | | | | |
| AT4G38850        | Farnesylated protein 6 | 0.017 | 0.4 | ns |
| AT3G15660        | Glutaredoxin 4 | 0.011 | ns | 6.0 |
| AT2G27950        | Glycosylphosphatidylinositol-anchored lipid protein transfer 1 | 0.027 | 16.0 | 44.0 |
| AT1G07670        | Endomembrane-type CA-ATPase 4 | 0.0026 | 0.1 | 5.0 |
| AT4G27200        | Proton pump interaction 1 | 0.006 | ns | 2.4 |
| AT4G39080        | Vacuolar proton ATPase A3 | 0.0041 | 0.5 | ns |
| AT4G25720        | Vacuolar proton PUMP D subunit | 0.044 | 0.4 | ns |
| AT1G15500        | TLC ATP/ADP transporter | 0.0099 | 0.3 | ns |
### Table 1. Cont.

| Accession Number | Protein Name                  | ANOVA (p-Value) | FC 5 min | FC 20 min |
|------------------|-------------------------------|-----------------|----------|-----------|
| AT4G28390        | ADP/ATP carrier 3             | 0.014           | 0.1      | ns        |
| AT5G60460        | Preprotein translocase Sec, Sec61-β subunit | 0.0081          | 4.7      | 4         |
| AT3G51890        | Clathrin light chain protein  | 0.019           | ns       | 0.5       |
| AT2G08530        | Clathrin, heavy chain         | 0.0021          | 0.5      | 0.2       |
| AT3G11130        | Clathrin, heavy chain         | 0.00044         | 0.5      | 0.4       |
| AT5G19760        | Mitochondrial substrate carrier protein | 0.038          | ns       | 2.2       |
| AT5G40810        | Cytochrome C1                 | 0.021           | 0.3      | ns        |

#### 8. Intracellular traffic

| Accession Number | Protein Name                  | ANOVA (p-Value) | FC 5 min | FC 20 min |
|------------------|-------------------------------|-----------------|----------|-----------|
| AT3G49560        | Mit. import inner membrane translocase Tim17 | 0.0031          | 0.1      | ns        |
| AT1G61570        | Mit. import inner membrane translocase 13 | 0.0044          | 17       | 23        |
| AT1G27390        | Translocase outer membrane 20-2 | 0.0015          | 0.1      | 2.1       |
| AT3G60600        | Vesicle associated protein    | 0.051           | ns       | 3         |
| AT4G34660        | SH3 domain-containing protein | 0.039           | 4.7      | ns        |

#### 9. Cell structure

| Accession Number | Protein Name                  | ANOVA (p-Value) | FC 5 min | FC 20 min |
|------------------|-------------------------------|-----------------|----------|-----------|
| AT5G209810       | Actin 7                        | 0.0038          | 0.5      | ns        |
| AT5G19770        | Tubulin alpha-3               | 0.016           | 0.1      | ns        |
| AT5G62690        | Tubulin beta chain 2          | 0.006           | 0.4      | 0.4       |
| AT2G29550        | Tubulin beta-7 chain          | 0.0034          | 0.3      | 0.4       |
| AT2G29860        | Tubulin/βtubulin family protein | 0.036          | 0.4      | 0.5       |
| AT3G09895        | Electron transport SCO1/SenC protein | 0.045          | 0.1      | 4.3       |

#### 10. Signal transduction

| Accession Number | Protein Name                  | ANOVA (p-Value) | FC 5 min | FC 20 min |
|------------------|-------------------------------|-----------------|----------|-----------|
| AT3G14840        | LRR transmembrane protein kinase | 0.025          | ns       | 11        |
| AT3G59840        | Ras-related small GTP-binding protein | 0.0084        | 0.1      | 2.7       |
| AT3G51800        | Metallopeptidase M24           | 0.0015          | 5.1      | ns        |

#### 11. Disease/Defence

| Accession Number | Protein Name                  | ANOVA (p-Value) | FC 5 min | FC 20 min |
|------------------|-------------------------------|-----------------|----------|-----------|
| AT3G57280        | Transmembrane proteins 14C    | 0.014           | 0.1      | ns        |
| AT3G12500        | Basic chitinase               | 0.035           | ns       | 2.1       |
| AT3G32980        | Peroxidase superfamily protein | 0.012          | ns       | 3         |
| AT4G36430        | Peroxidase superfamily protein | 0.015          | 0.1      | ns        |

#### 12. Unclassified

| Accession Number | Protein Name                  | ANOVA (p-Value) | FC 5 min | FC 20 min |
|------------------|-------------------------------|-----------------|----------|-----------|
| AT2G40765        | Unknown protein               | 0.019           | 2        | ns        |
| AT2G46540        | Unknown protein               | 0.033           | ns       | 3.5       |
| AT4G12590        | Transmembrane protein, DUFI06 | 0.033           | 0.3      | ns        |
| AT1G08480        | Unknown protein               | 0.048           | 0.2      | 0.4       |
| AT3G20370        | TRAF-like family protein      | 0.0058          | 0.2      | 3         |
| AT2G9720         | Unknown protein               | 0.016           | 0.1      | 0.3       |
| AT2G24330        | DUFI602, unknown protein      | 0.0077          | 0.1      | 2.2       |
| AT2G33585        | Unknown protein               | 0.019           | 0.1      | 13        |

Notes: no significant change against the mock treated samples; dep.: dependent; comp.: component; cyt.: cytosolic; ANOVA: Analysis Of Variance.

### Table 2. Proteins responsive to abscisic acid (ABA) treatment.

| Accession Number | Protein Name                  | ANOVA (p-Value) | FC 5 min | FC 20 min |
|------------------|-------------------------------|-----------------|----------|-----------|
| AT2G26400        | Acireductone dioxygenase 3    | 0.035           | 3.9      | 6.8       |
| AT4G24200        | D-3-phosphoglycerate dehydrogenase 1 | 0.017          | 0.5      | ns        |
| AT1G17745        | D-3-phosphoglycerate dehydrogenase 2 | 0.029          | 0.1      | 0.2       |
| AT4G19340        | S-adenosyl-L-homocysteine hydrolase | 0.028          | 2.5      | 2.0       |
| AT3G17820        | Glutamine synthetase 1.3      | 0.023           | 3.7      | 3.5       |
| AT5G17770        | NADH-cytochrome B5 reductase 1 | 0.046           | 2.3      | 2.3       |
| AT5G15730        | Phospholipase D α 1           | 0.014           | 3.2      | 3.8       |

#### 2. Energy

| Accession Number | Protein Name                  | ANOVA (p-Value) | FC 5 min | FC 20 min |
|------------------|-------------------------------|-----------------|----------|-----------|
| AT2G01140        | Aldolase superfamily protein  | 0.0075          | 5.0      | 5.1       |
| AT5G43940        | GroES-like zinc-binding dehydrogenase | 0.014          | 4.5      | 5.2       |
| AT4G34870        | Rotamase cyclophilin 5        | 0.0015          | 3.1      | 2.7       |
| AT2G05710        | Aconitase 3                   | 0.035           | 8.0      | 9.9       |
| AT1G15120        | Ubiquitin-cytochrome C reductase hinge | 0.041          | 15.0     | 11.0      |
| AT2G33220        | GRIM-19 protein               | 0.017           | 2.9      | 7.1       |

#### 3. Cell growth/division

| Accession Number | Protein Name                  | ANOVA (p-Value) | FC 5 min | FC 20 min |
|------------------|-------------------------------|-----------------|----------|-----------|
| AT5G43070        | WPP domain protein 1          | 0.042           | 0.4      | 0.2       |
3.1. $H_2O_2$-Responsive Microsomal Proteins

Of the 86 $H_2O_2$-dependent significantly changing proteins, 24 proteins increased and 42 proteins decreased in abundance at 5 min, while, 38 proteins increased and 18 proteins decreased in abundance at 20 min. These proteins were classified into 12 major functional categories [31] (Table 1, in depth detail in Table S2). The most represented functional categories include transporters (17%), energy (15%) and protein synthesis (14%) (Table 1). Gene ontology (GO) analysis of the $H_2O_2$ responsive proteins revealed enrichment in biological processes such as ‘cellular process’, ‘metabolic process’, ‘response to stress’ and ‘transport’ (Table S2). In addition, enriched molecular function categories included ‘structural molecule activity’, ‘transporter activity’ and ‘oxidoreductase activity’ (Table S2).
Further, we performed metabolic pathway analyses using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to find out if any pathways were represented. The ‘purine metabolism’ (13 proteins), ‘thiamine biosynthesis’ (11 proteins), and ‘citrate cycle (TCA cycle)’ (six proteins) (Table S2) were the most represented. The abundance of five of the six proteins associated with the TCA cycle increased, particularly at 20 min after H$_2$O$_2$ treatment (Figure 1). The responsive proteins included pyruvate dehydrogenase (AT1G24180), an essential precursor that links glycolysis to the TCA cycle, citrate synthase (AT2G44350), aconitate 3 (AT2G05710), isocitrate dehydrogenase (AT1G65930), succinyl-CoA ligase, alpha subunit (AT5G08300), and ATP citrate lyase/succinyl-CoA synthetase (AT2G20420), an enzyme that cleaves citrate to oxaloacetate and acetyl CoA in the presence of ATP and CoA, and is implicated in carbohydrate metabolism and production of fatty acids (Table 1; Figure 1). Citrate synthase catalyses the first committed step in the TCA cycle, and has been shown to decrease in activity by 54% upon oxidation by H$_2$O$_2$ [8]. Site-directed mutagenesis of six cysteine residues of citrate synthase showed that the mutant proteins could convert acetyl CoA to oxaloacetate with decreased efficiencies compared to the unmodified citrate synthase. The Cys108Ser and Cys325Ser had their activities decreased by 98%, suggesting that the Cys108 and Cys325 are important for citrate synthase activity. These two nearly inactive forms showed high insensitivity to H$_2$O$_2$, while other mutants just like the unmodified citrate synthase had decreased activities upon H$_2$O$_2$ treatment [8].

Similar to the observed change in citrate synthase upon H$_2$O$_2$ treatment [8], we observed citrate synthase together with pyruvate dehydrogenase significantly decreasing in abundance, particularly 20 min after treatment. Furthermore, it has been shown that pyruvate dehydrogenase and the TCA enzymes, including citrate synthase and aconitase, are sensitive to H$_2$O$_2$ [9]. Of these, aconitase is the most sensitive to the H$_2$O$_2$ effect [9]. Aconitase is one of the five enzymes that exerted the most influence on controlling the TCA, with a control coefficient of 0.964 (the control coefficient is the measure of the ratio of relative change in flux and the relative change in enzyme amount [33]), the second highest after malate dehydrogenase (1.76) [34]. Other enzymes identified that changed in response to H$_2$O$_2$ had a lower control coefficient; for example citrate synthase $-0.4$, isocitrate dehydrogenase $-0.123$ and succinyl CoA ligase 0.0008 [34]. Aconitase converts citrate to isocitrate and contains a Fe-S cluster that is one of the primary target sites for ROS effect [35]. In animals, aconitase activity is suppressed by treatment with 50 µM H$_2$O$_2$, that in turn causes reduced TCA cycle activity in the cardiac cells [36]. In plants, aconitase affects superoxide dismutase transcription, and has been implicated in regulating resistance to oxidative stress, cell death and salt stress in Arabidopsis and Nicotiana benthamiana [37]. This phenomenal role of aconitase in regulating oxidative stress was also supported by our data, in which the abundance of aconitase increased in response to H$_2$O$_2$ as well as ABA (Table 2). The latter may have been an ABA-dependent response or potentially an ABA-dependent H$_2$O$_2$ response. Aconitase has also been observed to increase in abundance in response to cyclic guanosine 3’, 5’-monophosphate (cGMP), a second messenger that also showed a time- and concentration-dependent ROS production [28]. It is important to note that the observed increase in aconitase 3 did not necessarily reflect an increase in its activity that consequently could lead to a TCA flux. This is so because it has been shown that aconitase activity is strongly reduced by H$_2$O$_2$ [9–11]. Rather, the increase may have been channelled towards its role as RNA-binding protein, where it regulates transcription and stability of mRNA transcripts, including its own [38,39]. Besides, the role of aconitase in RNA-binding and oxidative stress requires further analysis. Overall, a total of 36 H$_2$O$_2$-responsive proteins were experimentally linked to potential RNA interaction, including the other TCA cycle enzymes, isocitrate dehydrogenase, succinyl CoA ligase, and succinyl CoA synthase, that increased in abundance at 20 min post H$_2$O$_2$ treatment [39]. Taken together, an increase in the aconitase in response to ABA, cGMP or H$_2$O$_2$ may suggest a potential role of these signalling molecules in modulating oxidative stress as well as energy supplies.
Fifteen proteins decreased in abundance at both 5 and 20 min after ABA treatment, and these included six cytoskeleton-associated proteins (Table 2). Three of these were transporter-associated proteins namely clathrin, heavy chains (AT3G08530 and AT3G11130) and a plasma membrane intrinsic protein 2A (PIP2A, AT3G53420). The latter has been linked to other stress-responses, including temperature and salt [40,41], in addition to a potential role in mRNA binding [39]. The abundance of PIP2A, in particular, has been shown to decrease in response to salt stress [41,42], and much like in the current study, the abundance of PIP2A decreased after 5 and 20 min (although at 20 min it was only 0.6 fold change, and hence less than the cutoff of two-fold) of ABA-treatment. PIP2A is a member of the PIP2 subfamily proteins. PIP2A has water transport activity as depicted in Figure 1.

Figure 1. Tricarboxylic acid (TCA) cycle highlighted proteins changing in abundance after H$_2$O$_2$ treatment of cells. Maroon arrows represent proteins changing at 5 min after treatment and green arrows at 20 min after treatment. “−” signifies no change or a change of less than two-fold, “↑” signifies an increase in abundance, and “↓” signifies a decrease in abundance. Respective protein fold changes are indicated on top of each arrow.

### 3.2. ABA-Responsive Microsomal Proteins

A total of 52 proteins showed differential accumulation in response to ABA and 26 of these proteins have been identified as either RNA-binding proteins or candidate RNA interactors [39]. Abundance of 31 proteins increased and 18 decreased at 5 min after treatment with ABA, while at 20 min, 28 proteins increased and 17 proteins decreased (Table 2). The 52 proteins were classified into 11 functional categories [31], and the most represented functional categories were protein synthesis (19%), metabolism (13%), cell structure (13%), and energy (12%) (Table 2; extended summary in Table S3). A functional enrichment analysis of the ABA-responsive proteins revealed significant enrichment of biological processes, including ‘primary metabolic process’, ‘developmental process’, ‘response to stress’, ‘transport’, and ‘translation’ (Table S3). In addition, the molecular functions were also enriched, and these included ‘structural molecule activity’ and ‘transporter activity’ (Table S3). A metabolic pathway analysis was also performed on the ABA-responsive proteins using KEGG, and ‘purine metabolism’ (seven proteins), ‘thiamine metabolism’ (six proteins), and ‘methane metabolism’ (four proteins) were the most represented pathways (Table S3).

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ABA-dependent phosphoproteomics analysis detected four members of the aquaporin family including PIP2 decreasing in phosphorylation state at a carboxyl-terminal serine that is anticipated to instigate closure of the water-transporting aquaporin gate [46]. PIP2 dephosphorylation prevents rehydration during ABA-regulated seed germination and dormancy, and decreases water flux in response to drought [46]. Arabidopsis knockout mutants lacking the PIP2:1 aquaporin have a stomatal closure defect particularly in response to ABA [47]. In pip2:1 plants, ABA treatment induced an increase in osmotic water permeability of guard cell protoplasts while abolishing accumulation of ROS [47], supporting the involvement of aquaporins in ABA-dependent stomatal movements. The work by Grondin et al. [47] implies that PIP2:1 does significantly contribute to guard cell water permeability in the presence of ABA. Moreover, just like Kline et al. [46], Grondin et al. [47] showed that PIP2 phosphorylation is critical for responses to ABA, in particular in guard cell movements. In the current study, differential analysis of microsomal proteins showed that accumulation of PIP2 decreased in the presence of ABA, suggesting a potential direct or indirect regulation of aquaporins in ABA signalling. This may correlate with the decrease in phosphorylation of PIP2 upon ABA treatment [46].

Among the microsomal proteins that increased in abundance after ABA treatment, D-mannose-binding lectin protein (AT1G78850) and LRR transmembrane protein kinase (LTPK, AT3G14840) (Table 2) showed the greatest increase specifically at 20 min. The two proteins play a role in innate immunity against microbial attack. D-mannose-binding lectin recognises certain carbohydrate moieties on pathogen surfaces [48]. It is involved in the regulation of gene expression and cellular activities in response to increasing levels of hormones such as ABA and jasmonate, or biotic and abiotic (e.g., osmotic) stresses [49–53]. On the other hand, LTPK [also known as LYSM RLK1-INTERACTING KINASE 1 (LIK1)], a plasma membrane localised protein, also increased in abundance at 20 min after H2O2 treatment. Direct interaction between chitin elicitor receptor kinase (AtCERK1) and LIK1, both in vitro and in vivo studies showed that LIK1 is directly phosphorylated by AtCERK1 [54]. In Arabidopsis, AtCERK1 mutants show impairment in chitin responses including the activation of a MAPK cascade, ROS production and expression of chitin-induced genes consequently leading to failure of chitin-induced pathogen resistance [55,56]. Furthermore, Lik1 T-DNA mutant plants produced significantly more ROS in response to chitin and showed an increased resistance to Pseudomonas syringae pv. tomato [54]. The increase in abundance of LTPK in response to the ABA and H2O2 treatments we observed was consistent with the role of LTPK in ROS responses for regulating ROS accumulation that, if in excess, can facilitate necrotrophic infection and promotes programmed cell death [57].

3.3. The Microsomal Proteome of the ABA and H2O2 Responses Show Similarity

A total of 21 proteins were common among proteins significantly changing in response to ABA and H2O2 treatments. Ten of these proteins have been linked in RNA biology [39]. Changes in abundance for most of these proteins were similar (Table 3). Expression of six of these proteins increased in abundance at both 5 and 20 min post-treatment, while six decreased after either ABA or H2O2 treatment (Table 3). The other nine proteins showed dissimilar regulation patterns between the two treatments.
### Table 3. Comparison differential protein accumulation in response to ABA and \( \text{H}_2\text{O}_2 \).

| Accession Number | Protein Name                                      | \( \text{H}_2\text{O}_2 \) 5 min | \( \text{H}_2\text{O}_2 \) 20 min | ABA 5 min | ABA 20 min |
|------------------|--------------------------------------------------|----------------------------------|----------------------------------|-----------|------------|
| **1. Metabolism**|                                                  |                                  |                                  |           |            |
| AT1G17745        | D-3-phosphoglycerate dehydrogenase 2             | 0.1                              | 0.1                              | 0.1       | 0.2        |
| AT5G17770        | NADH cytochrome B5 reductase 1                   | 0.4                              | 2.5                              | 2.3       | 2.3        |
| **2. Energy**    |                                                  |                                  |                                  |           |            |
| AT2G05710        | Aconitase 3                                      | 2.5                              | 12                               | 8         | 9.9        |
| AT1G15120        | Ubiquinol-cytochrome C reductase hinge           | 19                               | 20                               | 15        | 11         |
| **3. Transcription** |                                              |                                  |                                  |           |            |
| AT1G14850        | Nucleoporin 155                                  | ns                               | 0.3                              | 0.1       | 0.2        |
| **4. Protein synthesis** |                                |                                  |                                  |           |            |
| AT5G04400        | Ribosomal protein L14p/L23e                      | 3.8                              | 3                                | 3.5       | 2.6        |
| ATG45030         | Ribosomal protein S10p/S20e                     | ns                               | 2.2                              | 2.3       | ns         |
| AT1G01100        | 60S acidic ribosomal protein family              | ns                               | 0.4                              | 0.4       | 0.4        |
| **5. Protein destination and storage** |                                      |                                  |                                  |           |            |
| AT1G14980        | Chaperonin 10                                    | ns                               | 2.9                              | 2.5       | 3          |
| AT5G56030        | Heat shock protein 81-2                          | 0.4                              | ns                               | 0.4       | 0.5        |
| **6. Transporters** |                                              |                                  |                                  |           |            |
| AT4G27900        | Proton pump interactor 1                         | ns                               | 2.4                              | ns        | 2.3        |
| AT3G08530        | Clathrin, heavy chain                            | 0.5                              | 0.2                              | 0.2       | 0.4        |
| AT3G11130        | Clathrin, heavy chain                            | 0.5                              | 0.4                              | 0.3       | 0.4        |
| **7. Intracellular traffic** |                                      |                                  |                                  |           |            |
| AT1G61570        | Mit. import inner membrane 13 translocase       | 17                               | 23                               | 19        | 11         |
| **8. Cell structure** |                                              |                                  |                                  |           |            |
| AT5G09810        | Actin 7                                          | 0.5                              | ns                               | 0.5       | 0.5        |
| AT4G14960        | Tubulin/FtsZ family protein                      | 0.4                              | 0.5                              | 0.4       | 0.3        |
| AT5G62690        | Tubulin beta chain 2                             | 0.4                              | 0.4                              | 0.3       | 0.4        |
| AT2G29550        | Tubulin beta-7 chain                             | 0.3                              | 0.4                              | 0.3       | 0.4        |
| AT5G19770        | Tubulin alpha-3                                  | 0.1                              | ns                               | 0.5       | 0.3        |
| **9. Signal transduction** |                                      |                                  |                                  |           |            |
| AT3G51800        | Metalloproteidase M24 family protein             | 5.1                              | ns                               | 3.4       | ns         |
| AT3G14840        | LRR transmembrane protein kinase                 | 11                               | 11                               | 8.3       | 14         |

Three proteins, ubiquinol-cytochrome C reductase hinge (UQCRH, AT1G15120), D-3-phosphoglycerate dehydrogenase (AT1G17745) and NADH:cytochrome B5 reductase 1 (Cytb5R, AT5G17770), belonging to the molecular function category ‘oxidoreductase activity’ (Table S2), showed differential accumulation in response to either ABA or \( \text{H}_2\text{O}_2 \) (Table 3). Oxidoreductase-related proteins have been shown to control responses to calcium homeostasis in organelles such as endoplasmic reticulum [58]. Besides, amongst the identified ABA and \( \text{H}_2\text{O}_2 \)–responsive proteins, UQCRH was one of the proteins increasing in abundance the most at both time points. It is a component of the mitochondrial respiratory chain complex III. Cytb5R is an integral membrane-bound flavoprotein that is localised mainly in the endoplasmic reticulum and the outer mitochondrial membrane [59]. It is an electron donor for Cytb5, a membrane-haeme-containing protein [60]. Non-cytotoxic concentration of \( \text{H}_2\text{O}_2 \) (24 \( \mu \text{M} \)) has been shown to induce a significant up-regulation of Cytb5R, suggesting a redox regulation [61]. However, in this study, we observed that a higher concentration of \( \text{H}_2\text{O}_2 \) (50 \( \mu \text{M} \)) can cause a decrease of Cytb5R abundance as an early response, and an increase at 20 min. This may suggest concentration-dependent redox regulation by Cytb5R in response \( \text{H}_2\text{O}_2 \) as well as ABA.

Within the common proteins set, the most represented functional category was the cell structure, comprising of four tubulins and one actin. Tubulin/FtsZ protein (AT4G14960), an \( \alpha \)-tubulin isoform that is required for right handed helical growth, is part of the tubulin complex or structural constituent of the cytoskeleton [62]. Tubulins showed differential expression in response to salt stress, for example, abundance of tubulin \( \beta \)-chain 2 (AT5G62690) decreased after salt treatment [63]. Tubulin has also been shown to regulate low nitrate-induced anthocyanin biosynthesis and plant nitrate response regulatory network in collaboration with other signalling pathways including ABA and \( \text{H}_2\text{O}_2 \) [64]. The four tubulins and actin 7 identified in the current study were all down-regulated in response to ABA and \( \text{H}_2\text{O}_2 \), and showed reduction in transcriptional level during senescence suggesting that the cytoskeletal
organization-related proteins are affected by oxidative stress. Further, the cytoskeleton has been shown to be essential for mitochondrial morphology, movement and immobilization. The actin cytoskeleton, for example, is involved in the immobilization of mitochondria at the cortex in cultured tobacco cells, and the cytoskeleton may be critical for retaining mitochondria at sites of high ATP demand (for review see [65]).

To further examine the mutual effect of ABA and H$_2$O$_2$, in particular to shed light on the common protein cohort whether the ABA effect is a result of the H$_2$O$_2$ signalling initiated via ABA pathway, we performed an ABA assay following H$_2$O$_2$ treatment of Arabidopsis cell suspension cultures and vise-versa. We noted no significant change in ABA quantity at 5 and 20 min post-H$_2$O$_2$ treatment. This may be attributed to the short treatment times aimed at observing early stimuli responses, or potentially from the low concentration of H$_2$O$_2$ used. Previously, it has been revealed that treating Arabidopsis guard cells with 100 µM H$_2$O$_2$ for 25 and 30 min induced an ABA-mediated nitric oxide production, which in turn is dependent on ABA-induced H$_2$O$_2$ synthesis [66]. Furthermore, a ROS assay was performed after treating Arabidopsis cell suspension cultures with 50 µM ABA. Here, we observed an increase in ROS accumulation at 20 min when compared with the controls (collected at 0 and 20 min) (Figure 2). This observation is not new as previously, ABA has been shown to induce H$_2$O$_2$ production through NADPH oxidase [67] and in guard cells of *Vicia faba*, ABA was also observed to generate H$_2$O$_2$ [68]. Additionally, in Arabidopsis, ROS accumulation was noted to occur upon ABA-induced stomatal closure [69]. Therefore, this suggests that ABA directly and/or indirectly induce ROS production in the cells that may lead to the observed common regulation of downstream processes with H$_2$O$_2$.

![Figure 2](image_url) Reactive oxygen species (ROS) assay. OxiSelect™ Intracellular ROS assay kit (Cell Biolabs, Inc., San Diego, CA, USA) was used in the in vivo oxidation experiments using cultured Arabidopsis (Col-0) cells according to the assay protocol provided by the manufacturer. Each bar represents data from four biological replicates ($n = 4$) with a calculated standard error. Treatment of cells with ABA at the final concentration of 50 µM induces a statistically significant increase in H$_2$O$_2$ production, $p < 0.001$ (represented by two asterisks (**)) symbols) using a two-sample *t*-test.

4. Conclusions

Shotgun proteomics approaches can distinguish common and specific responses linked to ABA or H$_2$O$_2$ treatments. The analysed proteomics data also revealed over 30 microsomal proteins associated with RNA interaction [70]. Furthermore, amongst the common set of proteins, we detected three proteins, namelyaconitase 3, UQCRH, and inner mitochondrial membrane translocase 13, to be the
most responsive and potentially as promising signatures for both ABA and H$_2$O$_2$ although they require further investigation to determine their mode of action. Besides, the fact that most of the common proteins changed in abundance in a similar manner is consistent with ABA-induced ROS production. Finally, we also show that a significant protein subset responds specifically to either ABA or H$_2$O$_2$, suggesting that these molecules also modulate independent complex intracellular responses.

**Supplementary Materials:** The following are available online http://www.mdpi.com/2227-7382/5/3/22/s1, Table S2: H$_2$O$_2$ differentially expressed proteins and GO analysis, Table S3: ABA differentially expressed proteins and GO analysis.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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