



Proteomic analysis reveals dynamic regulation of fruit ripening in response to exogenous ethylene in kiwifruit cultivars

Mi Hee Shin¹ · Sowbiya Muneer^{1,7} · Yun-Hee Kim^{2,6} · Jeung Joo Lee^{3,6} · Dong Won Bae⁴ · Yong-Bum Kwack^{5,8} · H. M. Prathibhani C. Kumarihami¹ · Jin Gook Kim^{1,6} 

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Abstract

Understanding the fruit ripening mechanism is critical for fruit quality improvement. Although postharvest ethylene application is known to enhance the onset of fruit ripening, the exact mechanisms remain unclear. In this study, a gel-based proteomic analysis was performed to investigate the changes in protein profiles during the ripening of exogenous-ethylene-treated kiwifruit (*Actinidia deliciosa*) cultivars ‘Hayward’ and ‘Garmrok’. Based on comparative two-dimensional gel electrophoresis, most of the proteins were aggregated in exogenous-ethylene-treated kiwifruit compared to the untreated kiwifruit. Consequently, 90 and 106 proteins were differentially expressed in ‘Hayward’ and ‘Garmrok’ kiwifruit, respectively. Among the successfully identified proteins by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry, the 50% in ‘Hayward’ kiwifruit and 60% in ‘Garmrok’ kiwifruit were associated with ripening. Also, 18% and 10% of proteins were associated with defense response in ‘Hayward’ and ‘Garmrok’ kiwifruit, respectively. The other major proteins were related to protein biosynthesis and photosynthesis/Calvin cycle during kiwifruit ripening. We used bioinformatics analysis to determine the interactions between identified proteins, and this proteomic approach provided insights into biological pathways and molecular functions in postharvest ripening of exogenous-ethylene-treated kiwifruit.

Keywords *Actinidia deliciosa* · Ethylene · Fruit ripening · Mass spectrometry · Postharvest · Proteomics

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✉ Jin Gook Kim
jgkim119@gnu.ac.kr

¹ Department of Horticulture, College of Agriculture and Life Sciences, Gyeongsang National University, Jinju 52828, Republic of Korea

² Department of Biology Education, College of Education, Gyeongsang National University, Jinju 52828, Republic of Korea

³ Department of Agricultural Plant Science, College of Agriculture and Life Sciences, Gyeongsang National University, Jinju 52828, Republic of Korea

⁴ Center for Research Facilities, Gyeongsang National University, Jinju 52828, Republic of Korea

1 Introduction

Two major species of kiwifruit (*Actinidia* spp.) have been commercialized worldwide: *Actinidia deliciosa* and *Actinidia chinensis*. At present, the predominant kiwifruit cultivar is *A. deliciosa* ‘Hayward’, owing to its larger fruit, better appearance, high vitamin C content, long storage life, and

⁵ Namhae Sub-Station, National Institute of Horticultural and Herbal Science, Rural Development Administration, Namhae 52430, Republic of Korea

⁶ Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea

⁷ Present Address: School of Agricultural Innovations and Advanced Learning, Vellore Institute of Technology, Vellore 632014, India

⁸ Present Address: Department of Fruits, Korea National College of Agriculture and Fisheries, Jeonju 54874, Republic of Korea

excellent flavour. In Korea, most kiwifruit cultivations are scattered in the southeast region, and the most prominent commercial kiwifruit cultivar has been ‘Hayward’ since the 1990s, although there are several other newly released commercial kiwifruit cultivars from Korea, i.e., *A. deliciosa* ‘Garmrok’, ‘Jecy Green’, and ‘Jecy Sweet’ and *A. chinensis* ‘Jecy Gold’ and ‘Goldone’ (Kwack and Park 2007; Kwack et al. 2017).

Kiwifruit is harvested at the physiologically mature, but unripe, stage, and ripening can be initiated by exposure to exogenous ethylene (Tanou et al. 2015). Fruit ripening has been typically defined as either “climacteric” or “non-climacteric” depending on the presence or absence of the climacteric rise in respiration and autocatalytic ethylene production (Giovannoni 2004; Minas et al. 2016). In climacteric fruit, the gaseous plant hormone ethylene is the major cue that controls most aspects of ripening. Further, climacteric fruit exhibits a sudden burst in the respiration rate and the ethylene biosynthesis at the onset of ripening (Giovannoni 2004; Antunes 2007; Yin et al. 2008; Minas et al. 2012, 2016; Tanou et al. 2015). Kiwifruit also has been classified as a climacteric fruit and is believed to be extremely sensitive to ethylene, which accelerates ripening markedly when exposed to even minute concentrations of ethylene (i.e., $0.01 \mu\text{L L}^{-1}$) (Antunes 2007). Exogenous ethylene treatment increased endogenous ethylene production in kiwifruit by enhancing activity of two enzymes involved in ethylene synthesis, i.e., 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO), characterized by a period of autocatalytic ethylene production, a well-known phenomenon in climacteric fruit (Antunes 2007; Minas et al. 2016). Further, kiwifruit ripening has been characterized by physiological and biochemical processes, including ethylene biosynthesis, conversion of starch to sugar, changes in cell wall constituents, organic acid accumulation, and production of characteristic volatiles, resulting in changes in fruit traits such as color, texture, flavor, and aroma, which are desired by consumers. In general, fruit ripening is a unique physiological and biochemical process that is predominantly regulated by ripening-related genes and proteins through a network of signaling pathways (Minas et al. 2016). Further, the expression of specific genes and the subsequent synthesis of proteins and enzymes required for the onset and progression of ripening are affected by internal and external factors (Giovannoni 2004).

In particular, the ripening behavior of kiwifruit is well documented, but until recently, it was limited primarily to the significant molecular understanding of the role and regulation of ethylene biosynthesis. Nevertheless, recently, the proteomic approach became widely recognized as a powerful tool that enables a more systematic investigation of fruit development and fruit ripening. The particular proteins related to fruit ripening undergo various post-translational

modifications during ethylene biosynthesis and elaborated most of the fruit sensory and nutritional quality traits at the fruit ripening stage (Minas et al. 2012). In recent years, proteomic approaches have furthered our understanding of biological processes during ripening of many types of fruit, such as grape (Deytieux et al. 2007; Negri et al. 2008), tomato (Faurobert et al. 2007; Page et al. 2010), citrus (Muccilli et al. 2009), banana (Li et al. 2015a), muskmelon (Li et al. 2015b), strawberry (Bianco et al. 2009), mango (Andrade et al. 2012), papaya (Huerta-Ocampo et al. 2012; Nogueira et al. 2012), and apple (Costa et al. 2010), which has paved the way for their use in kiwifruit. Proteomics in combination with phenotypical and physiological data offers a promising approach to further characterize fruit development and quality traits. In kiwifruit, comparative proteomic approaches already have been applied to elucidate important aspects of plant–microbe interactions by revealing substantial changes in the proteome composition of infected plant organs (Petriccione et al. 2013, 2014; Liu et al. 2018). Particular attention was also paid to the prediction of the protein–protein interaction networks in kiwifruit development and ripening (Minas et al. 2012, 2016; Tanou et al. 2015; Ainalidou et al. 2016; Lin et al. 2017), but this was mostly limited to the kiwifruit cultivar ‘Hayward’. These proteomic investigations provide information on both protein quantity and activity and identify protein–protein interactions during kiwifruit development and ripening (Lin et al. 2017). In this study, a gel-based proteomic analysis was used to investigate the changes in protein profiles during kiwifruit ripening after exogenous ethylene treatment in two kiwifruit cultivars, *A. deliciosa* ‘Hayward’ and ‘Garmrok’, and the proteins were sequenced using matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS) and bioinformatics analysis.

2 Materials and methods

2.1 Fruit material

Kiwifruit (*Actinidia deliciosa*) cultivars ‘Hayward’ and ‘Garmrok’, grown under conventional cultural practices, were harvested on November 4, 2016 from the Namhae Branch, National Institute of Horticultural and Herbal Science, RDA, Republic of Korea, at physiologically mature stage as determined by the days after full bloom (170 DAFB) and soluble solids content ($\geq 7\%$). After harvest, all kiwifruit were packaged immediately in 10 kg corrugated cardboard boxes and moved to the laboratory of fruit science, Gyeongsang National University, Republic of Korea. Uniformly sized fruit, free from defects, were selected randomly for each cultivar.

The green-fleshed kiwifruit cultivar ‘Hayward’ was introduced to Korea in the 1970s. The kiwifruit cultivar ‘Garmrok’ was bred and released by the Rural Development Administration, Republic of Korea, in mid-2013, and this cultivar has a good taste, is resistant to chilling injuries, and can be harvested about 1 week earlier compared to ‘Hayward’ kiwifruit grown in Korea (Kwack and Park 2007; Kwack et al. 2017). Up to now, the kiwifruit cultivar ‘Hayward’ has been extensively researched in numerous aspects, whereas there have been few studies on newly introduced cultivars such as ‘Garmrok’, one of the prominent kiwifruit cultivars grown and commercialized in Korea. Hence, in this study, we selected *A. deliciosa* ‘Hayward’ and ‘Garmrok’ to enhance our understanding of the biological pathways and molecular functions in postharvest kiwifruit ripening in response to exogenous ethylene application.

2.2 Exogenous ethylene treatment

Kiwifruit were exposed to ethylene at a concentration of $1000 \mu\text{L L}^{-1}$ for 24 h at 20°C in an airtight acrylic box. This concentration of ethylene was applied based on our previous experiments, as optimum kiwifruit ripening occurred at this concentration (Shin 2018; Shin et al. 2019). Ethylene-treated kiwifruit were stored at 20°C for 9 days, and the data were recorded at 3 day intervals (0, 3, 6, and 9 days). Simultaneously, nontreated kiwifruit were evaluated as a control.

2.3 Physiochemical analysis

2.3.1 Soluble solids content

The soluble solids content (SSC) was assayed in 10 biological replicates. The fruit were wrapped in four layers of cheese cloth and squeezed, and the absorbance was read using a handheld refractometer (Pocket Refractometer, PAL-1, Atago Co. LTD, Tokyo, Japan) and was expressed in %.

2.3.2 Titratable acidity

The titratable acidity (TA) was assayed by titrating with 0.05 mol L^{-1} NaOH to an endpoint of pH 8.1 using a professional benchtop BP3001 pH meter (Trans Instruments, Singapore). TA was measured in three replicates, each a combined extract obtained from 10 fruit. The results were recalculated and presented as anhydrous citric acid equilibrium, expressed as percentages.

2.3.3 Firmness

Fruit firmness (flesh and core firmness) was measured on 10 biological replicates using a rheometer (RHEO TEX SD-700, Sun Scientific Inc, Japan) fitted with an 8 mm

round flat probe. Each piece of fruit was compressed to a 3 mm depth at the crosshead speed of 120 mm min^{-1} at room temperature ($20 \pm 2^\circ\text{C}$), and the maximum force developed during the test was recorded and expressed in Newton (N).

2.3.4 Ethylene production and respiration rates

The ethylene production and respiration rates were determined with three biological replicates. Three pieces of fruit were weighed, and each piece was placed in a separate 680 mL volume airtight polypropylene container (HPL851-2.1L, Locknlock, China) fitted with a rubber septum for 4 h at room temperature ($20 \pm 2^\circ\text{C}$). Ethylene production was measured by withdrawing a 1 mL headspace gas sample from each container through the rubber septum using a 1 mL syringe and injecting it into a gas chromatograph (GC-7890B; Agilent Technologies, USA) equipped with a stainless steel column ($2.0 \text{ m} \times 3.0 \text{ mm i.d.}$) packed with Porapak Q (Shinwa, Kyoto, Japan) and a flame ionization detector (FID). Respiration rate was measured by means of CO_2 emission in the gas phase of the container. A 1 mL headspace gas sample from each container was withdrawn through the rubber septum using a 1 mL syringe, and the CO_2 concentration was determined using a gas chromatograph (GC 6890, Agilent Technologies, USA) equipped with a stainless steel column ($2.0 \text{ m} \times 3.0 \text{ mm i.d.}$) packed with Shincarbon ST (Shinwa, Kyoto, Japan) and a thermal conductivity detector (TCD).

2.4 Protein extraction

For proteomic analysis, three biological replicates of three fruit were used after exogenous ethylene treatment. The ethylene-treated kiwifruit were peeled off and separated into flesh and core samples. Then, kiwifruit flesh and core samples were separately ground in liquid nitrogen and immediately stored at -80°C until further analysis. The total proteins from kiwifruit flesh and core samples were extracted according to the phenol-based method described by Lee et al. (2012) with slight modifications. Ten grams of frozen powder samples were suspended directly in 20 mL of extraction buffer (pH 8.8) containing 0.9 M sucrose, 0.1 M Tris-HCl, 10 mM EDTA, and 0.4% (v/v) β -mercaptoethanol and homogenized by vigorous vortexing. The samples were incubated at 4°C for 10 min, and an equal volume (5 mL) of Tris-buffered phenol (pH 8.8) was added followed by vigorous vortexing. The samples were then centrifuged at 3500 rpm for 20 min to separate the insoluble materials. The resulting supernatant (upper phenolic phase) was collected, and the same procedure was repeated three to four times to increase the protein extractability. Thereafter, the total protein was precipitated from the phenol phase overnight at -20°C using 100 mM ammonium acetate mixed in

methanol, and the collected protein pellet was washed four to five times with precipitation solution and 80% acetone. The acetone-washed protein pellet was dried at room temperature and dissolved in lysis buffer containing 9 M urea, 2% CHAPS, 0.2% (w/v) Phamalyte, pH 3–10, and 50 mM dithiothreitol (DTT) immediately prior to isoelectric focusing (IEF). Finally, the extracted proteins were quantified according to the Bradford (1976) method.

2.5 Isoelectric focusing and two-dimensional gel electrophoresis

Isoelectric focusing (IEF) and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed according to the method described by Muneer et al. (2016). Total protein extract (400 µg) was loaded on 17 cm immobilized pH gradient (IPG) gel strips (Bio-Rad; pH 4–7) passively rehydrated overnight. The IEF conditions were as follows: 250 V for a conditioning step of 15 min, followed by a slow ramping step to 10,000 V for 3 h, and finally 10,000 V for 9 h. After IEF, the IPG strips were equilibrated by gentle shaking for 15 min in 2–3 mL of equilibration buffer (8 M urea, 2% SDS, 50 mM Tris-HCl (pH 8.8), and 20% (v/v) glycerol) with 1% DTT added for protein reduction, followed by another 15 min in 2–3 mL of the same equilibration buffer with 2.5% iodoacetamide (IAA) added for protein alkylation. Second-dimension SDS-PAGE was performed with 12.5% (w/v) SDS–polyacrylamide gels using a PROTEAN II system (Bio-Rad, Hercules, CA, USA) at a voltage gradient of 70–120 V for 8–9 h. The molecular weights of proteins were determined by running a commercial pre-stained molecular marker (Intron Biotechnology, Seongnam City, Republic of Korea) on one side of the SDS-PAGE gels. The two-dimensional electrophoresis (2-DE) gels were stained with colloidal Coomassie Brilliant Blue. All 2-DE analyses were repeated three times as replicates.

2.6 Image and data analysis

In each treatment, three independent biological replicate were analyzed. Digital images of 2-DE gels were acquired and analyzed under constant settings by a photo imager using PDQuest 2-D image analysis software (version 7.2.0; Bio-Rad, Hercules, CA, USA).

2.7 Protein in-gel digestion and identification by matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-TOF-MS)

Excised protein spots were subjected to in-gel digestion according to the method described by Muneer et al. (2014)

with slight modifications. The digested peptide solution was spotted onto the MALDI-TOF MS target plate using a pipette, and MALDI-MS analysis was performed with a Voyager DE-STR mass spectrometer (ABI 4800, Applied Biosystems, Framingham, MA, USA).

2.8 Data processing, functional annotations, and protein–protein interactions

Hierarchical clustering of differentially expressed protein spots was performed using hierarchical clustering explorer software (HCE 3.5 Interactive Power Analysis). Functional annotations of the identified proteins were conducted using Panther gene analysis (<http://pantherdb.org/>). To determine the functions and interactions of the identified proteins, a protein–protein interaction network (PPI) was analyzed using the online tool STRING 9.0 (<http://string-db.org>).

2.9 RNA isolation, cDNA preparation, and qRT-PCR

Isolation of RNA from kiwifruit flesh was performed using an RNA isolation kit according to the manufacturer's instructions (Intron Biotechnology, Seongnam-City, Republic of Korea). The isolated RNA was quantified using a NanoDrop spectrophotometer. One microgram of DNAase-treated RNA was reverse transcribed using a reverse transcriptase kit (Promega, Madison, WI, USA) to synthesize first-strand cDNA. Quantitative real-time PCR was performed with a Rotor-Gene Q 2plex HRM platform using SYBR green as a reference dye. All quantifications were normalized to actin with three replicates. The gene-specific primers used in this study are as follows: *ACS1* (accession number U86865), F-5'-CGGTACTGTTTTAGCTCTCCAGACT-3' and R-5'-TGGAGTGGCTACTGTCCTTTAGAA-3'; *ACO3* (accession number HQ293206), F-5'-GGCAACCAAAGGCCTAGAG-3' and R-5'-GGTGCTTTCCAGTCCAAATC-3'; and *ACT1* (accession number DQ682826), F-5'-TCCTTCGTCTTGACCTTGCT-3' and R-5'-AATTGTGATGAAGTACCAT-3'.

2.10 Statistical analysis

For physiochemical parameters, a complete randomized design was used with 10 (SSC and flesh and core firmness) and three (TA, ethylene production, and respiration rates) biological replicates. For proteomic analysis, three biological replicates of three fruit were used. Experimental data represent means with standard error bars ($n = 10$ and $n = 3$), and values with different letters are significantly different at $p \leq 0.05$ according to Tukey's studentized range test performed with SAS software (SAS Inst., Cary, N.C., USA).

3 Results

3.1 Physiochemical analysis of kiwifruit's response to exogenous ethylene treatment

Figure 1 shows the progression of kiwifruit ripening after exogenous ethylene treatment. The typical phenotype changes during kiwifruit ripening were observed in both control and ethylene-treated kiwifruit, which enabled the definition of two well-characterized groups for differential proteome profiling. The physiochemical changes in exogenous-ethylene-treated kiwifruit, stored at 20 °C were evaluated over 9 days (Fig. 2a–f). The link between the kiwifruit physiology and kiwifruit proteome changes was made based on the physiochemical measurements, i.e., SSC, TA, flesh and core firmness, ethylene production, and respiration rates during the ripening of exogenous-ethylene-treated kiwifruit.

Figure 2a shows the changes in SSC of two kiwifruit cultivars during storage at 20 °C. The exogenous-ethylene-treated ‘Garmrok’ and ‘Hayward’ kiwifruit showed a rapid increase in SSC compared to the untreated kiwifruit. After

9 days of storage, the exogenous-ethylene-treated ‘Garmrok’ kiwifruit recorded the highest SSC value (15.8%) and a bigger increase in SSC compared to that of ‘Hayward’ kiwifruit treated with exogenous ethylene (12.5%) (Fig. 2a). The TA of exogenous-ethylene-treated ‘Garmrok’ and ‘Hayward’ decreased more rapidly than that of untreated kiwifruit (Fig. 2b). After 9 days of storage, the TA of exogenous-ethylene-treated ‘Hayward’ kiwifruit reached 0.9%, while it was 0.6% in ‘Garmrok’ (Fig. 2b). Flesh firmness of exogenous-ethylene-treated kiwifruit declined gradually from 34.4 to 3.1 N in ‘Garmrok’ and from 56.7 to 4.6 N in ‘Hayward’ (Fig. 2c), while the core firmness declined gradually from 76.9 to 4.8 N in ‘Garmrok’ and from 95.9 to 10.6 N in ‘Hayward’ (Fig. 2d).

The exogenous ethylene treatment significantly accelerated kiwifruit ripening with typical climacteric changes in ethylene production and respiration 3 days after treatment (Fig. 2e, f). According to the physiochemical measurements, both cultivars were ready to eat 6 days after ethylene treatment. Kiwifruit after 9 days of ethylene treatment were not used for further analysis due to over-ripening, which made them inedible.



Fig. 1 The progression of ‘Garmrok’ and ‘Hayward’ kiwifruit ripening after exogenous ethylene treatment and the typical phenotype changes during ripening in control and ethylene-treated kiwifruit,

which enabled the definition of two well-characterized groups for differential proteome profiling. DAT days after treatment

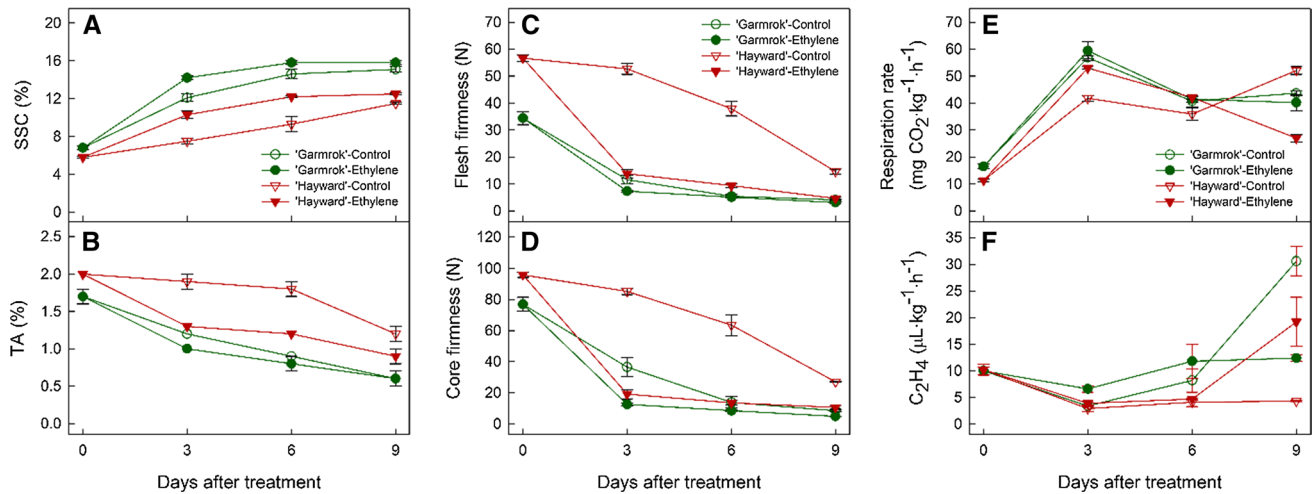


Fig. 2 Physiochemical changes in ‘Garmrok’ and ‘Hayward’ kiwifruit in response to exogenous ethylene treatment: **a** SSC, **b** TA, **c** flesh firmness, **d** core firmness, **e** respiration rate, and **f** ethylene production rate. Vertical bars indicate SE with $n = 10$ (**a**, **c**, and **d**) and $n = 3$ (**b**, **e** and **f**)

3.2 2-DE and image analysis during kiwifruit ripening in response to exogenous ethylene treatment

The exogenous-ethylene-treated kiwifruit exhibited rapid softening compared to untreated kiwifruit. A comparative 2-DE analysis was performed to detect the differentially expressed proteins in ethylene-treated kiwifruit (Fig. 3 and

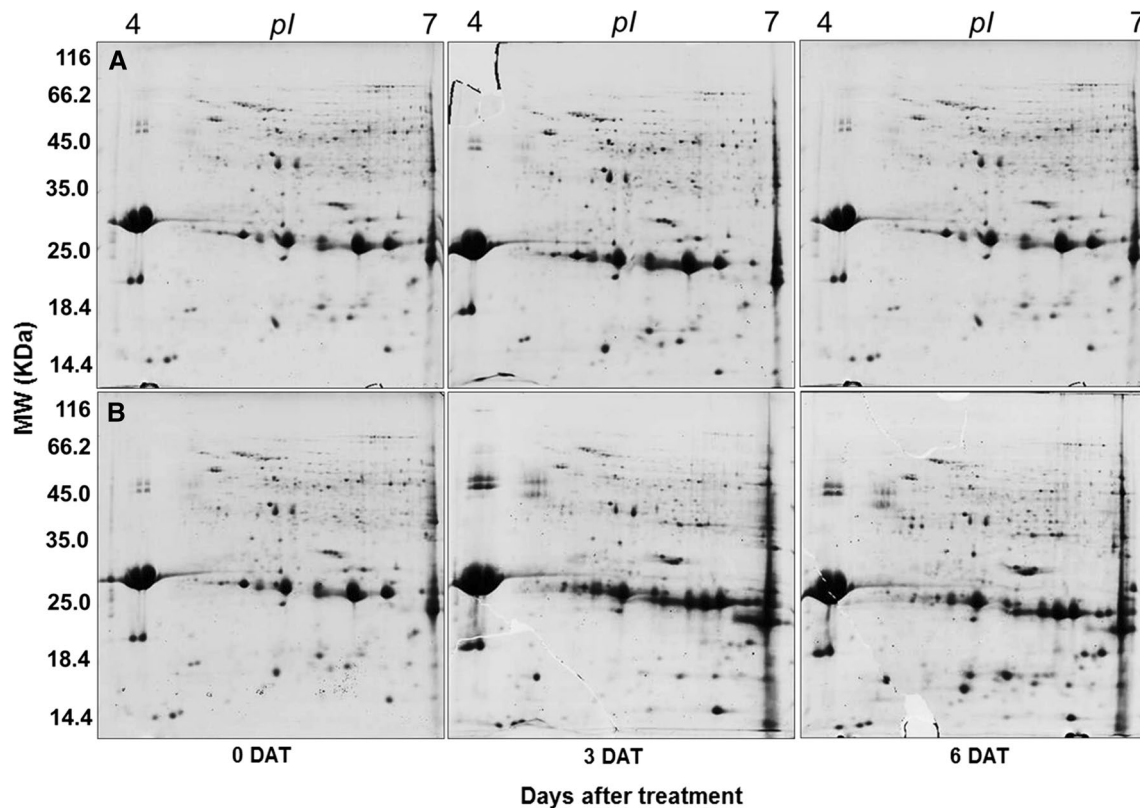


Fig. 3 Representative 2-DE gel electrophoresis maps with the total protein profile of **a** ‘Hayward’ and **b** ‘Garmrok’ kiwifruit cultivars at 0, 3, and 6 days after exogenous ethylene treatment. Total proteins were extracted by phenol, and 400 μg of protein samples was sepa-

rated by IEF using 17 cm IPG strips with a pH range of 4–7. Focused strips were processed for 2DE-PAGE and stained with colloidal Coomassie Brilliant Blue

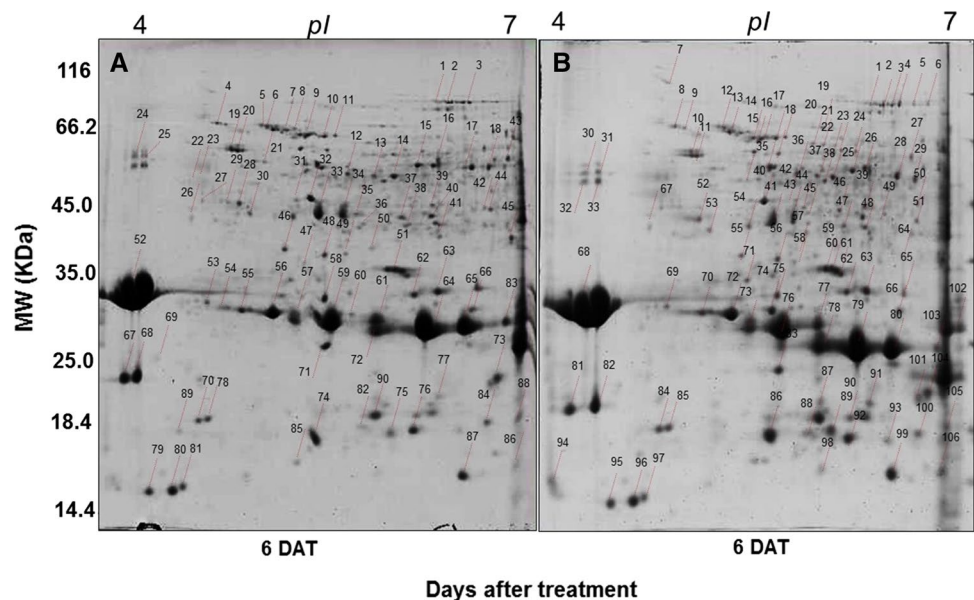
Supplementary Fig. 1). The comparison of kiwifruit 2-DE maps at 0, 3, and 6 days after ethylene treatment was done using Progenesis SameSpots TotalLab (Supplementary Tables 1 and 2). Accordingly, the 600 and 700 protein spots were detected in the 2-DE maps related to ‘Hayward’ kiwifruit at 3 and 6 days after ethylene treatment, respectively, while 500 protein spots were observed for the untreated ‘Hayward’ kiwifruit (Fig. 6a). ‘Garmrok’ kiwifruit at both 3 and 6 days after ethylene treatment showed 700 protein spots in the 2-DE maps, while 600 protein spots were observed for the untreated ‘Garmrok’ kiwifruit (Fig. 6b). After the comparative analysis, the kiwifruit at 6 days after ethylene treatment were used to generate a high-resolution 2-DE map to select the protein spots for further analysis of the differentially expressed proteins (Fig. 4). Consequently, 90 proteins in ‘Hayward’ kiwifruit and 106 in ‘Garmrok’ kiwifruit were differentially expressed (Figs. 4, 6a, b) in response to exogenous ethylene treatment. Moreover, a hierarchical clustering was performed to separate the overall expression profiles of the differentially expressed proteins (Fig. 5). The results of hierarchical cluster analysis clearly demonstrated a dynamic change in protein population in both ‘Hayward’ and ‘Garmrok’ kiwifruit through ripening in response to exogenous ethylene treatment. Accordingly, the most significant protein changes occurred 6 days after ethylene treatment. In addition, a statistically clear quantitative difference between each differentially expressed protein was observed using Progenesis (Supplementary Tables 1 and 2).

3.3 Protein identification and functional classifications during kiwifruit ripening in response to exogenous ethylene treatment

The identification of kiwifruit protein spots was difficult due to the lack of genome sequence data. The interest or differentially expressed proteins in the protein maps of two kiwifruit cultivars were excised and analyzed using MALDI-TOF/TOF MS (Tables 1, 2, Supplementary Tables 3 and 4). The 90 and 106 differentially expressed proteins were analyzed using MALDI TOF/TOF for the ‘Hayward’ and ‘Garmrok’ kiwifruit, respectively, while 80 and 90 differentially expressed proteins were successfully identified for each cultivar, respectively. Most of the identified proteins were successfully annotated in a universal protein database (Uniprot/Swissport/NCBI).

In ‘Hayward’ kiwifruit, 10 major proteins related to defense response were identified, i.e., chloroplast heat shock protein 70–20 (no. 4), heat shock protein 70 (no. 5), putative defensive-like protein (nos. 6, 84, and 85), protein-like kinase (no. 7), ATP synthase subunit alpha (no. 15), NADP quinone reductase (no. 16), actin (no. 50), and metallothionein-like protein type 3 (no. 87) (Table 1). Also, nine major proteins related to defense response were identified in ‘Garmrok’ kiwifruit, i.e., heat shock protein 70 (nos. 8 and 12), GDP-L-galactose phosphorylase (nos. 40 and 54), calmodulin (no. 44), ACC oxidase (no. 44), metallothionein-like protein type 3 (nos. 57 and 99), and putative glycine-rich RNA binding protein type 3 (no. 99) (Table 2). Another six key proteins related to protein biosynthesis/translation were identified in ‘Hayward’ kiwifruit, i.e., elongation factor elongation factor 2-like (no. 1), ribosomal protein S12 (no. 30), and 30S ribosomal protein S7 (nos. 33, 38, 57, and 72)

Fig. 4 A high-resolution 2-DE map generated to select the protein spots for further analysis of the differentially expressed proteins 6 days after exogenous ethylene treatment in **a** ‘Hayward’ and **b** ‘Garmrok’ kiwifruit cultivars using Progenesis SameSpots TotalLab (New Castle, UK). Consequently, 90 proteins in **a** ‘Hayward’ kiwifruit and 106 in **b** ‘Garmrok’ kiwifruit were differentially expressed in response to exogenous ethylene treatment. For descriptive quantification analysis of each spot, please refer Supplementary Tables 1 and 2



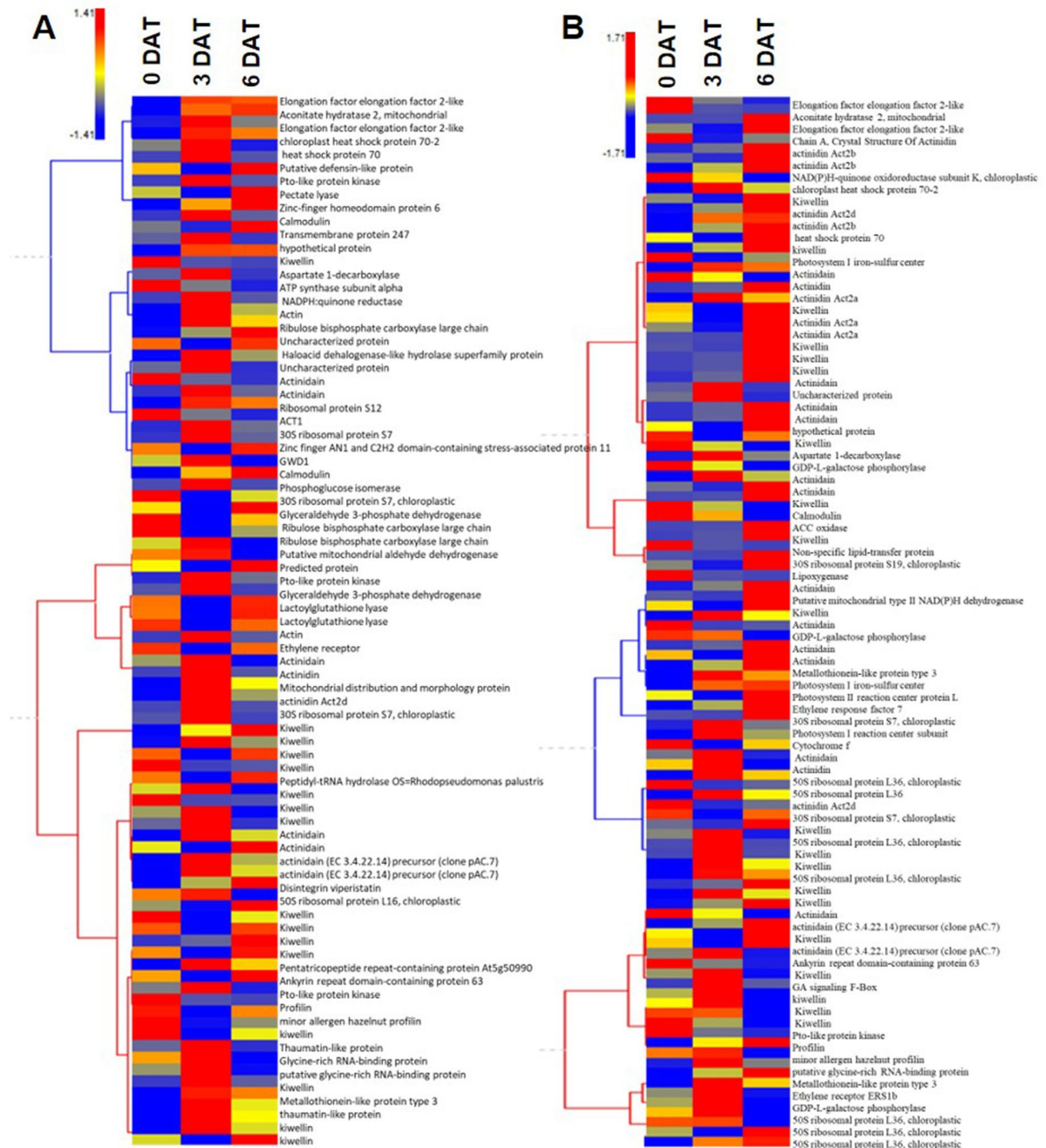


Fig. 5 Hierarchical clustering analysis of the protein spots that distinguish the overall expression profiles of the differentially expressed proteins in **a** ‘Hayward’ and **b** ‘Garmrok’ kiwifruit 0, 3, and 6 days after exogenous ethylene treatment. Mean values of three independent analysis for each treatment were expressed as ratios between 0, 3,

and 6 days after exogenous ethylene treatment using hierarchical clustering explorer (HCE 3.5 interactive power analysis) software. Relative quantification for each protein spot is provided in Supplementary Tables 1 and 2

Table 1 Protein identification from ‘Hayward’ kiwifruit flesh following the exogenous ethylene treatment by 2DE-PAGE and MALDI-TOF/TOF MS

Spot no.	Protein name	Organism	Accession no.	Protein score	Biological function	Mr value	Calcu. pI/Exp. pI	Sequence coverage
1	Elongation factor elongation factor 2-like	<i>Cicer arietinum</i>	gil502088409	297	Protein biosynthesis/Translation	95,002	5.7/6.0	37
2	Aconitate hydratase 2, mitochondrial	<i>Arabidopsis thaliana</i>	ACO2M_ARATH	107	Glycoxylate bypass	108,760	6.7/6.1	14
3	Elongation factor elongation factor 2-like	<i>Cicer arietinum</i>	gil502088409	221	Protein biosynthesis/Translation	95,002	5.7/6.3	33
4	Chloroplast heat shock protein 70-2	<i>Ipomoea nil</i>	gil166919372	357	Defense response	74,811	5.2/4.5	18
5	Heat shock protein 70	<i>Cucumis sativus</i>	gil6911,549	331	Defense response	73,599	5.0/4.9	33
6	Putative defensin-like protein	<i>Arabidopsis thaliana</i>	DEF53_ARATH	39	Defense response	6682	7.4/5.5	69
7	Pto-like protein kinase	<i>Actinidia deliciosa</i>	A0A0N9E1F7	50	Defense response	20,886	8.3/5.8	13
8	Pectate lyase	<i>Actinidia deliciosa</i>	E7D4U0	8	Pectin catabolic process	25,018	7.3/5.9	14
9	Zinc-finger homeodomain protein 6	<i>Oryza sativa</i>	ZHD6_ORYSJ	30	Transcription	28,284	8.9/6.0	22
10	Calmodulin	<i>Actinidia deliciosa</i>	B1NDP5	18	Signaling pathway	16,528	3.1/6.2	19
11	Transmembrane protein 247	<i>Bos taurus</i>	TM247_BOVIN	32	Signaling pathway	24,769	4.8/5.3	5
12	Hypothetical protein	<i>Streptomyces ipomoeae</i>	gil496696994	61	–	8755	5.0/6.4	44
13	Kiwellingin	<i>Actinidia deliciosa</i>	L7TRW4	16	Allergen/Fruit ripening	23,023	5.7/5.6	24
14	Aspartate 1-decarboxylase	<i>Synechocystis sp.</i>	PAND_SYNY3	46	Metabolism/Amino acids	16,404	5.4/6.8	23
15	ATP synthase subunit alpha	<i>Convolvulus assyricus</i>	Q6E2Y9	364	ATP synthesis	46,575	5.9/5.9	15
16	NADPH: quinone reductase	<i>Desulfovibrio sp. Dsv1</i>	gil550895832	62	Defense response	49,376	7.6/6.1	12
17	Actin	<i>Actinidia deliciosa</i>	Q0PVN2	20	Defense response	4059	10.8/6.4	68
18	Ribulose biphosphate carboxylase large chain	<i>Actinidia deliciosa</i>	Q5S855	693	Calvin cycle/Photosynthesis	53,159	6.2/6.9	57
19	Uncharacterized protein	<i>Rhizobium meliloti</i>	I2E1I1	46	–	5427	5.5/4.6	100
20	Haloacid dehalogenase-like hydrolase superfamily protein	<i>Klebsormidium flaccidum</i>	A0A0U9I6A1	47	Metabolism/Amino acids	31,542	6.6/4.7	20
21	Uncharacterized protein	<i>Setaria italica</i>	K3XWK8	166	–	54,167	5.1/5.6	33
24	Actinidain	<i>Actinidia chinensis</i>	P00785	102	Allergen/Fruit ripening	42,545	4.9/4.0	21

Table 1 (continued)

Spot no.	Protein name	Organism	Accession no.	Protein score	Biological function	Mr value	Calcu. pI/Exp. pI	Sequence coverage
28	Actinidain	<i>Actinidia deli-ciosa</i>	A5HII1	366	Allergen/Fruit ripening	42,483	4.9/4.1	47
30	Ribosomal protein S12	<i>Olea europaea</i>	E3TJL4	13	Protein biosynthesis/Translation	10,278	11.0/5.7	22
32	ACT1	<i>Actinidia deli-ciosa</i>	A7YVW7	137	Allergen/Fruit ripening	41,865	5.1/6.2	44
33	30S Ribosomal protein S7	<i>Actinidia deli-ciosa</i>	A0A0C5CDH4	5	Protein biosynthesis/Translation	17,468	5.2/5.2	44
34	Zinc finger AN1 and C2H2 domain-containing stress-associated protein 11	<i>Arabidopsis thaliana</i>	Q8VZ42	158	Transcription	32,316	5.5/5.3	12
35	GWD1	<i>Actinidia deli-ciosa</i>	A0A1S5RRZ4	28	Allergen/Fruit ripening	164,550	6.0/5.6	11
36	Calmodulin	<i>Actinidia deli-ciosa</i>	B1NDP5	24	Hormone signaling pathway	16,678	3.1/6.2	19
37	Phosphoglucose isomerase	<i>Drosophila pri-maeva</i>	A0A096VHF7	70	Glycolysis	18,003	6.2/5.9	46
38	30S Ribosomal protein S7, chloroplastic	<i>Actinidia deli-ciosa</i>	A0A0C5CDH4	150	Protein biosynthesis/Translation	17,468	6.0/6.0	38
41	Glyceraldehyde 3-phosphate dehydrogenase	<i>Actinidia deli-ciosa</i>	B7SFB1	180	Glycolysis	13,869	6.0/6.3	18
42	Ribulose biphosphate carboxylase large chain	<i>Actinidia deli-ciosa</i>	A0A0C5CDD3	149	Calvin cycle/Photosynthesis	53,159	6.2/6.9	37
43	Ribulose biphosphate carboxylase large chain	<i>Guatteria heterotricha</i>	Q5S855	150	Calvin cycle/Photosynthesis	51,018	5.8/6.9	37
44	Putative mitochondrial aldehyde dehydrogenase	<i>Populus canadensis</i>	Q2EHP9	65	Calvin cycle/Photosynthesis	16,642	5.1/7.0	21
45	Predicted protein	<i>Chlamydomonas reinhardtii</i>	A8HNE2	119	Secondary metabolism	85,820	6.0/7.0	26
46	Pto-like protein kinase	<i>Actinidia deli-ciosa</i>	A0A0N6WZX8	114	Allergen/Fruit ripening	21,936	9.0/8.9	14
47	Glyceraldehyde 3-phosphate dehydrogenase	<i>Actinidia deli-ciosa</i>	B7SFB1	126	Glycolysis	13,869	6.0/5.2	15
48	Lactoylglytathione lyase	<i>Lupinus angustifolius</i>	A0A1J7GBZ8	137	Metabolism/Amino acids	35,596	5.3/5.3	18
49	Lactoylglytathione lyase	<i>Lupinus angustifolius</i>	A0A1J7GBZ9	134	Metabolism/Amino acids	35,596	5.3/5.5	18
50	Actin	<i>Gossypium hirsutum</i>	Q7XZJ5	147	Defense response	41,902	5.2/5.7	41
51	Ethylene receptor	<i>Actinidia deli-ciosa</i>	Q6S5L7	77	Fruit ripening	24,861	5.9/6.0	28
52	Actinidain	<i>Actinidia deli-ciosa</i>	A5HII1	577	Allergen/Fruit ripening	42,483	4.9/4.0	23

Table 1 (continued)

Spot no.	Protein name	Organism	Accession no.	Protein score	Biological function	Mr value	Calcu. pI/Exp. pI	Sequence coverage
54	Actinidain	<i>Actinidia eriantha</i>	gil146215986	72	Allergen/Fruit ripening	42,971	6.3/4.8	16
55	Mitochondrial distribution and morphology protein	<i>Clavispora lusitanae</i>	MDM10_CLAL4	26	Protein biogenesis	51,595	6.2/4.9	26
56	Actinidain Act2d	<i>Actinidia eriantha</i>	gil146215986	365	Allergen/Fruit ripening	42,971	6.3/5.5	16
57	30S Ribosomal protein S7, chloroplastic	<i>Actinidia deliciosa</i>	A0A0C5CDH4	238	Protein biosynthesis/Translation	42,018	6.2/5.3	19
58	Kiwellin	<i>Actinidia deliciosa</i>	L7TV12	216	Allergen/Fruit ripening	23,027	5.28/5.5	52
59	Kiwellin	<i>Actinidia deliciosa</i>	gil441482352	216	Allergen/Fruit ripening	23,027	5.2/5.5	52
60	Kiwellin	<i>Actinidia deliciosa</i>	L7TV12	425	Allergen/Fruit ripening	23,027	5.2/5.6	66
61	Kiwellin	<i>Actinidia deliciosa</i>	gil441482352	425	Allergen/Fruit ripening	23,027	5.2/6.0	66
62	Peptidyl-tRNA hydrolase	<i>Rhodopseudomonas palustris</i>	PTH_RHOPB	62	Secondary metabolism	21,834	9.3/6.1	44
63	Kiwellin	<i>Actinidia deliciosa</i>	L7TRW4	245	Allergen/Fruit ripening	23,023	5.7/6.3	19
64	Kiwellin	<i>Actinidia deliciosa</i>	L7TRW4	158	Allergen/Fruit ripening	23,023	5.7/6.3	19
65	Kiwellin	<i>Actinidia deliciosa</i>	gil441482346	245	Allergen/Fruit ripening	22,997	5.9/6.4	19
66	Actinidain	<i>Actinidia deliciosa</i>	Q96228	181	Allergen/Fruit ripening	20,324	4.4/6.4	17
67	Actinidain	<i>Actinidia chinensis</i>	ACTN_ACTCH	177	Allergen/Fruit ripening	42,545	4.9/4.0	8
68	Actinidain (EC 3.4.22.14) precursor (clone pAC.7)	<i>Actinidia chinensis</i>	gil81543	181	Allergen/Fruit ripening	20,324	4.4/4.1	17
70	Actinidain (EC 3.4.22.14) precursor (clone pAC.7)	<i>Actinidia chinensis</i>	gil81544	143	Allergen/Fruit ripening	20,324	4.4/4.8	17
71	Disintegrin viperistatin	<i>Daboia palaestinae</i>	DIS_DABPA	25	Secondary metabolism	4914	4.2/5.5	16
72	50S Ribosomal protein L16, chloroplastic	<i>Actinidia deliciosa</i>	A0A0C5CHP6	5	Protein biosynthesis/Translation	15,292	6.6/6.0	15
73	Kiwellin	<i>Actinidia deliciosa</i>	L7TRW4	358	Allergen/Fruit ripening	23,023	5.7/7.0	40
74	Kiwellin	<i>Actinidia deliciosa</i>	L7TRW4	274	Allergen/Fruit ripening	23,023	5.7/6.0	35
75	Kiwellin	<i>Actinidia deliciosa</i>	gil441482354	369	Allergen/Fruit ripening	23,011	5.6/6.0	33
76	Kiwellin	<i>Actinidia deliciosa</i>	L7TY87	390	Allergen/Fruit ripening	22,997	5.9/6.0	52

Table 1 (continued)

Spot no.	Protein name	Organism	Accession no.	Protein score	Biological function	Mr value	Calcu. pI/Exp. pI	Sequence coverage
77	Pentatricopeptide repeat-containing protein	<i>Arabidopsis thaliana</i>	PP428_ARATH	63	RNA modification	60,739	9.0/6.4	25
78	Ankyrin repeat domain-containing protein 63	<i>Mus musculus</i>	ANR63_MOUSE	50	Secondary metabolism	59,705	9.4/6.5	21
79	Pto-like protein kinase	<i>Actinidia deliciosa</i>	A0A0N9E1N1	173	Allergen/Fruit ripening	21,854	6.8/4.4	8
80	Profilin	<i>Beta vulgaris</i>	PROF_BETVU	103	Actin binding	2519	4.0/4.5	100
81	Minor allergen hazelnut profilin	<i>Corylus avellana</i>	gil12659208	109	Actin binding	14,118	4.7/4.6	100
82	Kiwellin	<i>Actinidia deliciosa</i>	gil441482346	342	Allergen/Fruit ripening	22,997	5.9/4.7	52
83	Thaumatococcal-like protein	<i>Actinidia deliciosa</i>	TLP_ACTDE	222	Allergen/Fruit ripening	25,175	8.2/7.0	24
84	Glycine-rich RNA-binding protein	<i>Sinapis alba</i>	GRP1_SINAL	157	Defense response	16,063	5.2/7.0	22
85	Putative glycine-rich RNA-binding protein	<i>Chorisporea bungeana</i>	gil209976406	165	Defense response	16,796	5.5/5.5	30
86	Kiwellin	<i>Actinidia deliciosa</i>	KIWEL_ACTDE	311	Allergen/Fruit ripening	20,753	5.8/7.1	34
87	Metallothionein-like protein type 3	<i>Actinidia deliciosa</i>	P43389	311	Defense response	20,753	5.8/6.9	34
88	Thaumatococcal-like protein	<i>Actinidia chinensis</i>	gil441482370	78	Allergen/Fruit ripening	25,221	5.9/7.1	33
89	Kiwellin	<i>Actinidia deliciosa</i>	gil441482346	87	Allergen/Fruit ripening	22,997	5.9/4.5	44
90	Kiwellin	<i>Actinidia deliciosa</i>	L7TRW4	206	Allergen/Fruit ripening	23,023	5.7/5.0	13

Mr value: Molecular weight

(Table 1). Similarly, in ‘Garmrok’ kiwifruit, the 12 key proteins related to protein biosynthesis/translation were identified, i.e., elongation factor elongation factor 2-like (nos. 1 and 3), 30S ribosomal protein S19, chloroplastic (nos. 48 and 61), and 50S ribosomal protein (nos. 70, 71, 73, 75, 78, 102, 103, and 105) (Table 2). Moreover, the proteins related to Calvin cycle/photosynthesis were identified in both kiwifruit cultivars as four proteins from ‘Hayward’, i.e., ribulose biphosphate carboxylase (RuBisCO) large chain (nos. 18, 42, and 43) and putative mitochondrial aldehyde dehydrogenase (no. 44) (Table 1); and six proteins from ‘Garmrok’, i.e., chloroplastic (no. 7), photosystem I iron-sulfur center (nos. 17 and 58), photosystem II reaction center protein L (no. 59), photosystem I reaction center subunit (no. 62), and cytochrome f (no. 66) (Table 2).

Thereafter, the identified kiwifruit proteins were classified into functional categories using Panther gene analysis

(<http://pantherdb.org/>) (Fig. 6c, d). In ‘Hayward’ kiwifruit, the proteins were mainly classified into 11 categories, i.e., allergen/fruit ripening (50%), defense response (18%), protein biosynthesis/translation (10%), signaling pathway (2%), metabolism/amino acid (5%), glycolysis (3%), transcription (2%), ATP synthesis (1%), pectin/catabolic process (1%), Calvin cycle/photosynthesis (4%), and secondary metabolism (4%) (Fig. 6c). Likewise, the identified proteins from ‘Garmrok’ kiwifruit were classified into 10 categories, i.e., allergen/fruit ripening (60%), defense response (10%), protein biosynthesis/translation (13%), Calvin cycle/photosynthesis (6%), metabolism/amino acids (1%), actin binding (2%), lipid transport (1%), metal binding (2%), glyoxylate bypass (1%), and uncharacterized protein/unknown protein (4%) (Fig. 6d).

Table 2 Protein identification from ‘Garmrok’ kiwifruit flesh following the exogenous ethylene treatment by 2DE-PAGE and MALDI-TOF/TOF MS

Spot no.	Protein name	Organism	Accession no.	Protein score	Biological function	Mr value	Calcu. pI/Exp. pI	Sequence coverage
1	Elongation factor elongation factor 2-like	<i>Cicer arietinum</i>	gil502088409	297	Protein biosynthesis/Translation	95,002	5.7/6.0	37
2	Aconitate hydratase 2, mitochondrial	<i>Arabidopsis thaliana</i>	ACO2M_ARATH	107	Glycoxylate bypass	108,760	6.7/6.1	14
3	Elongation factor elongation factor 2-like	<i>Cicer arietinum</i>	gil502088409	221	Protein biosynthesis/Translation	95,002	5.7/6.3	33
4	Chain A, Crystal Structure of Actinidin	<i>Actinidia chinensis</i>	gil157829826	735	Allergen/Fruit ripening	23,942	4.2/6.7	43
5	Actinidin Act2b	<i>Actinidia eriantha</i>	gil146215982	242	Allergen/Fruit ripening	42,316	5.8/6.8	17
6	Actinidin Act2b	<i>Actinidia eriantha</i>	gil146215982	314	Allergen/Fruit ripening	42,316	5.8/6.9	19
7	NAD(P)H-quinone oxidoreductase subunit K, chloroplastic	<i>Actinidia deliciosa</i>	A0A0C5CHE8	19	Calvin cycle/Photosynthesis	32,729	8.9/4.5	17
8	Chloroplast heat shock protein 70-2	<i>Ipomoea nil</i>	gil166919372	357	Defense response	74,811	5.2/4.5	18
9	Kiwellin	<i>Actinidia deliciosa</i>	KIWEL_ACTDE	126	Allergen/Fruit ripening	20,753	5.8/5.5	39
10	Actinidin Act2d	<i>Actinidia deliciosa</i>	gil146215986	489	Allergen/Fruit ripening	42,971	6.3/5.5	16
11	Actinidin Act2b	<i>Actinidia eriantha</i>	gil146215982	222	Allergen/Fruit ripening	42,316	5.8/5.6	13
12	Heat shock protein 70	<i>Cucumis sativus</i>	gil6911549	331	Defense response	73,599	5.0/4.9	33
13	Kiwellin	<i>Actinidia deliciosa</i>	gil441482352	622	Allergen/Fruit ripening	23,027	5.2/5.5	61
17	Photosystem I iron-sulfur center	<i>Actinidia deliciosa</i>	A0A0C5CKX4	22	Calvin cycle/Photosynthesis	9545	6.6/5.5	30
21	Actinidain	<i>Actinidia deliciosa</i>	A5HII1	697	Allergen/Fruit ripening	42,483	4.9/5.5	34
22	Actinidin	<i>Actinidia deliciosa</i>	A5HII4	183	Allergen/Fruit ripening	42,018	8.8/5.5	11
23	Actinidin Act2a	<i>Actinidia deliciosa</i>	A5HII4	247	Allergen/Fruit ripening	42,018	8.2/5.6	15
24	Kiwellin	<i>Actinidia deliciosa</i>	L7TV12	432	Allergen/Fruit ripening	20,753	5.2/6.0	66
25	Actinidin Act2a	<i>Actinidia deliciosa</i>	A5HII4	270	Allergen/Fruit ripening	42,018	8.0/6.0	15
26	Actinidin Act2a	<i>Actinidia deliciosa</i>	A5HII4	179	Allergen/Fruit ripening	42,018	8.0/6.5	9
27	Kiwellin	<i>Actinidia deliciosa</i>	L7TV12	622	Allergen/Fruit ripening	23,027	5.2/6.8	61
28	Kiwellin	<i>Actinidia deliciosa</i>	L7TV12	144	Allergen/Fruit ripening	23,027	5.2/6.7	66
29	Kiwellin	<i>Actinidia deliciosa</i>	L7TY87	187	Allergen/Fruit ripening	22,997	5.9/6.8	22

Table 2 (continued)

Spot no.	Protein name	Organism	Accession no.	Protein score	Biological function	Mr value	Calcu. pI/Exp. pI	Sequence coverage
30	Actinidain	<i>Actinidia chinensis</i>	P00785	102	Allergen/Fruit ripening	42,545	4.9/4.0	21
31	Uncharacterized protein	<i>Setaria italica</i>	K3XWK8	166	–	54,167	5.1/5.6	33
32	Actinidain	<i>Actinidia chinensis</i>	P00785	102	Allergen/Fruit ripening	42,545	4.9/4.0	21
33	Actinidain	<i>Actinidia chinensis</i>	P00786	103	Allergen/Fruit ripening	42,546	4.9/4.1	22
36	Hypothetical protein	<i>Streptomyces ipomoeae</i>	gil496696994	61	–	8755	5.0/6.4	44
38	Kiwelin	<i>Actinidia deliciosa</i>	L7TRW4	16	Allergen/Fruit ripening	23,023	5.7/5.6	24
39	Aspartate 1-decarboxylase	<i>Synechocystis</i> sp.	PAND_SYNY3	46	Metabolism/Amino acids	16,404	5.4/6.8	23
40	GDP-L-galactose phosphorylase	<i>Actinidia deliciosa</i>	D3JYW8	12	Defense response	50,587	4.8/5.8	9
41	Actinidain	<i>Actinidia deliciosa</i>	A5HII1	11	Allergen/Fruit ripening	42,483	4.9/5.9	12
42	Actinidain	<i>Actinidia deliciosa</i>	Q7DMV2	11	Allergen/Fruit ripening	1409	8.5/6.0	61
43	Kiwelin	<i>Actinidia deliciosa</i>	L7TY87	34	Allergen/Fruit ripening	22,997	5.9/6.2	40
44	Calmodulin	<i>Actinidia deliciosa</i>	B1NDI6	18	Defense response	16,735	6.5/6.2	20
45	ACC oxidase	<i>Actinidia deliciosa</i>	G3DSB9	11	Defense response	36,470	5.4/6.3	8
46	Kiwelin	<i>Actinidia deliciosa</i>	L7TRW4	250	Allergen/Fruit ripening	23,023	5.7/6.5	59
47	Non-specific lipid-transfer protein	<i>Actinidia deliciosa</i>	P86137	15	Lipid Transport	9908	9.1/6.5	30
48	30S Ribosomal protein S19, chloroplastic	<i>Actinidia deliciosa</i>	A0A0C5CHH0	60	Protein biosynthesis/Translation	10,493	10/6.8	11
49	Lipoxygenase	<i>Actinidia deliciosa</i>	Q0ZDG2	60	Metal binding	51,450	5.9/6.9	5
50	Actinidain	<i>Actinidia deliciosa</i>	Q7DMV2	11	Allergen/Fruit ripening	1409	8.5/6.9	61
51	Putative mitochondrial type II NAD(P)H dehydrogenase	<i>Actinidia deliciosa</i>	C3VXG0	70	Metal binding	24,927	5.8/6.9	5
52	Kiwelin	<i>Actinidia deliciosa</i>	L7TRW4	59	Allergen/Fruit ripening	23,023	5.7/4.5	13
53	Actinidain	<i>Actinidia deliciosa</i>	Q7DMV2	11	Allergen/Fruit ripening	1409	8.5/4.6	61
54	GDP-L-galactose phosphorylase	<i>Actinidia deliciosa</i>	D3JYW8	12	Defense response	50,587	4.8/5.5	9
55	Actinidain	<i>Actinidia deliciosa</i>	Q7DMV2	11	Allergen/Fruit ripening	1409	8.5/5.5	61
56	Actinidain	<i>Actinidia deliciosa</i>	Q7DMV2	11	Allergen/Fruit ripening	1409	8.5/5.6	61
57	Metallothionein-like protein type 3	<i>Actinidia deliciosa</i>	P43389	18	Defense response	7070	4.4/5.6	30

Table 2 (continued)

Spot no.	Protein name	Organism	Accession no.	Protein score	Biological function	Mr value	Calcu. pI/Exp. pI	Sequence coverage
58	Photosystem I iron-sulfur center	<i>Actinidia deliciosa</i>	A0A0C5CKX4	15	Calvin cycle/Photosynthesis	9545	6.6/6.5	33
59	Photosystem II reaction center protein L	<i>Actinidia deliciosa</i>	A0A0C5CKT7	9	Calvin cycle/Photosynthesis	4467	4.5/6.5	39
60	Ethylene response factor 7	<i>Actinidia deliciosa</i>	D8VD34	6	Fruit ripening	25,516	9.3/6.0	9
61	30S Ribosomal protein S7, chloroplastic	<i>Actinidia deliciosa</i>	A0A0C5CDH4	5	Protein biosynthesis/Translation	17,468	11/6.1	9
62	Photosystem I reaction center subunit	<i>Cucumis sativus</i>	PSAN_CUCSA	32	Calvin cycle/Photosynthesis	2791	4.5/6.2	45
66	Cytochrome f	<i>Actinidia deliciosa</i>	A0A0C5CDD8	21	Calvin cycle/Photosynthesis	35,426	9.8/6.7	15
67	Actinidain	<i>Actinidia deliciosa</i>	A5HII1	577	Allergen/Fruit ripening	42,483	4.9/4.0	23
68	Actinidin	<i>Actinidia eriantha</i>	gil146215986	72	Allergen/Fruit ripening	42,971	6.3/4.8	16
70	50S Ribosomal protein L36, chloroplastic	<i>Actinidia deliciosa</i>	A0A0C5CYN0	8	Protein biosynthesis/Translation	4615	11.9/5.5	29
71	50S Ribosomal protein L36	<i>Actinidia deliciosa</i>	A0A0C5CYN0	8	Protein biosynthesis/Translation	4615	11.9/5.8	29
72	Actinidin Act2d	<i>Actinidia eriantha</i>	gil146215986	365	Allergen/Fruit ripening	42,971	6.3/5.5	16
73	30S Ribosomal protein S7, chloroplastic	<i>Actinidia deliciosa</i>	A0A0C5CDH4	238	Protein biosynthesis/Translation	42,018	6.2/5.3	19
74	Kiwelin	<i>Actinidia deliciosa</i>	L7TV12	216	Allergen/Fruit ripening	23,027	5.28/5.5	52
75	50S Ribosomal protein L36, chloroplastic	<i>Actinidia deliciosa</i>	A0A0C5CDH4	238	Protein biosynthesis/Translation	4615	11.9/5.5	29
76	Kiwelin	<i>Actinidia deliciosa</i>	gil441482352	216	Allergen/Fruit ripening	23,027	5.2/5.5	52
77	Kiwelin	<i>Actinidia deliciosa</i>	gil441482352	425	Allergen/Fruit ripening	23,027	5.2/6.0	66
78	50S Ribosomal protein L36, chloroplastic	<i>Actinidia deliciosa</i>	A0A0C5CYN0	8	Protein biosynthesis/Translation	4615	11.9/6.0	29
79	Kiwelin	<i>Actinidia deliciosa</i>	L7TRW4	158	Allergen/Fruit ripening	23,023	5.7/6.3	19
80	Kiwelin	<i>Actinidia deliciosa</i>	gil441482346	245	Allergen/Fruit ripening	22,997	5.9/6.4	19
81	Actinidain	<i>Actinidia chinensis</i>	ACTN_ACTCH	177	Allergen/Fruit ripening	42,545	4.9/4.0	8
82	Actinidain	<i>Actinidia chinensis</i>	gil81543	181	Allergen/Fruit ripening	20,324	4.4/4.1	17
83	Kiwelin	<i>Actinidia deliciosa</i>	L7TRW4	63	Allergen/Fruit ripening	23,023	5.7/5.5	41
84	Actinidain	<i>Actinidia deliciosa</i>	gil81544	143	Allergen/Fruit ripening	20,324	4.4/4.8	17

Table 2 (continued)

Spot no.	Protein name	Organism	Accession no.	Protein score	Biological function	Mr value	Calcu. pI/Exp. pI	Sequence coverage
85	Ankyrin repeat domain-containing protein 63	<i>Mus musculus</i>	ANR63_MOUSE	50	Secondary metabolism	59,705	9.4/6.5	21
86	Kiwellin	<i>Actinidia deliciosa</i>	L7TRW4	274	Allergen/Fruit ripening	23,023	5.7/6.0	35
87	GA signaling F-Box	<i>Actinidia deliciosa</i>	V5QMV7	5	–	19,628	9.3/6.0	8
88	Kiwellin	<i>Actinidia deliciosa</i>	gil441482346	342	Allergen/Fruit ripening	22,997	5.9/4.7	52
89	Kiwellin	<i>Actinidia deliciosa</i>	gil441482354	369	Allergen/Fruit ripening	23,011	5.6/6.0	33
92	Kiwellin	<i>Actinidia deliciosa</i>	L7TY87	390	Allergen/Fruit ripening	22,997	5.9/6.0	52
95	Pto-like protein kinase	<i>Actinidia deliciosa</i>	A0A0N9E1N1	173	Allergen/Fruit ripening	21,854	6.8/4.4	8
96	Profilin	<i>Beta vulgaris</i>	PROF_BETVU	103	Actin binding	2519	4.0/4.5	100
97	Minor allergen hazelnut profilin	<i>Corylus avellana</i>	gil12659208	109	Actin binding	14,118	4.7/4.6	100
98	Putative glycine-rich RNA-binding protein	<i>Chorispora bungeana</i>	gil209976406	165	Defense response	16,796	5.5/5.5	30
99	Metallothionein-like protein type 3	<i>Actinidia deliciosa</i>	P43389	311	Defense response	20,753	5.8/6.9	34
100	Ethylene receptor ERS1b	<i>Actinidia deliciosa</i>	B3FIA8	8	Fruit ripening	71,118	6.0/6.9	5
101	GDP-L-galactose phosphorylase	<i>Actinidia deliciosa</i>	D3JYW8	8	–	50,587	4.8/6.8	7
102	50S Ribosomal protein L36, chloroplastic	<i>Actinidia deliciosa</i>	A0A0C5CYN0	8	Protein biosynthesis/Translation	4615	11.9/7.0	29
103	50S Ribosomal protein L36, chloroplastic	<i>Actinidia deliciosa</i>	A0A0C5CYN0	8	Protein biosynthesis/Translation	4615	11.9/6.0	29
104	50S Ribosomal protein L36, chloroplastic	<i>Actinidia deliciosa</i>	A0A0C5CYN0	8	Protein biosynthesis/Translation	4615	11.9/6.0	29

Mr value: Molecular weight

3.4 Regulatory protein–protein interaction networks in response to exogenous ethylene treatment

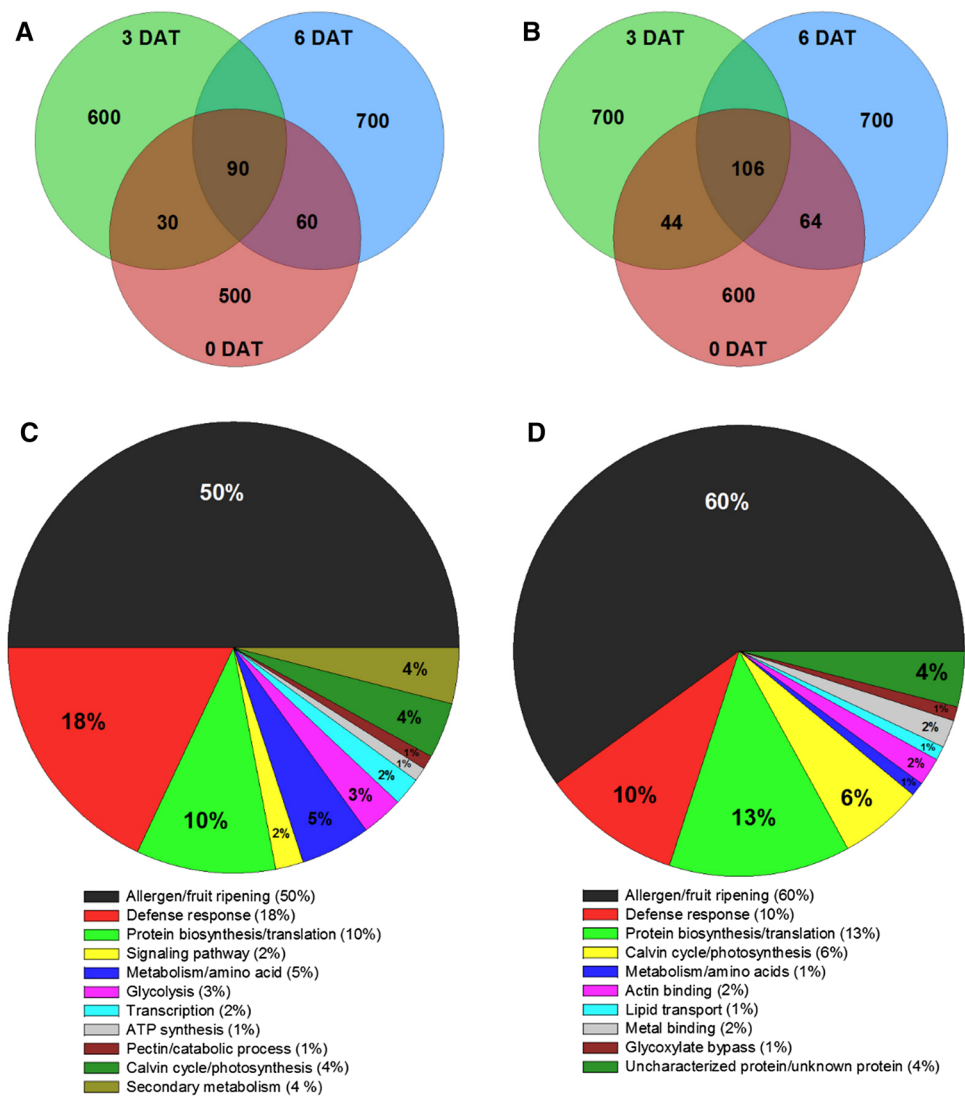
The functional links between different proteins in ‘Hayward’ and ‘Garmrok’ kiwifruit cultivars in response to ethylene treatment were determined by the protein–protein interaction network generated using STRING 9.0 (Fig. 7). The highly interactive protein clusters included the proteins associated with defense response and Calvin cycle/photosynthesis and are highlighted by circles in Fig. 7. In ‘Garmrok’ kiwifruit, slight interactions were observed in one of the major identified

protein categories, i.e., allergen/fruit ripening, but substantial interactions with other proteins were not observed in this category (Fig. 7). There is no database on proteins specially related to fruit ripening, which may be why we failed to detect interactions between fruit ripening proteins.

3.5 Gene level confirmation of differentially expressed proteins in response to exogenous ethylene treatment

In this study, a number of differentially expressed proteins were identified as being associated with fruit ripening, but

Fig. 6 Comparative analysis of **a** 'Hayward' and **b** 'Garmrok' created from three standard replicates 0, 3, and 6 days after ethylene treatment using Progenesis SameSpots TotalLab (New Castle, UK). **c** Functional classification of identified proteins in 'Hayward' and **d** 'Garmrok' created using Panther gene analysis



they were previously reported as being associated with defense response (Minas et al. 2012, 2016; Tanou et al. 2015). Hence, gene level confirmation was conducted with two fruit-ripening-related proteins, i.e., ACC synthase and ACC oxidase (Tables 1, 2), to validate the proteomic data (Fig. 8). The ethylene biosynthetic genes *ACS1* and *ACO1*, which encode ACC synthase and ACC oxidase, respectively are closely related to the climacteric ethylene production (Ilina et al. 2010). The relative expressions of *ACS1* and *ACO3* increased concomitantly with the increase in days after ethylene treatment in both 'Hayward' and 'Garmrok' kiwifruit cultivars. Consequently, the gene expression results with a correlation to MS-based quantification verified the reliability of current proteomic approaches. Moreover, the ethylene-induced ripening in kiwifruit is confirmed on the basis of *ACS1* and *ACO3* gene expression levels.

4 Discussion

Fruit ripening is a sophisticated developmental process in higher plants, involves a number of stages from immature to mature fruit, depending on the plant species, and is affected by various signaling pathways and environmental conditions (Tanou et al. 2015). The ripening process of kiwifruit, as a climacteric fruit, is affected by the rate of ethylene production (Yin et al. 2008; Minas et al. 2016). During kiwifruit ripening, a series of biochemical, physiological, and structural modifications occur that affect the final fruit quality attributes (Giovannoni 2004; Yin et al. 2008; Minas et al. 2016; Shin 2018; Shin et al. 2019). The biochemical changes include an increase in respiration rate and ethylene synthesis, conversion of starch to sugar, changes in cell wall constituents, and production of

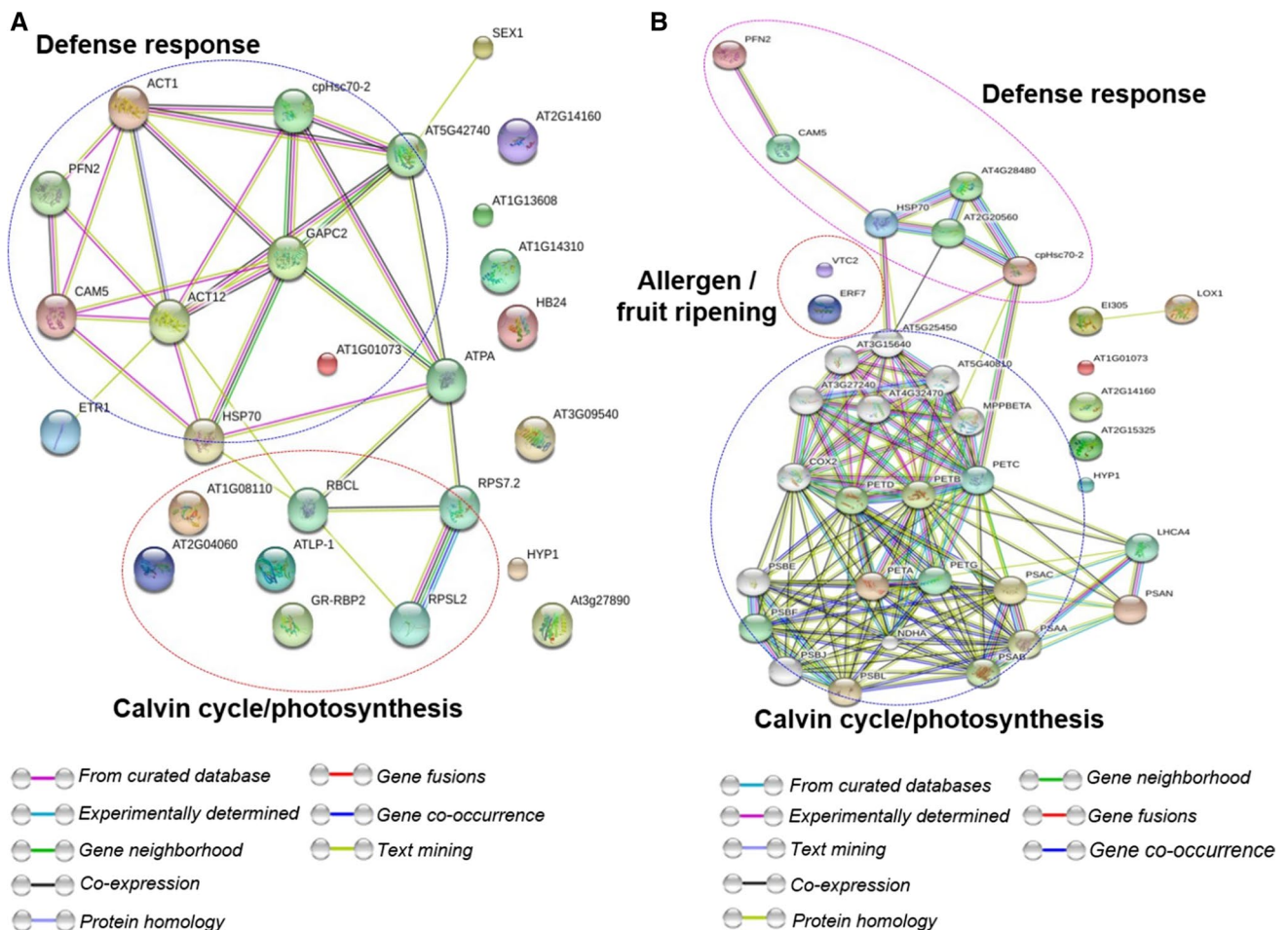


Fig. 7 Analysis of protein interaction network by STRING 9.1. TAIR homologous proteins from identified proteins were mapped by searching the STRING 9.1 software with a confidence level of 0.4 using *Arabidopsis thaliana*. Colored lines between the proteins indi-

cate the various types of interactions. The clusters of highly interacting protein nodes are marked circled and include proteins involved in defense response, Calvin cycle/photosynthesis, and allergen/fruit ripening

characteristic volatiles, which contribute to the taste, texture, and aroma (Giovannoni 2004; Yin et al. 2008; Minas et al. 2016; Shin 2018; Shin et al. 2019). Hence, a global evaluation of fruit ripening process is desirable since there are numerous metabolic changes during fruit ripening. Recently, a number of different “omics” technologies, i.e., genomics, transcriptomics, epigenomics, proteomics, metabolomics, and phenomics, have allowed researchers to access the global data sets of fruit maturation, ripening, and senescence-related metabolites and the corresponding metabolic pathways in a distinctive way (Faurobert et al. 2007; Janssen et al. 2008; Pech et al. 2008; Palma et al. 2011; Shi et al. 2014).

The characterization of protein maps during fruit ripening has been well documented in many fleshy fruits, i.e., apple, banana, muskmelon, papaya, mango, and citrus (Muccilli et al. 2009; Costa et al. 2010; Andrade et al. 2012; Huerta-Ocampo et al. 2012; Nogueira et al. 2012; Li et al. 2015a,

b). However, the comprehensive studies on discrepancies between exogenous ethylene treatment and fruit ripening are still lacking, though several studies on the effects of exogenous ethylene treatment on fruit characteristics, such as taste, softening, and soluble sugars, have been reported (Bower et al. 2003; Giribaldi et al. 2007; Jabbar and East 2016; Lim et al. 2017).

In this study, a large-scale analysis of proteins and their interactions in response to exogenous ethylene treatment in ‘Hayward’ and ‘Garmrok’ kiwifruit cultivars was performed by constructing 2-DE maps with subsequent protein identification. A considerable difference in the protein maps of exogenous-ethylene-treated kiwifruit was observed. Moreover, among the identified proteins, the kiwellin and actinidain proteins, which are responsible for the initiation of fruit ripening, were abundant in both kiwifruit cultivars treated with exogenous ethylene. In ‘Hayward’ kiwifruit, 50% of proteins were identified as kiwellin or actinidain (nos. 13,

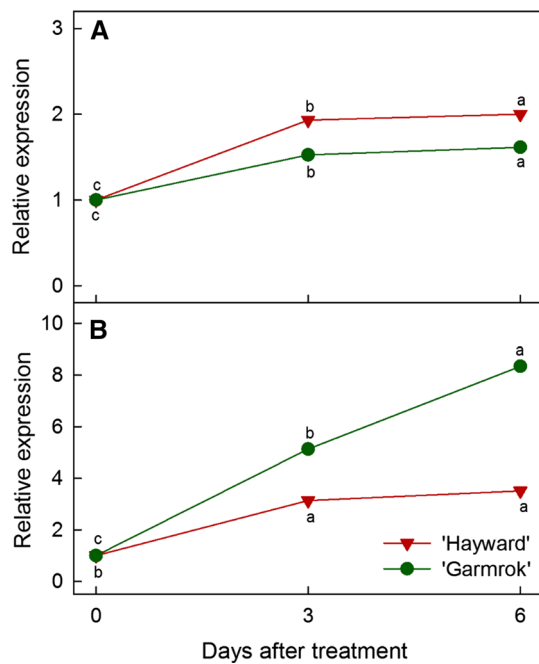


Fig. 8 Relative transcript level of **a** ACC synthase (*ACST*) and **b** ACC oxidase (*ACO3*) in 'Hayward' and 'Garmrok' kiwifruit cultivars 0, 3, and 6 days after exogenous ethylene treatment. The results were normalized to the Actin housekeeping gene. Experimental data are shown as means with standard error bars ($n=3$), and values with different letters are significantly different at $p \leq 0.05$ according to Tukey's studentized range test performed with SAS software (SAS Inst., Cary, N.C., USA)

24, 28, 52, 54, 56, 58, 59, 60, 61, 63, 64, 65, 66, 67, 68, 70, 73, 74, 75, 76, 82, 86, 89, and 90) (Table 1 and peptide information in Supplementary Table 3), while in 'Garmrok', 60% of proteins were similarly identified as kiwellin or actinidin (nos. 5, 6, 9, 10, 11, 13, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 38, 41, 42, 43, 46, 50, 52, 53, 55, 56, 67, 68, 72, 74, 76, 77, 79, 80, 81, 82, 83, 84, 86, 88, 89, and 92) (Table 2 and peptide information in Supplementary Table 4). Accordingly, current proteomic analysis in ripening kiwifruit in response to exogenous ethylene treatment provides an insightful quantitative overview on kiwifruit ripening and the role of ethylene in that process. The significant role of ethylene in kiwifruit ripening has been well documented through previous proteomic analyses (Minas et al. 2012, 2016). Our proteomic findings provide a broad range of quantitative data regarding the possible interaction between ethylene and fruit ripening and, hence, the importance of exogenous ethylene treatment in commercial kiwifruit ripening. Several other proteomic studies of the effect of ethylene treatment on fruit ripening have identified and classified the most differentially expressed proteins as energy/metabolism and defense response proteins (Deytieu et al. 2007; Negri et al. 2008; Muccilli et al. 2009; Li et al. 2015a, b). However, in this study, in addition to defense response proteins,

differentially expressed proteins were identified and classified under a few other categories, i.e., protein biosynthesis, Calvin cycle/photosynthesis, and allergen/fruit ripening proteins. The majority of the differentially expressed proteins were classified under the allergen/fruit ripening category, and the key role of ethylene in fruit ripening was revealed, in agreement with Giribaldi et al. (2007) and Lim et al. (2017). Also, climacteric fruit, such as tomato, banana, apple, mango, and kiwifruit, display a well-characterized peak in respiration with a concomitant burst of ethylene at the onset of ripening (Sapitnitskaya et al. 2006). There are several proteomic studies on fruit ripening that quantified the proteins related to energy metabolism. However, to the best of our knowledge, this study has depicted the maximum number of proteins related to the fruit ripening.

Additionally, in accordance with Szklarczyk et al. (2011), the bioinformatics approaches implemented in this study using STRING enable the characterization of protein–protein interaction with main clusters related to defense response (the second most commonly classified proteins in kiwifruit) and the Calvin cycle/photosynthesis (the third most commonly classified proteins in kiwifruit) (Fig. 7). Moreover, the protein–protein interaction data provided strong evidence that these proteins were abundantly synthesized during fruit ripening in response to exogenous ethylene; hence, the identified proteins during ripening in exogenous-ethylene-treated 'Hayward' and 'Garmrok' kiwifruit were classified into three major protein categories, i.e., allergen/fruit ripening, defense response, and protein biosynthesis.

Allergen or fruit-ripening proteins regulated by ethylene play an important role in fruit ripening. Kiwellin and actinidin are the most commonly present allergen proteins in kiwifruit due to their stable and active characteristics (Nieuwenhuizen et al. 2007). Actinidin protein has been extensively characterized in multiple forms, and this has contributed to the breeding of new kiwifruit cultivars with altered actinidin levels (Alexander and Grierson 2002). In this study, the two key identified allergen proteins, i.e., kiwellin and actinidin, were significantly increased/aggregated in ethylene-treated 'Hayward' and 'Garmrok' kiwifruit cultivars (Figs. 3, 4) compared to the untreated kiwifruit.

The defense-responsive proteins are well known to mitigate the several biotic and abiotic stresses in fruit via the detoxification of reactive oxygen species (ROS) (Aharoni and O'Connell 2002; Muccilli et al. 2009). Nevertheless, a developmental role of the defense response proteins has been determined recently in the fruit ripening process as well (Jimenez et al. 2002; Gorovits et al. 2007). Also, in this study, the defense response proteins with chaperone function, i.e., HSP70, actin, metallothionein, and ethylene-related regulated enzyme (ACC oxidase), were recognized as the second major functional group in the identified differentially expressed proteins (Fig. 6). All the defense response proteins

were up-regulated in exogenous-ethylene-treated kiwifruit compared to the untreated kiwifruit (Fig. 6). The increased abundance of heat shock proteins and metallothioneins is well known to be associated with the detoxification of ROS and HSP enzymes, which are also known to be involved in interaction and folding of protein kinase (Negri et al. 2008; Muccilli et al. 2009). The up-regulation of defense proteins, i.e., HSPs and ACC oxidase, has also been observed in several other fruits, i.e., apple (Li et al. 2016), tomato (Gorovits et al. 2007), and peach (Nilo et al. 2010). The defense response proteins identified in exogenous-ethylene-treated ‘Hayward’ and ‘Garmrok’ kiwifruit cultivars are involved in fruit ripening and senescence and provide protection against harmful external stimuli, particularly in response to exogenous ethylene treatment. The third major group of differentially expressed proteins identified in exogenous-ethylene-treated ‘Hayward’ and ‘Garmrok’ kiwifruit cultivars was categorized as biosynthesis/translation proteins, i.e., elongation factor (nos. 1 and 3) and ribosomal proteins (30S and 50S) (Tables 1, 2). In contrast to previous proteomic studies on several other fruits (Muccilli et al. 2009; Costa et al. 2010; Andrade et al. 2012; Huerta-Ocampo et al. 2012; Nogueira et al. 2012; Li et al. 2015a, b, 2016), our study identified many proteins related to biosynthesis and translation that have also been reported in studies on pineapple (Moyle et al. 2005) and tomato (Fujisawa et al. 2014). The biosynthesis and translation proteins are highly involved in plant physiology in response to plant stress conditions (Muneer et al. 2014, 2016). According to our findings, biosynthesis and translation proteins are involved in kiwifruit softening in response to exogenous ethylene treatment. In addition, RuBisCO, PSI, PSII, and cytochrome proteins are the key proteins in the Calvin cycle/photosynthesis category (Tables 1, 2). The expression of photosynthetic proteins, i.e., chloroplast oxygen-evolving enhancer protein, during fruit ripening has been reported in several other fruits, i.e., tomato (Faurobert et al. 2007), grape (Terrier et al. 2001; Martinez-Esteso et al. 2013), and date palm (Marondedze et al. 2014). The identification of photosynthetic proteins in this study implies the involvement of ethylene in the Calvin cycle/photosynthesis of kiwifruit cultivars.

In summary, ethylene-elicited kiwifruit ripening was studied along with quantitative data on the protein expression associated with kiwifruit ripening. The identification of proteins related to allergens/fruit ripening, defense response, the Calvin cycle/photosynthesis, and biosynthesis/translation provides insight into the fruit ripening mechanisms, including a wide range of pathways. Moreover, a rudimentary insight of protein–protein interactions depicted based on exogenous-ethylene-treated ‘Hayward’ and ‘Garmrok’ kiwifruit ripening will provide a significant set of proteome data for the future studies on protein–protein interactions related to fruit ripening. Finally, the difference in abundance

of major fruit ripening initiation proteins, i.e., kiwellin and actinidain, in ‘Hayward’ (50%) and ‘Garmrok’ (60%) kiwifruit shows the differences between the late harvest ‘Hayward’ and early harvest ‘Garmrok’ cultivars and their ripening behavior in response to exogenous ethylene treatment, as ‘Garmrok’ kiwifruit exhibited more softening than the ‘Hayward’. Overall, this study provides insight into the role and regulation of proteins and pathways during kiwifruit ripening and will contribute to the development of innovative practices for kiwifruit quality improvements during pre-harvest and postharvest practices.

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Author contributions SMH and MS performed the experiments and analyzed the data. BDW assisted with proteomic measurements and KHMPC helped carry out the results. KJG designed and wrote the manuscript in consultation with KYH and LJJ. KYB helped supervise the experiment. All authors discussed the results and contributed to the final manuscript.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

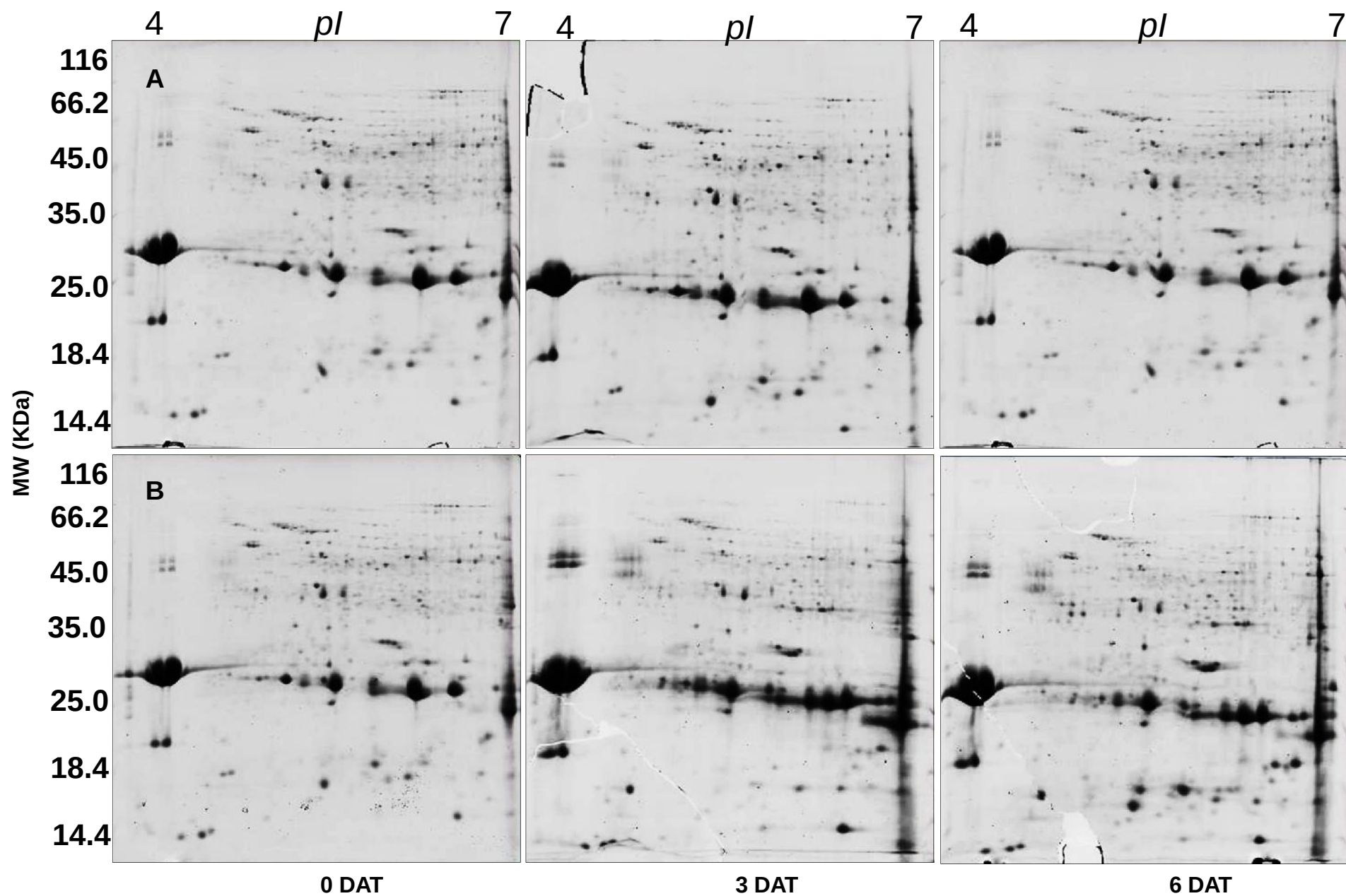
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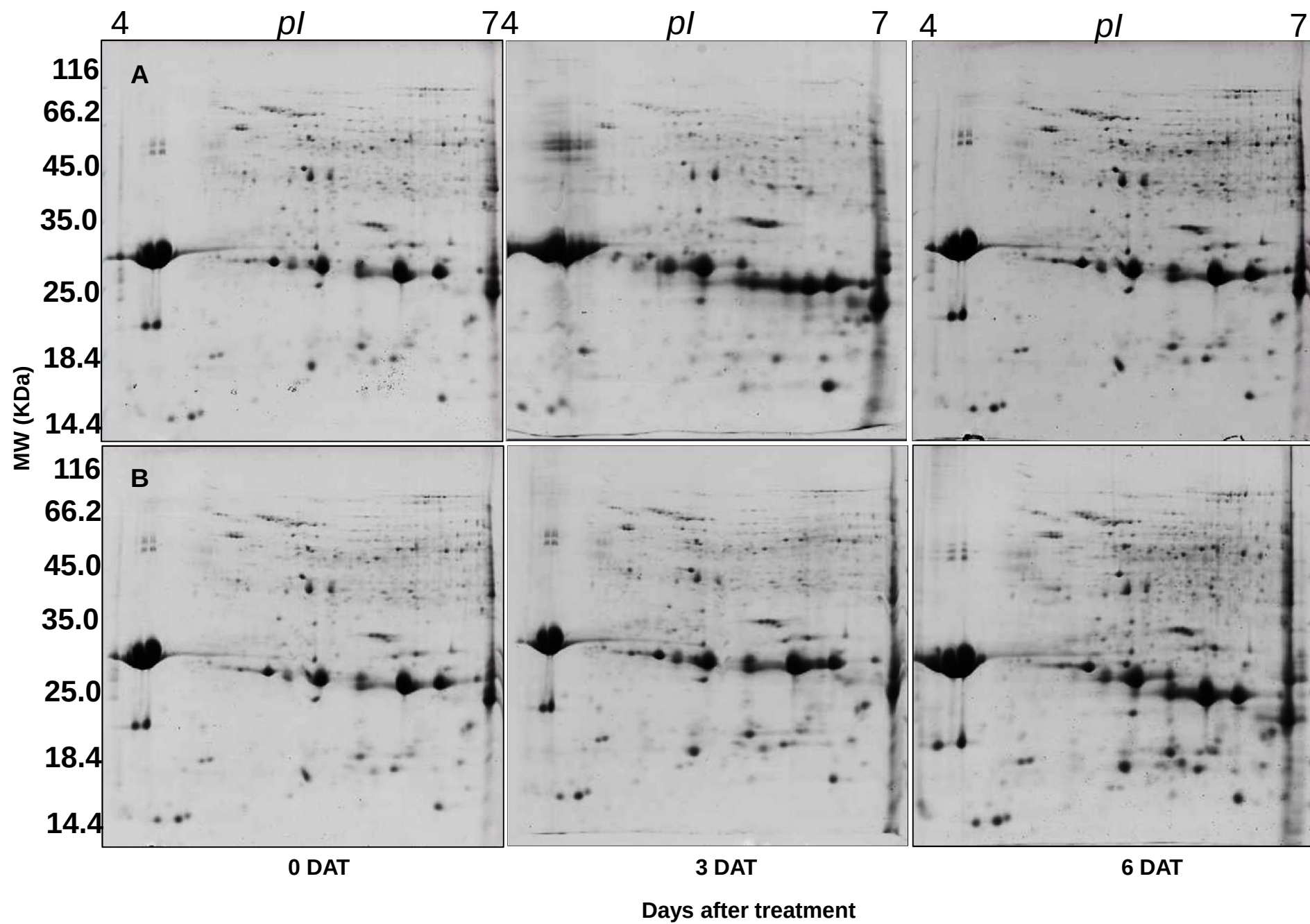
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