Proteomic Analysis Reveals Dynamic Regulation of Fruit Ripening in Response to Exogenous Ethylene in Kiwifruit Cultivars

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Abstract

An understanding of the mechanism underlying fruit ripening is critical for fruit quality improvement. Although post-harvest ethylene application is known to enhance the onset of fruit ripening, exact mechanisms remain unclear. To characterize the fruit ripening process and mechanism, we investigated the effects of exposing kiwifruit cultivars 'Hayward' and 'Gamrok' to exogenous ethylene treatment post-harvest using comprehensive proteomic analyses. Comparative two-dimensional gel electrophoresis showed that most of the proteins aggregated in ethylene-treated samples compared to the control (non-treated). We observed that among all ethylene treatments, 95 proteins from 'Hayward' and 106 from 'Gamrok' were differentially expressed. Interestingly, among the elicited protein successfully identified by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry, 50% from "Hayward' and 60% from 'Gamrok' are associated with fruit ripening. Furthermore, 18% and 10% of proteins, respectively, are associated with defense response, whereas other major proteins are related to protein biosynthesis and photosynthesis/Calvin cycle. Interactions between identified proteins were demonstrated by bioinformatic analysis, providing insights into biological pathways and molecular functions in post-harvest kiwifruit ripening elicited by ethylene application. The present proteomic study in accordance with physiological analysis provides a quantitative evaluation of fruit ripening in response to exogenous ethylene in post-harvest kiwifruit.

Keywords: *Actinidia deliciosa*; ethylene; fruit ripening; mass spectrometry; post-harvest; proteomics

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1. Introduction

Fruit ripening is an extremely complex process during which a large number of biochemical modifications occur. These biochemical changes, including cell wall softening, conversion of starch to sugar, and accumulation of aromatic and volatile compounds [1] contribute to make fruits attractive, consumable, and also determine their nutritional values such as fiber and vitamin contents and antioxidative properties [2]. Other major changes that occurs during fruit ripening involve the regulation of genes and proteins or their interaction in order to create the physiological conditions that allow fruit to accomplish its final objective [3-6]. During the last 25–30 years, the methods that divide fleshy fruit into climacteric or non-climacteric have depended on the peak of respiration rate and ethylene production [3]. In climacteric fruit, increased respiration rate and ethylene biosynthesis were believed to be characteristic of the ripening process; however, recent studies have commonly discussed the onset of ethylene production for the ripening process [7-10].

Ethylene is a small hydrocarbon gas and is synthesized in almost all fruits to promote the ripening process [11]. The biosynthesis of ethylene is known to involve two key biosynthetic enzymes, namely ACC synthase (ACS), which converts SAM (*S*-adenosyl-L-methionine) to 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC oxidase (ACO), which further converts ACC to ethylene (11]. Ethylene is the most commonly commercially produced compound in the world and is used in many pomology industries for fruit ripening. Ethylene has long been used for its properties in fruit ripening, being used in ancient Egyptian and Chinese practices to promote ripening of gashing figs and pears, respectively. A significant amount of research has been conducted on the effects of ethylene on fruit ripening; moreover, fruit ripening ethylene is also reported to play a vital role in ameliorating certain biotic and abiotic stresses [12-16]. The strategies used to manage the external or internal sources of ethylene are key practices in commercial optimization of the storage life and eating quality of many fruits. Understanding the fundamental mechanisms underlying ethylene-induced fruit ripening is thus important for managing harvesting, storage, and distribution processes. Although several recent studies have reported on the biochemical pathways of ethylene and the fruit ripening process [8-10, 17], the precise underlying mechanisms remain unclear.

Proteomic approaches are increasing being adopted to investigate and establish complex pathways, including fruit ripening. Indeed, there have been a number of large-scale proteomic investigations on the ripening of several fruits, including grapes [18-20], tomato [21-24], citrus [25], banana [7, 26], apricot [17], muskmelon [8], strawberry [27], mango [28], papaya [29-30], apple [31-32], and kiwifruit [10]. Nevertheless, despite all these studies, there is still a lack of information on the mechanisms by which ethylene induces fruit ripening.

Kiwifruit is an economically important fruit and is widely consumed for its beneficial nutritional properties, including antioxidative properties, fiber, and taste. The ripening behavior of kiwifruit

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is depended on the biosynthesis of ethylene and respiration rate [33-35]. Moreover, kiwifruit is a warm-temperate fruit and often suffers freezing damage during winter, when the temperature drops below -12°C. In Korea, kiwifruit farms are located in southern coastal regions to prevent freezing injury. Among the kiwifruit cultivars, 'Hayward' is the cultivar predominantly grown throughout the world. This cultivar, was released in the late 1920s in New Zealand, similar to the 'Fuji' apple, but is not appropriate for cultivation in Korea due to chilling injury. To resolve the chilling injury problem, the cultivar 'Gamrok' was bred in Korea in the mid-1980s. 'Gamrok' is harvested in late October and does not suffer frost damage. Previous proteomic studies on kiwifruit have either provided data only for the 'Hayward' cultivar or only quantitatively analyzed the proteins related to sugar content, metabolism, and plant defense, whereas no data related to fruit ripening has been presented [10, 36], nor on less cultivated kiwifruit cultivars. Proteins specifically related to fruit ripening undergo various post-translational modifications during the biosynthesis of ethylene, which are related to the subsequent development of fruit nutritional characteristics. Given these current gaps in our knowledge, the present study was undertaken to examine the mechanisms underlying ethylene-induced ripening in two kiwifruit cultivars, 'Hayward' and 'Gamrok', using gel-based proteomic analysis followed by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF TOF MS) and bioinformatic analysis. Besides, physiological analysis was analyzed to support and select optimal time point for proteomic analysis.

2. Material and Methods

2.1. Fruit Material and Experimental Design

Kiwifruit (*Actinidia deliciosa*) cultivars 'Hayward' and 'Gamrok' were harvested from the National Horticultural Research Institute, Republic of Korea. Trees were maintained using a T-bar system and managed using general cultivation practices. The fruits from both cultivars were harvested physiologically at mature stage with stable flesh/core firmness and were packaged in low-vent plastic clamshell container (HPL826M, LocknLock, China), 20 fruit each container and were immediately moved to the laboratory of fruit science, Gyeongsang National University, Korea. After screening of fruits, only healthy fruits were used for experimental analysis with ten biological replicates (n=10) for physiological analysis and three biological (n=3) replicates for proteomic analysis. Primarily kiwifruits were exposed to 1000 ppm of exogenous ethylene in an airtight plastic container containing a vent for air circulation, while CO_2 was maintained with NaOH solution. The ethylene concentration selected for the present study was based on our initial observations as 1000 ppm showed best ripening compared to lower concentrations in *Actinidia deliciosa*. The ethylene treatment was given to kiwifruits for 0 (control, without treatment), 3, 6, and 9 days at 20°C. Thereafter, the fruits were analyzed for physiological measurements and based on those results proteomic analysis was performed at two selected time points viz., 3 and 6 days. Because these two-time

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points showed best edibility of kiwifruit (**Figure 1**). For proteomic analysis the ethylene treated kiwifruits were peeled off and separated into core and flesh, grinded in liquid nitrogen and immediately stored at -80°C until use.

In recent years, major studies have been conducted on 'Hayward' while no such studies have been reported on other cultivars such as 'Gamrok' which is widely used in Asian countries such as Korea. Gamrok cultivar bred by Korean Rural Development Administration has a good taste and can be harvested about 1 week earlier compared to 'Hayward' kiwifruit in Korea. Early maturation could be free of frost injury in Korea and can produce a good quality fruit, Consequently, due to these reasons kiwifruit cultivars viz., 'Hayward' and 'Gamrok' were used for the present study.

2.2. Physiological Measurements

The soluble sugar content (SSC) was observed in a fruit juice. The fruit was wrapped in a four-layer cheese cloth, and absorbance was read using portable refractometer (Pocket Refractometer, PAL-1, Atago, Japan).

The titratable acidity (TA) of fruit juice was assayed by titration with 0.05 mol·L^{-1} NaOH. The TA content was expressed as citric acid equilibrium. The fruit firmness was measured with a probe diameter of 3mm at a horizontal axis using a rheometer (RHEO TEX SD-700, Sun Scientific Inc, Japan) (3mm depth).

For measurement of respiration rate (CO_2) and ethylene evolution (C_2H_4) the polypropylene airtight container (HPL851-2.1L, Locknlock, China) containing kiwifruit were allowed to incubate for 4 hours at room temperature. After incubation 1 mL of gas from each container were then analyzed for CO_2 and C_2H_4 gas by injecting into (GC-FID and TCD) gas chromatograph with flame ionization detector (GC 6890, Agilent Technologies, USA). The setting of GC-FID & TCD was as follows: oven temperature 100°C, front inlet 100°C, back inlet 375°C, front detector 250°C, and back detector 150°C. The standard used for CO_2 and C_2H_4 analysis was 285 µL·L^{-1} and 5.2 µL·L^{-1} commercial standards.

2.3. Protein Extraction

Total proteins from kiwifruit flesh were extracted using a phenol-based method with minor modifications [37]. In brief, the fruit material was ground to a fine powder in liquid nitrogen and transferred to 50-mL falcon tubes. The powdered tissues were immediately suspended in protein extraction buffer, containing 0.9 M sucrose, 0.1 M Tris-HCl, pH 8.8, 10 mM EDTA, and 0.4% (v/v) β-mercaptoethanol, and incubated on ice for 10 min. Thereafter, an equal volume of Tris-buffered phenol, pH 8.8, was added and the mixture was vigorously vortexed. To separate the insoluble material, for aqueous and organic phases, the samples were centrifuged for 20 min at 3,500 rpm. The resulting upper phenolic phase was carefully transferred to a new tube and then back extracted three to four times in extraction buffer. The resulting

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supernatant was precipitated overnight at -20°C in four volumes of precipitation solution containing 100 mM ammonium acetate prepared in methanol. The protein pellet collected was washed four to five times in precipitation solution and cold acetone. The resulting protein pellet was dried and solubilized in lysis buffer, containing 9 M urea, 2% CHAPS, 0.2% (w/v) Pharmalyte, pH 3–10, and 50 mM dithiothreitol (DTT), prior to isoelectric focusing (IEF). The extracted proteins were quantified using the Bradford method (Bradford, 1976).

2.4. Isoelectric Focusing and Two-Dimensional Gel Electrophoresis

IEF and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed, as described previously [38]. Four hundred micrograms of total protein extract were rehydrated on Bio-Rad 17 cm immobilized pH gradient gel strips (pH 4–7). 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 incubating on a shaker in 2–3 mL of freshly prepared equilibration buffer 1 [8 M urea, 2% SDS, 50 mM Tris-HCl (pH 8.8), 20% (v/v) glycerol, and 1% DTT] for 15 min, followed by incubation in 2–3 mL of equilibration buffer 2 [the same content as equilibration buffer 1 with the exception that DTT was replaced by 2.5% iodoacetamide] for 15 min. Separation in the second dimension was performed on 12.5% (w/v) SDS-polyacrylamide gels using a PROTEAN II system (Bio-Rad, Hercules, CA, USA) using a gradient voltage 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, South Korea) on one side of the SDS-PAGE gels. The two-dimensional electrophoresis (2-DE) gel was stained with colloidal Coomassie brilliant blue. For each treatment three independent replicates were run simultaneously for further analysis.

2.5. Image and Data Analysis

For each treatment, three independent biological replicates were analyzed. Gels were imaged under constant settings using a photo imager as described by [39]

2.6. MALDI-TOF/TOF MS Identification of Protein Spots

Excised protein spots were subjected to in-gel digestion according to Kim *et al.* [40] with minor modifications. The tryptic-digested proteins (peptides) were subjected to MALDI-TOF/TOF-MS (ABI 4800, Applied Biosystems, Framingham, MA, USA) [41]

2.7. Data Processing, Functional Annotations, and Protein–Protein Interactions

Hierarchical clustering of differentially expressed protein spots was carried out using hierarchical clustering explorer (HCE 3.5 Interactive Power Analysis) software. Functional annotations of the identified proteins were carried out using panther gene analysis (http://pantherdb.org/). To determine the functions

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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).

2.8. 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, South Korea). The isolated RNA was quantified by using Nano-drop spectrophotometer. One µg 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 (Rotor-Gene Q 2plex HRM Platform), using SYBR green as a reference dye provide by Qiagen qPCR kit (QIAGEN OneStep RT-PCR Kit, Westburg, Netherland) for 5 min at 95 °C, followed by 25 cycles consisting of 20 sec at 95 °C, 30 sec at 50-57 °C (gradient) and 30 sec at 72 °C, then 10 min at 72 °C. 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'-CGGTACTGTTTTTAGCTCTCCAGACT-3'; R-5'-TGGAGTGGCTACTGTCCTTTAGAA-3'; *ACO3:* (accession number: HQ293206) F-5'-GGCAACCAAAGGCCTAGAG-3'; R-5'-GGTGCTTTCCCAGTCCAAATC-3'; *ACT1:* (accession number DQ682826) F-5'-TCCTTCGTCTTGACCTTGCT-3'; R-5'- AATTGTGATGAACTGACCAT-3'

3. Results and Discussion

3.1. Physiological Analysis

The soluble sugar content (°Brix), titratable acidity (TA), firmness, respiration rate (CO_2) and ethylene evolution (C_2H_4) in kiwifruit cultivars 'Hayward' and 'Gamrok' underwent significant changes to exogenous ethylene (**Figure 2**). In accordance with previous reports [10], 'Hayward' exhibited a continuous decrease in firmness, titratable acidity (%) and increase in soluble sugar content, respiration rate and ethylene content exposed to exogenous ethylene treatment compared to control (**Figure 2**). These results were though different at different time points of ethylene treatment and indicated that day 3 and 6 are best time point for fruit ripening (ready to eat). The physiological results were concomitant to morphological differences of kiwifruit exposed to ethylene (**Figure 1**). Although not much difference was observed even after 9 of ethylene treatment but overall the results indicated that after 3 and 6 day of ethylene treatment 'Hayward' cultivar was ready to eat. While, 'Gamrok' cultivar is known to mature early than 'Hayward' and our physiological and morphological differences specified that 3 and 6 days ethylene treatment are optimal for its ripening. Indeed after 9 days of ethylene treatment 'Gamrok' was observed to be over ripened (**Figures 1, 2**).

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3.2. 2-DE and Image Analysis of kiwifruits in Response to Exogenous Ethylene

Based on our physiological data 3 and 6-day time point was selected for proteomic analysis along with 0 day (without ethylene treatment) as a main control. Kiwifruits exposed to exogenous ethylene generally exhibited rapid softening compared to the control. To detect differentially expressed proteins in kiwifruits treated with exogenous ethylene, a comparative 2-DE analysis was performed (**Figure 3, Supplementary Figure 1**). The comparison of kiwifruit 2-DE maps between control and ethylene treatments (3 and 6 day) was analyzed using Progenesis SameSpots TotalLab (**Supplementary Tables 1 and 2**). The comparative analysis showed that 600 and 700 protein spots were detected in the 2DE maps of 'Hayward' cultivar post ethylene treatment (3 and 6 days) (**Figure 6A**), whereas only 500 protein spots were observed in the controls. Similarly, 700 protein spots were aggregated in the 2DE maps of 'Gamrok' cultivar treated with exogenous ethylene (3 and 6 days), whereas 600 were observed in the control samples (**Figure 6B**). After comparative analysis, a high-resolution 2DE map was generated to select protein spots for further analysis of differentially expressed proteins (**Figure 4**). We observed that among all ethylene treatments, 90 proteins from 'Hayward' and 106 from 'Gamrok' were differentially expressed (**Figure 4 and Figures 6A and 6B**). In addition, hierarchical clustering was performed to separate overall expression profiles of differentially expressed proteins (**Figure 5**). Inspection of the cluster analysis results clearly reveals the differences in different ethylene treatments in both kiwifruit cultivars ('Hayward' and 'Gamrok'. These results indicated that most important protein changes occurred at 6 days after ethylene treatment. Furthermore, a clear quantitative difference between each differentially expressed spot was observed statistically using Progenesis (**Supplementary Tables 1 and 2**).

We observed a considerable difference in the protein maps of kiwifruit subjected to exogenous ethylene treatment. The characterization of protein maps during fruit ripening has been well documented in many fleshy fruits such as apple, banana, apricot, muskmelon, papaya, mango, and citrus [7-8, 17, 25, 28, 29-31]; however, studies on discrepancies between ethylene exposure and fruit ripening are still lacking. There have, nevertheless, been a number of studies on the effects of ethylene exposure on fruit characteristics, such as increases in taste, softening, and soluble sugars [42-46]. In the present study, the 2DE maps of kiwifruit cultivars ('Hayward' and 'Gamrok') represent a large-scale analysis of proteins and indicate the possible interactions involved in ethylene-induced fruit ripening, which were subsequently confirmed by protein identification.

3.3. *Protein Identification and Functional Distribution*

The identification of kiwifruit protein spots was challenging due to the lack of genome sequence data. The proteins of interest (differentially expressed protein spots) in the protein maps of kiwifruit cultivars were excised and analyzed by MALDI-TOF/TOF MS (**Tables 1 and 2, supplementary Tables 3 and 4**). For the kiwifruit cultivars 'Hayward' and 'Gamrok', 90 and 106 protein spots, respectively, were

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analyzed by MALDI TOF/TOF, 80 and 90 of which, respectively, were successfully identified. Most of the proteins identified were successfully annotated in a universal protein database (Uniport/Swissport/NCBI).

It was interesting to observe that most of the protein spots identified from both kiwifruit cultivars were two important proteins, kiwellin and actinidain, both of which are involved in the initiation of fruit ripening. In 'Hayward', 50% of proteins were identified as kiwellin or actinidain (protein spot nos. 13, 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, For peptide information please see supplementary Table 3**), whereas in 'Gamrok', 60% of proteins were similarly identified (protein spot 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 For peptide information please see supplementary Table 4**). Thus, our proteomic analysis of the response to exogenous ethylene gives a broad quantitative overview of the relationship between ethylene and fruit ripening, indicating that ethylene is mostly responsible for the ripening of fruits rather than energy and metabolism as described in previous proteomic analyses of kiwifruit [10, 35] and other fruits. Furthermore, these identifications also provide a broad range of quantitative data regarding the possible interaction between ethylene hormone and fruit ripening. Overall, we can predict that application of ethylene can be beneficial for the promotion of ripening in fleshy fruits, particularly kiwifruit, for commercial purposes.

In 'Hayward' kiwifruit, a further 10 major proteins related to defense response were identified as chloroplast heat shock protein 70-20 (protein spot no. 4), heat shock protein 70 (no. 5), putative defensive-like protein (nos. 6, 84, and 85), protein-like kinase (no. 7), ATP synthase sub unit alpha (no. 15), NADP quinone reductase (no. 16), actin (no. 50), and metallothionein-like protein type 3 (no. 87) (**Table 1**). Similarly, in 'Gamrok', nine protein spots related to defense response were identified as 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 (no. 57 and 99), and putative glycine-rich RNA binding protein type 3 (spot no. 99) (**Table 2**).

Another six key proteins in 'Hayward' kiwifruit is related to protein biosynthesis/translation and were identified as elongation factor elongation factor 2-like (no. 1), ribosomal protein S12 (no. 30), and 30S ribosomal protein S7 (nos. 33, 38, 57, and 72). Similarly, 12 key proteins from 'Gamrok' are related to protein/translation and were identified as 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).

In addition, four proteins from 'Hayward' and six proteins from 'Gamrok' are related to the Calvin cycle/photosynthesis, which were identified as ribulose bisphosphate carboxylase (RuBisCO) large chain

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(nos. 18, 41, 42, 43, and 44 in Hayward and no. 7 and 17 in Gamrok), and photosystem I (PSI), photosystem II (PSII) (nos. 58, 59, and 62) and cytochrome (no. 66) in Gamrok.

The identified kiwifruit proteins were then classified into functional categories (**Figures 6C and 6D**), using panther gene analysis (http://pantherdb.org/). Identified kiwifruit proteins from 'Hayward' were mainly classified into general categories of 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%) (**Figure 6C**). Identified proteins from 'Gamrok' were classified into following categories: 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%), glycoxylate bypass (1%), and uncharacterized protein/unknown protein (4%) (**Figure 6D**).

Previous proteomic data on the response of fruits to exogenous ethylene treatment have indicated that most of the differentially expressed proteins were classified as being related to energy/metabolism and defense response [7-8, 19-20, 25]. In addition to defense response, our proteome data also identified differentially expressed proteins related to protein biosynthesis and the Calvin cycle/photosynthesis, whereas the maximum number of proteins fall under category of allergen/fruit ripening, indicating that ethylene is a key hormone for kiwifruit ripening. Thus, on the basis of these results, it is evident that identified proteins of kiwifruit ('Hayward' and 'Gamrok') are mainly classified into three major categories: allergen/fruit ripening, defense response, and protein biosynthesis.

3.3.1 Proteins Related to Allergen/Fruit Ripening

Several "omics" technologies, such as genomics, transcriptomics, proteomics, metabolomics, have been used recently to obtain information on the global changes that occur during fruit maturation, ripening, and senescence [22-23, 26, 47-51]. Allergen proteins, which are regulated by ethylene hormone, play an important role in the ripening of fruits. Among allergen or fruit-ripening proteins, kiwellin and actinidin are commonly present in large amounts in kiwifruits due to their stable and active characteristics [52]. Actinidin has been extensively characterized in multiple forms [53], and this has contributed to the breeding of new kiwifruit cultivars with altered actinidin levels. In our studies, the two main identified allergen proteins (kiwellin/actinidain) were significantly increased/aggregated in ethylene-treated kiwifruit cultivars ('Hayward' and 'Gamrok') (**Figures 3 and 4**) compared to non-ethylene treated samples. Indeed, the majority of the differentially expressed proteins were classified under the allergen/fruit ripening category, indicating that ethylene plays a prominent role in fruit ripening, as has also been indicated by several physiological analyses [45-46]. It is also well known that ethylene plays a critical role in the ripening process of climacteric fruits, whereas, an ethylene burst during fruit ripening is followed by a respiratory

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peak [54]. Although a number of proteomic studies on fruit ripening have quantified the proteins related to energy metabolism, the present study is the first to observe a maximum number proteins related fruit ripening.

3.3.2. Proteins Related to Defense Response

It is well known that defense responsive proteins play a key role in mitigating several biotic and abiotic stresses in fruits via the detoxification of reactive oxygen species (ROS) [25, 55]. Recently, it has been proposed that defense response proteins may play a developmental role in the ripening process [22, 56-57]. In the present study, we found that a second major functional group of differentially expressed proteins consisted of proteins involved in defense response (**Figures 6A and 6B**) including proteins with chaperone function, such as HSP70, actin, metallothionine, and ethylene-related regulated enzyme (ACC oxidase). All the defense response proteins were up-regulated in kiwifruit treated with ethylene compared with non-treated fruit (control) (**Figures 6A and 6B**). The increased abundance of heat shock proteins and metallothioneins is well known to be associated with the detoxification of ROS, and HSP enzymes are also known to be involved in interaction and folding of protein kinase [20, 25]. The up-regulation of defense proteins such as HSPs and ACC oxidase has also been observed in several other fruits, such as apple [51], tomato [58], and peach [6]. Our defense response protein category results indicate that, in addition to their role in fruit ripening and senescence, 'Hayward' and 'Gamrok' kiwifruit cultivars synthesize these proteins as protection against harmful external stimuli, and particularly in response to ethylene treatment.

3.3.3. Proteins Related to Protein Biosynthesis/Translation

Proteins belonging to the category protein biosynthesis/translation, such as elongation factor (protein spot no. 1 and 3) and ribosomal proteins (30S and 50S) (**Tables 1 and 2**) were the third major group of differentially expressed proteins identified in the present study. In contrast to previous proteomic studies on fruits [7-8, 17, 25, 28, 29-31, 58], the present study identified a large number of proteins related to biosynthesis and translation, the latter of which have also been reported in studies on pineapple [59] and tomato [60]. It is well known that proteins related to biosynthesis and translation play an important role in the physiology of plants in response to unfavorable conditions [38, 40]. The identification of these proteins in kiwifruit cultivars suggests their role in softening in response to exposure to exogenous ethylene.

3.3.4. Proteins Related to the Calvin Cycle/Photosynthesis

Proteins such as RuBisCO, PSI, PSII, and cytochrome are key components of the Calvin cycle/photosynthesis (**Tables 1 and 2**). It is well known that green fruits contain photosynthetically active chloroplasts that contribute to energy metabolism [61]. The expression of photosynthetic proteins such as chloroplast oxygen-evolving enhancer protein during the ripening process has been reported in several fruits, including tomato [23], apricot 17], grapes [62-63], and date palm fruits [64]. The identification of

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photosynthetic proteins in the present study indicates the involvement of ethylene in the Calvin cycle/photosynthesis of kiwifruit cultivars.

3.4. Regulatory Protein–Protein Interaction Networks

The protein–protein interaction network generated using STRING 9.0 revealed functional links between different proteins identified in kiwifruit cultivars ('Hayward' and 'Gamrok') exposed to ethylene (**Figure 7**). The major clusters of interacting proteins are highlighted in circles and contain proteins that are associated with defense response, and the Calvin cycle/photosynthesis. With the exception of a few interactions observed for 'Gamrok', proteins in the major identified category (allergen/fruit ripening) did not show interactions with other proteins. The failure to detect fruit ripening protein interactions can be attributed to the fact that there is currently no database for fruit proteins related specifically to ripening.

Bioinformatic approaches using STRING [65] enable characterization of protein–protein interaction with main clusters related to defense response (the second most commonly classified proteins in kiwifruits) and the Calvin cycle/photosynthesis (the third most commonly classified proteins) (**Figure 7**). The protein–protein interaction data thus strongly suggest that these proteins are synthesized abundantly during fruit ripening in response to exogenous ethylene.

3.5. Confirmation of Differentially Expressed Proteins at Gene Level

While many of the differentially abundant proteins found in the present study have earlier been shown in defense responsive proteome [9-10, 35], majority were found to be associated with fruit ripening in our study. Thus, two proteins related to fruit ripening viz., ACC synthase, and ACC oxidase (please see **Table 1** and **2**) were selected for gene expression level to validate proteomic data (**Figure 8**). Previous study on kiwifruit ripening showed that the ethylene biosynthetic genes *ACS1*, and *ACO1*, which encodes ACC synthase and ACC oxidase respectively are closely related with climacteric ethylene production [66]. In our study, relative expression showed *ACS1* (ACC synthase), and *ACO3* (ACC oxidase) were at least two-fold higher in 3-day ethylene treated kiwifruits whereas, the fold change increased at 6-day compared to 0 day (control). As expected, gene expression levels showed a correlation with MS-based quantification supporting the reliability of proteomic approaches. Moreover, the gene expression levels of *ACS1*, and *ACO3* also confirmed that ethylene induced fruit ripening process.

4. Conclusion

In summary, we observed that exogenous ethylene elicited kiwifruit ripening and we also provide quantitative data on the associated changes in protein expression. Moreover, the quantitative proteomic data provides a novel insight into the mechanisms of fruit ripening involving a wide range of pathways, such as our identification of proteins related to allergens/fruit ripening, defense response, the Calvin cycle/photosynthesis, and biosynthesis/translation. Furthermore, we also anticipate that the two kiwifruit

cultivars ('Hayward' and 'Gamrok') analyzed in the present study will provide a large set of proteome data to further study of the protein–protein interactions related to fruit ripening, although in the present study, we could only obtain a rudimentary insight into protein–protein interactions related to either defense or photosynthesis. Additionally, the identification and classification of the major proteins kiwellin and actinidin (which represented 50% of the differentially expressed proteins in Hayward and 60% in Gamrok) indicates the differences between late (Hayward) and early (Gamrok) kiwifruit cultivars and suggests that exogenous ethylene application induces greater softening in 'Gamrok' cultivar that in 'Hayward'. The overall data could also be applied in the selection of specific cultivars for growth in particular seasons.

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Author contributions

The authors have made the following contributions. SM, and JGK conceived and designed the experiments. MHS performed and analyzed the physiological data. SM performed and analyzed the proteomic analysis. JJL, DWB, YBK, and JGK shared materials and technical instruments and discussed the study. SM and JGK wrote the paper.

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1 **Table 1.** Protein identification of kiwifruit flesh 'Hayward' following ethylene treatments by 2DE-PAGE and mass spectrometry MALDI-TOF TOF

Spot No.	Protein Name	Organisms	accession No.	Protein score	Biological function	Mr value	Calcu. *pI*/ Exp. *pI*	Sequence coverage
1	Elongation factor elongation factor 2-like	*Cicer arietinum*	gi|502088409	297	Protein biosynthesis/Translation	95002	5.7/6.0	37
2	Aconitate hydratase 2, mitochondrial	*Arabidopsis thaliana*	ACO2M_ARATH	107	Glycoxylate bypass	108760	6.7/6.1	14
3	Elongation factor elongation factor 2-like	*Cicer arietinum*	gi|502088409	221	Protein biosynthesis/Translation	95002	5.7/6.3	33
4	chloroplast heat shock protein 70-2	*Ipomoea nil*	gi|166919372	357	Defense response	74811	5.2/4.5	18
5	heat shock protein 70	*Cucumis sativus*	gi|6911549	331	Defense response	73599	5.0/4.9	33
6	Putative defensin-like protein	*Arabidopsis thaliana*	DEF53_ARATH	39	Defense response	6682	7.4/5.5	69
7	Pto-like protein kinase	*Actinidia deliciosa*	A0A0N9E1F7	50	Defense response	20886	8.3/5.8	13
8	Pectate lyase	*Actinidia deliciosa*	E7D4U0	8	Pectin catabolic process	25018	7.3/5.9	14
9	Zinc-finger homeodomain protein 6	*Oryza sativa*	ZHD6_ORYSJ	30	Transcription	28284	8.9/6.0	22
10	Calmodulin	*Actinidia deliciosa*	B1NDP5	18	Signaling pathway	16528	3.1/6.2	19
11	Transmembrane protein 247	*Bos taurus*	TM247_BOVIN	32	Signaling pathway	24769	4.8/5.3	5
12	hypothetical protein	*Streptomyces ipomoeae*	gi|496696994	61	-	8755	5.0/6.4	44
13	Kiwellin	*Actinidia deliciosa*	L7TRW4	16	Allergen/Fruit ripening	23023	5.7/5.6	24
14	Aspartate 1-decarboxylase	*Synechocystis sp.*	PAND_SYNY3	46	Metabolism/Amino acids	16404	5.4/6.8	23
15	ATP synthase subunit alpha	*Convolvulus assyricus*	Q6E2Y9	364	ATP synthesis	46575	5.9/5.9	15
16	NADPH: quinone reductase	*Desulfovibrio sp. Dsv1*	gi|550895832	62	Defense response	49376	7.6/6.1	12
17	Actin	*Actinidia deliciosa*	Q0PVN2	20	Defense response	4059	10.8/6.4	68
18	Ribulose bisphosphate carboxylase large chain	*Actinidia deliciosa*	Q5S855	693	Calvin cycle/Photosynthesis	53159	6.2/6.9	57
19	Uncharacterized protein	*Rhizobium meliloti*	I2E1I1	46	-	5427	5.5/4.6	100
20	Haloacid dehalogenase-like hydrolase superfamily protein	*Klebsormidium flaccidum*	A0A0U9I6A1	47	Metabolism/Amino acids	31542	6.6/4.7	20
21	Uncharacterized protein	*Setaria italica*	K3XWK8	166	-	54167	5.1/5.6	33
24	Actinidain	*Actinidia chinensis*	P00785	102	Allergen/Fruit ripening	42545	4.9/4.0	21
28	Actinidain	*Actinidia deliciosa*	A5HII1	366	Allergen/Fruit ripening	42483	4.9/4.1	47
30	Ribosomal protein S12	*Olea europaea*	E3TJL4	13	Protein biosynthesis/Translation	10278	11.0/5.7	22
32	ACT1	*Actinidia deliciosa*	A7YVW7	137	Allergen/Fruit ripening	41865	5.1/6.2	44
33	30S ribosomal protein S7	*Actinidia deliciosa*	A0A0C5CDH4	5	Protein biosynthesis/Translation	17468	5.2/5.2	44

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34	Zinc finger AN1 and C2H2 domain-containing stress-associated protein 11	*Arabidopsis thaliana*	Q8VZ42	158	Transcription	32316	5.5/5.3	12
35	GWD1	*Actinidia deliciosa*	A0A1S5RRZ4	28	Allergen/Fruit ripening	164550	6.0/5.6	11
36	Calmodulin	*Actinidia deliciosa*	B1NDP5	24	Hormone signaling pathway	16678	3.1/6.2	19
37	Phosphoglucose isomerase	*Drosophila primaeva*	A0A096VHF7	70	Glycolysis	18003	6.2/5.9	46
38	30S ribosomal protein S7, chloroplastic	*Actinidia deliciosa*	A0A0C5CDH4	150	Protein biosynthesis/Translation	17468	6.0/6.0	38
41	Glyceraldehyde 3-phosphate dehydrogenase	*Actinidia deliciosa*	B7SFB1	180	Glycolysis	13869	6.0/6.3	18
42	Ribulose bisphosphate carboxylase large chain	*Actinidia deliciosa*	A0A0C5CDD3	149	Calvin cycle/Photosynthesis	53159	6.2/6.9	37
43	Ribulose bisphosphate carboxylase large chain	*Guatteria heterotricha*	Q5S855	150	Calvin cycle/Photosynthesis	51018	5.8/6.9	37
44	Putative mitochondrial aldehyde dehydrogenase	*Populus canadensis*	Q2EHP9	65	Calvin cycle/Photosynthesis	16642	5.1/7.0	21
45	Predicted protein	*Chlamydomonas reinhardtii*	A8HNE2	119	Secondary metabolism	85820	6.0/7.0	26
46	Pto-like protein kinase	*Actinidia deliciosa*	A0A0N6WZX8	114	Allergen/Fruit ripening	21936	9.0/8.9	14
47	Glyceraldehyde 3-phosphate dehydrogenase	*Actinidia deliciosa*	B7SFB1	126	Glycolysis	13869	6.0/5.2	15
48	Lactoylglutathione lyase	*Lupinus angustifolius*	A0A1J7GBZ8	137	Metabolism/Amino acids	35596	5.3/5.3	18
49	Lactoylglutathione lyase	*Lupinus angustifolius*	A0A1J7GBZ9	134	Metabolism/Amino acids	35596	5.3/5.5	18
50	Actin	*Gossypium hirsutum*	Q7XZJ5	147	Defense response	41902	5.2/5.7	41
51	Ethylene receptor	*Actinidia deliciosa*	Q6S5L7	77	Fruit ripening	24861	5.9/6.0	28
52	Actinidain	*Actinidia deliciosa*	A5HII1	577	Allergen/Fruit ripening	42483	4.9/4.0	23
54	Actinidain	*Actinidia eriantha*	gi|146215986	72	Allergen/Fruit ripening	42971	6.3/4.8	16
55	Mitochondrial distribution and morphology protein	*Clavispora lusitaniae*	MDM10_CLAL4	26	Protein biogenesis	51595	6.2/4.9	26
56	Actinidain Act2d	*Actinidia eriantha*	gi|146215986	365	Allergen/Fruit ripening	42971	6.3/5.5	16
57	30S ribosomal protein S7, chloroplastic	*Actinidia deliciosa*	A0A0C5CDH4	238	Protein biosynthesis/Translation	42018	6.2/5.3	19
58	Kiwellin	*Actinidia deliciosa*	L7TV12	216	Allergen/Fruit ripening	23027	5.28/5.5	52
59	Kiwellin	*Actinidia deliciosa*	gi|441482352	216	Allergen/Fruit ripening	23027	5.2/5.5	52
60	Kiwellin	*Actinidia deliciosa*	L7TV12	425	Allergen/Fruit ripening	23027	5.2/5.6	66
61	Kiwellin	*Actinidia deliciosa*	gi|441482352	425	Allergen/Fruit ripening	23027	5.2/6.0	66
62	Peptidyl-tRNA hydrolase	*Rhodopseudomonas palustris*	PTH_RHOPB	62	Secondary metabolism	21834	9.3/6.1	44
63	Kiwellin	*Actinidia deliciosa*	L7TRW4	245	Allergen/Fruit ripening	23023	5.7/6.3	19
64	Kiwellin	*Actinidia deliciosa*	L7TRW4	158	Allergen/Fruit ripening	23023	5.7/6.3	19
65	Kiwellin	*Actinidia deliciosa*	gi|441482346	245	Allergen/Fruit ripening	22997	5.9/6.4	19
66	Actinidain	*Actinidia deliciosa*	Q96228	181	Allergen/Fruit ripening	20324	4.4/6.4	17

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67	Actinidain	*Actinidia chinensis*	ACTN_ACTCH	177	Allergen/Fruit ripening	42545	4.9/4.0	8
68	Actinidain (EC 3.4.22.14) precursor (clone pAC.7)	*kiwi fruit*	gi|81543	181	Allergen/Fruit ripening	20324	4.4/4.1	17
70	Actinidain (EC 3.4.22.14) precursor (clone pAC.7)	*kiwi fruit*	gi|81544	143	Allergen/Fruit ripening	20324	4.4/4.8	17
71	Disintegrin viperistatin	*Daboia palaestinae*	DIS_DABPA	25	Secondary metabolism	4914	4.2/5.5	16
72	50S ribosomal protein L16, chloroplastic	*Actinidia deliciosa*	A0A0C5CHP6	5	Protein biosynthesis/Translation	15292	6.6/6.0	15
73	Kiwellin	*Actinidia deliciosa*	L7TRW4	358	Allergen/Fruit ripening	23023	5.7/7.0	40
74	Kiwellin	*Actinidia deliciosa*	L7TRW4	274	Allergen/Fruit ripening	23023	5.7/6.0	35
75	Kiwellin	*Actinidia deliciosa*	gi|441482354	369	Allergen/Fruit ripening	23011	5.6/6.0	33
76	Kiwellin	*Actinidia deliciosa*	L7TY87	390	Allergen/Fruit ripening	22997	5.9/6.0	52
77	Pentatricopeptide repeat-containing protein	*Arabidopsis thaliana*	PP428_ARATH	63	RNA modification	60739	9.0/6.4	25
78	Ankyrin repeat domain-containing protein 63	*Mus musculus*	ANR63_MOUSE	50	Secondary metabolism	59705	9.4/6.5	21
79	Pto-like protein kinase	*Actinidia deliciosa*	A0A0N9E1N1	173	Allergen/Fruit ripening	21854	6.8/4.4	8
80	Profilin	*Beta vulgaris*	PROF_BETVU	103	Actin binding	2519	4.0/4.5	100
81	minor allergen hazelnut profilin	*Corylus avellana*	gi|12659208	109	Actin binding	14118	4.7/4.6	100
82	kiwellin	*Actinidia deliciosa*	gi|441482346	342	Allergen/Fruit ripening	22997	5.9/4.7	52
83	Thaumatin-like protein	*Actinidia deliciosa*	TLP_ACTDE	222	Allergen/Fruit ripening	25175	8.2/7.0	24
84	Glycine-rich RNA-binding protein	*Sinapis alba*	GRP1_SINAL	157	Defense response	16063	5.2/7.0	22
85	putative glycine-rich RNA-binding protein	*Chorispora bungeana*	gi|209976406	165	Defense response	16796	5.5/5.5	30
86	Kiwellin	*Actinidia deliciosa*	KIWEL_ACTDE	311	Allergen/Fruit ripening	20753	5.8/7.1	34
87	Metallothionein-like protein type 3	*Actinidia deliciosa*	P43389	311	Defense response	20753	5.8/6.9	34
88	thaumatin-like protein	*Actinidia chinensis*	gi|441482370	78	Allergen/Fruit ripening	25221	5.9/7.1	33
89	Kiwellin	*Actinidia deliciosa*	gi|441482346	87	Allergen/Fruit ripening	22997	5.9/4.5	44
90	Kiwellin	*Actinidia deliciosa*	L7TRW4	206	Allergen/Fruit ripening	23023	5.7/5.0	13

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1**Table 2.** Protein identification of kiwifruit flesh 'Gamrok' following ethylene treatments by 2DE-PAGE and mass spectrometry MALDI-TOF TOF

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	*Cicer arietinum*	gi|502088409	297	Protein biosynthesis/Translation	95002	5.7/6.0	37
2	Aconitate hydratase 2, mitochondrial	*Arabidopsis thaliana*	ACO2M_ARATH	107	Glycoxylate bypass	108760	6.7/6.1	14
3	Elongation factor elongation factor 2-like	*Cicer arietinum*	gi|502088409	221	Protein biosynthesis/Translation	95002	5.7/6.3	33
4	Chain A, Crystal Structure of Actinidin	*Actinidia chinensis*	gi|157829826	735	Allergen/Fruit ripening	23942	4.2/6.7	43
5	Actinidin Act2b	*Actinidia eriantha*	gi|146215982	242	Allergen/Fruit ripening	42316	5.8/6.8	17
6	Actinidin Act2b	*Actinidia eriantha*	gi|146215982	314	Allergen/Fruit ripening	42316	5.8/6.9	19
7	NAD(P)H-quinone oxidoreductase subunit K, chloroplastic	*Actinidia deliciosa*	A0A0C5CHE8	19	Calvin cycle/Photosynthesis	32729	8.9/4.5	17
8	Chloroplast heat shock protein 70-2	*Ipomoea nil*	gi|166919372	357	Defense response	74811	5.2/4.5	18
9	Kiwellin	*Actinidia deliciosa*	KIWEL_ACTDE	126	Allergen/Fruit ripening	20753	5.8/5.5	39
10	Actinidin Act2d	*Actinidia deliciosa*	gi|146215986	489	Allergen/Fruit ripening	42971	6.3/5.5	16
11	Actinidin Act2b	*Actinidia eriantha*	gi|146215982	222	Allergen/Fruit ripening	42316	5.8/5.6	13
12	Heat shock protein 70	*Cucumis sativus*	gi|6911549	331	Defense response	73599	5.0/4.9	33
13	kiwellin	*Actinidia deliciosa*	gi|441482352	622	Allergen/Fruit ripening	23027	5.2/5.5	61
17	Photosystem I iron-sulfur center	*Actinidia deliciosa*	A0A0C5CKX4	22	Calvin cycle/Photosynthesis	9545	6.6/5.5	30
21	Actinidain	*Actinidia deliciosa*	A5HII1	697	Allergen/Fruit ripening	42483	4.9/5.5	34
22	Actinidin	*Actinidia deliciosa*	A5HII4	183	Allergen/Fruit ripening	42018	8.8/5.5	11
23	Actinidin Act2a	*Actinidia deliciosa*	A5HII4	247	Allergen/Fruit ripening	42018	8.2/5.6	15
24	Kiwellin	*Actinidia deliciosa*	L7TV12	432	Allergen/Fruit ripening	20753	5.2/6.0	66
25	Actinidin Act2a	*Actinidia deliciosa*	A5HII4	270	Allergen/Fruit ripening	42018	8.0/6.0	15
26	Actinidin Act2a	*Actinidia deliciosa*	A5HII4	179	Allergen/Fruit ripening	42018	8.0/6.5	9
27	Kiwellin	*Actinidia deliciosa*	L7TV12	622	Allergen/Fruit ripening	23027	5.2/6.8	61
28	Kiwellin	*Actinidia deliciosa*	L7TV12	144	Allergen/Fruit ripening	23027	5.2/6.7	66
29	Kiwellin	*Actinidia deliciosa*	L7TY87	187	Allergen/Fruit ripening	22997	5.9/6.8	22
30	Actinidain	*Actinidia chinensis*	P00785	102	Allergen/Fruit ripening	42545	4.9/4.0	21

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31	Uncharacterized protein	*Setaria italica*	K3XWK8	166	-	54167	5.1/5.6	33
32	Actinidain	*Actinidia chinensis*	P00785	102	Allergen/Fruit ripening	42545	4.9/4.0	21
33	Actinidain	*Actinidia chinensis*	P00786	103	Allergen/Fruit ripening	42546	4.9/4.1	22
36	Hypothetical protein	*Streptomyces ipomoeae*	gi|496696994	61	-	8755	5.0/6.4	44
38	Kiwellin	*Actinidia deliciosa*	L7TRW4	16	Allergen/Fruit ripening	23023	5.7/5.6	24
39	Aspartate 1-decarboxylase	*Synechocystis sp.*	PAND_SYNY3	46	Metabolism/Amino acids	16404	5.4/6.8	23
40	GDP-L-galactose phosphorylase	*Actinidia deliciosa*	D3JYW8	12	Defense response	50587	4.8/5.8	9
41	Actinidain	*Actinidia deliciosa*	A5HII1	11	Allergen/Fruit ripening	42483	4.9/5.9	12
42	Actinidain	*Actinidia deliciosa*	Q7DMV2	11	Allergen/Fruit ripening	1409	8.5/6.0	61
43	Kiwellin	*Actinidia deliciosa*	L7TY87	34	Allergen/Fruit ripening	22997	5.9/6.2	40
44	Calmodulin	*Actinidia deliciosa*	B1NDI6	18	Defense response	16735	6.5/6.2	20
45	ACC oxidase	*Actinidia deliciosa*	G3DSB9	11	Defense response	36470	5.4/6.3	8
46	Kiwellin	*Actinidia deliciosa*	L7TRW4	250	Allergen/Fruit ripening	23023	5.7/6.5	59
47	Non-specific lipid-transfer protein	*Actinidia deliciosa*	P86137	15	Lipid Transport	9908	9.1/6.5	30
48	30S ribosomal protein S19, chloroplastic	*Actinidia deliciosa*	A0A0C5CHH0	60	Protein biosynthesis/Translation	10493	10/6.8	11
49	Lipoxygenase	*Actinidia deliciosa*	Q0ZDG2	60	Metal binding	51450	5.9/6.9	5
50	Actinidain	*Actinidia deliciosa*	Q7DMV2	11	Allergen/Fruit ripening	1409	8.5/6.9	61
51	Putative mitochondrial type II NAD(P)H dehydrogenase	*Actinidia deliciosa*	C3VXG0	70	Metal binding	24927	5.8/6.9	5
52	Kiwellin	*Actinidia deliciosa*	L7TRW4	59	Allergen/Fruit ripening	23023	5.7/4.5	13
53	Actinidain	*Actinidia deliciosa*	Q7DMV2	11	Allergen/Fruit ripening	1409	8.5/4.6	61
54	GDP-L-galactose phosphorylase	*Actinidia deliciosa*	D3JYW8	12	Defense response	50587	4.8/5.5	9
55	Actinidain	*Actinidia deliciosa*	Q7DMV2	11	Allergen/Fruit ripening	1409	8.5/5.5	61
56	Actinidain	*Actinidia deliciosa*	Q7DMV2	11	Allergen/Fruit ripening	1409	8.5/5.6	61
57	Metallothionein-like protein type 3	*Actinidia deliciosa*	P43389	18	Defense response	7070	4.4/5.6	30
58	Photosystem I iron-sulfur center	*Actinidia deliciosa*	A0A0C5CKX4	15	Calvin cycle/Photosynthesis	9545	6.6/6.5	33
59	Photosystem II reaction center protein L	*Actinidia deliciosa*	A0A0C5CKT7	9	Calvin cycle/Photosynthesis	4467	4.5/6.5	39
60	Ethylene response factor 7	*Actinidia deliciosa*	D8VD34	6	Fruit ripening	25516	9.3/6.0	9

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61	30S ribosomal protein S7, chloroplastic	*Actinidia deliciosa*	A0A0C5CDH4	5	Protein biosynthesis/Translation	17468	11/6.1	9
62	Photosystem I reaction center subunit	*Cucumis sativus*	PSAN_CUCSA	32	Calvin cycle/Photosynthesis	2791	4.5/6.2	45
66	Cytochrome f	*Actinidia deliciosa*	A0A0C5CDD8	21	Calvin cycle/Photosynthesis	35426	9.8/6.7	15
67	Actinidain	*Actinidia deliciosa*	A5HII1	577	Allergen/Fruit ripening	42483	4.9/4.0	23
68	Actinidin	*Actinidia eriantha*	gi|146215986	72	Allergen/Fruit ripening	42971	6.3/4.8	16
70	50S ribosomal protein L36, chloroplastic	*Actinidia deliciosa*	A0A0C5CYN0	8	Protein biosynthesis/Translation	4615	11.9/5.5	29
71	50S ribosomal protein L36	*Actinidia deliciosa*	A0A0C5CYN0	8	Protein biosynthesis/Translation	4615	11.9/5.8	29
72	actinidin Act2d	*Actinidia eriantha*	gi|146215986	365	Allergen/Fruit ripening	42971	6.3/5.5	16
73	30S ribosomal protein S7, chloroplastic	*Actinidia deliciosa*	A0A0C5CDH4	238	Protein biosynthesis/Translation	42018	6.2/5.3	19
74	Kiwellin	*Actinidia deliciosa*	L7TV12	216	Allergen/Fruit ripening	23027	5.28/5.5	52
75	50S ribosomal protein L36, chloroplastic	*Actinidia deliciosa*	A0A0C5CDH4	238	Protein biosynthesis/Translation	4615	11.9/5.5	29
76	Kiwellin	*Actinidia deliciosa*	gi|441482352	216	Allergen/Fruit ripening	23027	5.2/5.5	52
77	Kiwellin	*Actinidia deliciosa*	gi|441482352	425	Allergen/Fruit ripening	23027	5.2/6.0	66
78	50S ribosomal protein L36, chloroplastic	*Actinidia deliciosa*	A0A0C5CYN0	8	Protein biosynthesis/Translation	4615	11.9/6.0	29
79	Kiwellin	*Actinidia deliciosa*	L7TRW4	158	Allergen/Fruit ripening	23023	5.7/6.3	19
80	Kiwellin	*Actinidia deliciosa*	gi|441482346	245	Allergen/Fruit ripening	22997	5.9/6.4	19
81	Actinidain	*Actinidia chinensis*	ACTN_ACTCH	177	Allergen/Fruit ripening	42545	4.9/4.0	8
82	Actinidain	*Actinidia chinensis*	gi|81543	181	Allergen/Fruit ripening	20324	4.4/4.1	17
83	Kiwellin	*Actinidia deliciosa*	L7TRW4	63	Allergen/Fruit ripening	23023	5.7/5.5	41
84	Actinidain	*Actinidia deliciosa*	gi|81544	143	Allergen/Fruit ripening	20324	4.4/4.8	17
85	Ankyrin repeat domain-containing protein 63	*Mus musculus*	ANR63_MOUSE	50	Secondary metabolism	59705	9.4/6.5	21
86	Kiwellin	*Actinidia deliciosa*	L7TRW4	274	Allergen/Fruit ripening	23023	5.7/6.0	35
87	GA signaling F-Box	*Actinidia deliciosa*	V5QMV7	5	-	19628	9.3/6.0	8
88	kiwellin	*Actinidia deliciosa*	gi|441482346	342	Allergen/Fruit ripening	22997	5.9/4.7	52
89	Kiwellin	*Actinidia deliciosa*	gi|441482354	369	Allergen/Fruit ripening	23011	5.6/6.0	33
92	Kiwellin	*Actinidia deliciosa*	L7TY87	390	Allergen/Fruit ripening	22997	5.9/6.0	52
95	Pto-like protein kinase	*Actinidia deliciosa*	A0A0N9E1N1	173	Allergen/Fruit ripening	21854	6.8/4.4	8

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96	Profilin	*Beta vulgaris*	PROF_BETVU	103	Actin binding	2519	4.0/4.5	100
97	Minor allergen hazelnut profilin	*Corylus avellana*	gi|12659208	109	Actin binding	14118	4.7/4.6	100
98	Putative glycine-rich RNA-binding protein	*Chorispora bungeana*	gi|209976406	165	Defense response	16796	5.5/5.5	30
99	Metallothionein-like protein type 3	*Actinidia deliciosa*	P43389	311	Defense response	20753	5.8/6.9	34
100	Ethylene receptor ERS1b	*Actinidia deliciosa*	B3FIA8	8	Fruit ripening	71118	6.0/6.9	5
101	GDP-L-galactose phosphorylase	*Actinidia deliciosa*	D3JYW8	8	-	50587	4.8/6.8	7
102	50S ribosomal protein L36, chloroplastic	*Actinidia deliciosa*	A0A0C5CYN0	8	Protein biosynthesis/Translation	4615	11.9/7.0	29
103	50S ribosomal protein L36, chloroplastic	*Actinidia deliciosa*	A0A0C5CYN0	8	Protein biosynthesis/Translation	4615	11.9/6.0	29
104	50S ribosomal protein L36, chloroplastic	*Actinidia deliciosa*	A0A0C5CYN0	8	Protein biosynthesis/Translation	4615	11.9/6.0	29

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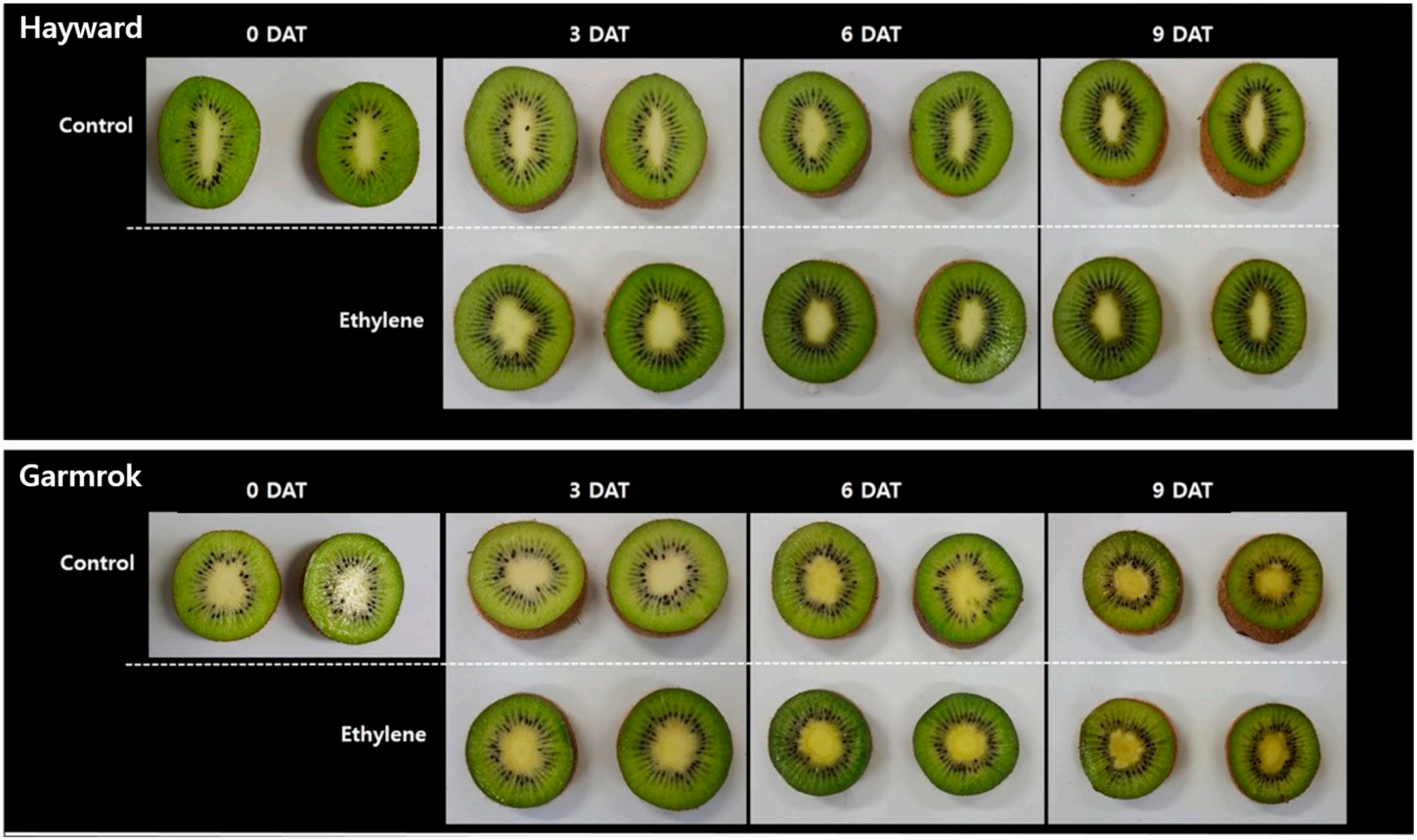

Figure 1. Morphological differences or ripening behavior in 'Gamrok' and 'Hayward' kiwifruit cultivars after ethylene treatment for 3, 6 and 9 days along with control (0 days. Without treatment). Figure depicts fruits from 'Gamrok' cultivar is ready to eat (fully ripened) after 6 days of ethylene treatment. While, fruits from 'Hayward' cultivar are ready to eat both at 6 and 9 days after ethylene treatment.

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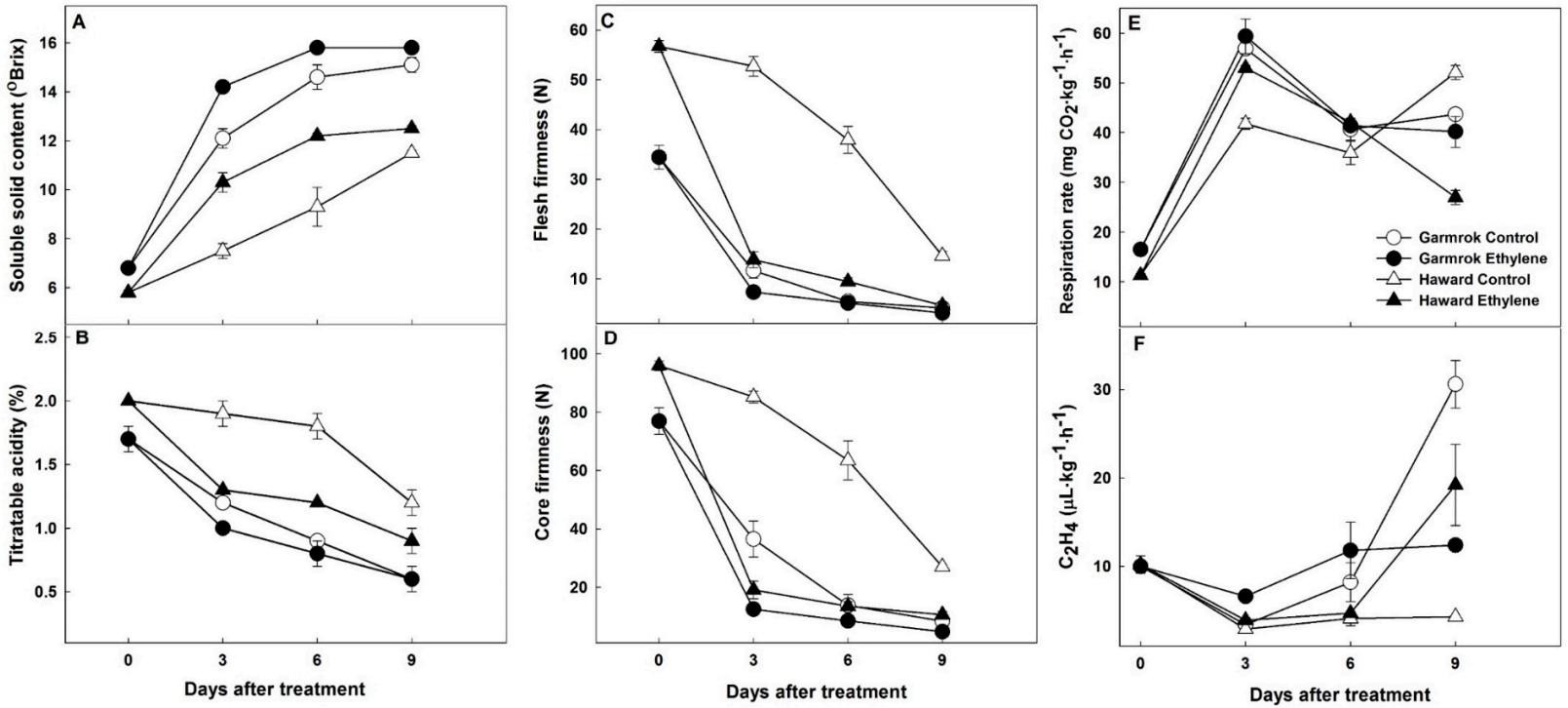

Figure 2. Changes of (A) soluble solid content, (B) titratable acidity, and (C) flesh firmness, and (D) core firmness, and (E) respiration rate, and (F) ethylene production in 'Hayward' and 'Gamrok' kiwifruit cultivars after ethylene treatment for 3, 6 and 9 days along with control (0 days. Without treatment). Lines indicate Mean±SE with ten biological replicates.

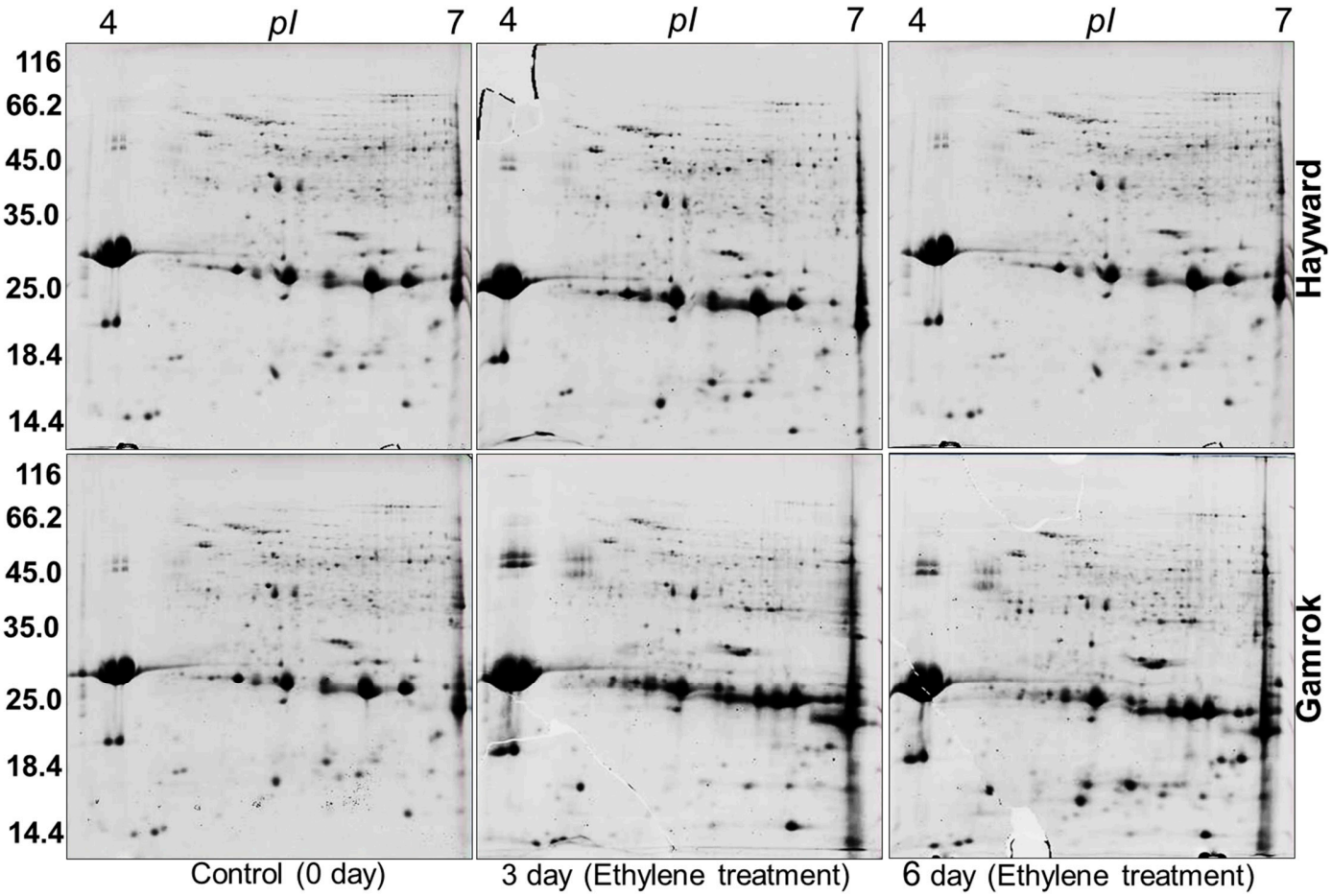

Figure 3. Representative second dimensional gel electrophoresis (2DE) maps of kiwifruit cultivars. 2DE maps show total protein profile from the control (0 day) and ethylene treated (3 and 6 day) 'Hayward' and 'Gamrok' kiwifruit cultivars. Total proteins were extracted by phenol and 400 µg of protein samples were separated by isoelectric focusing (IEF) using 17 cm IPG strips with pH range 4-7. Focused strips were processed for second dimension polyacrylamide gel electrophoresis for second dimension and stained with colloidal Coomassie brilliant blue.

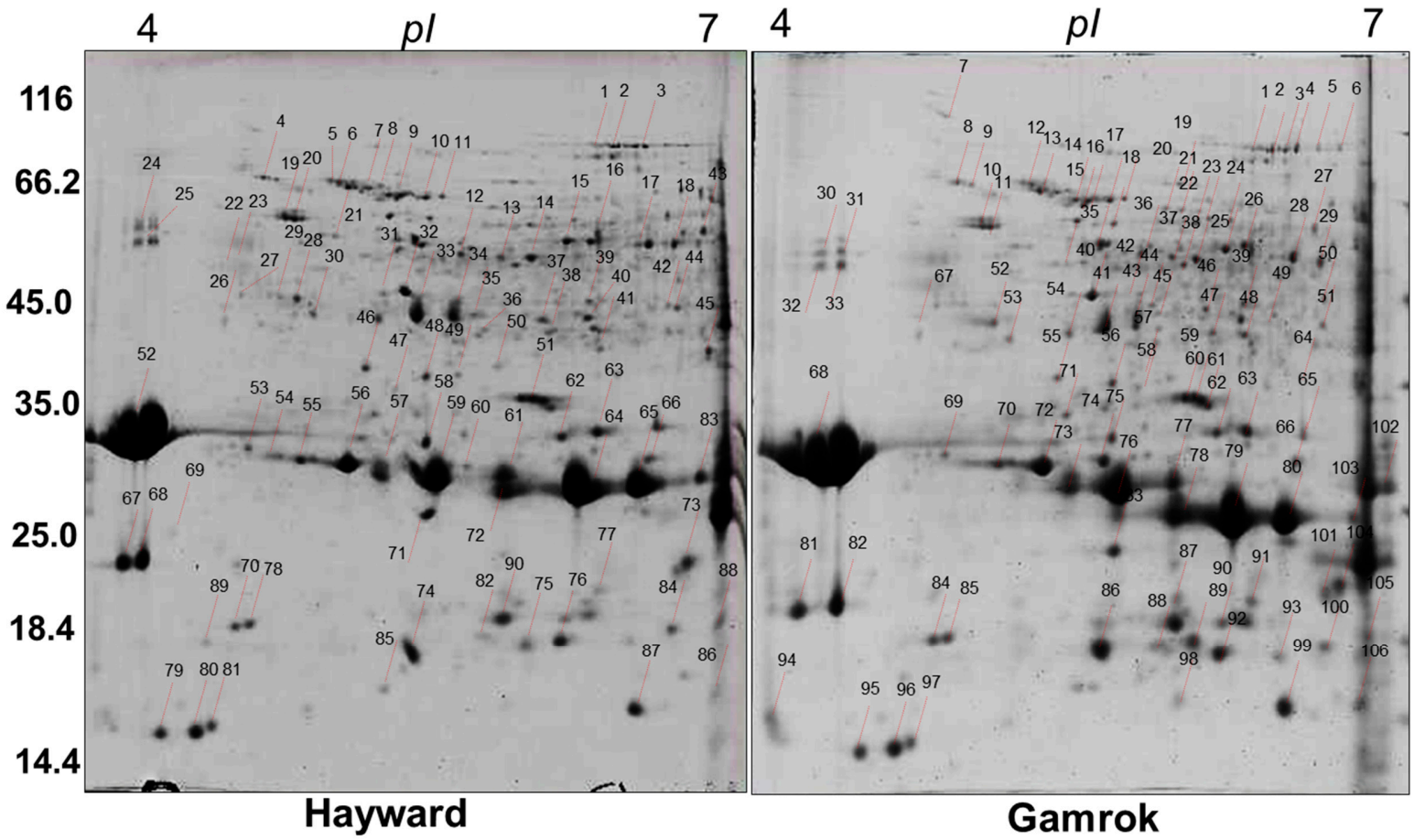

Figure 4. Higher level match set of protein spots detected on 2DE maps of kiwifruit cultivars. The matches were created from three standard replicates of control (0 day) and ethylene treated (3 and 6 day) 'Hayward' and 'Gamrok' kiwifruit cultivars using Progenesis SameSpots TotalLab (New Castle, UK). The numbers on 2DE gels indicate differentially expressed protein. For descriptive quantification analysis of each spot please refer Supplementary Table 1 and Table 2.

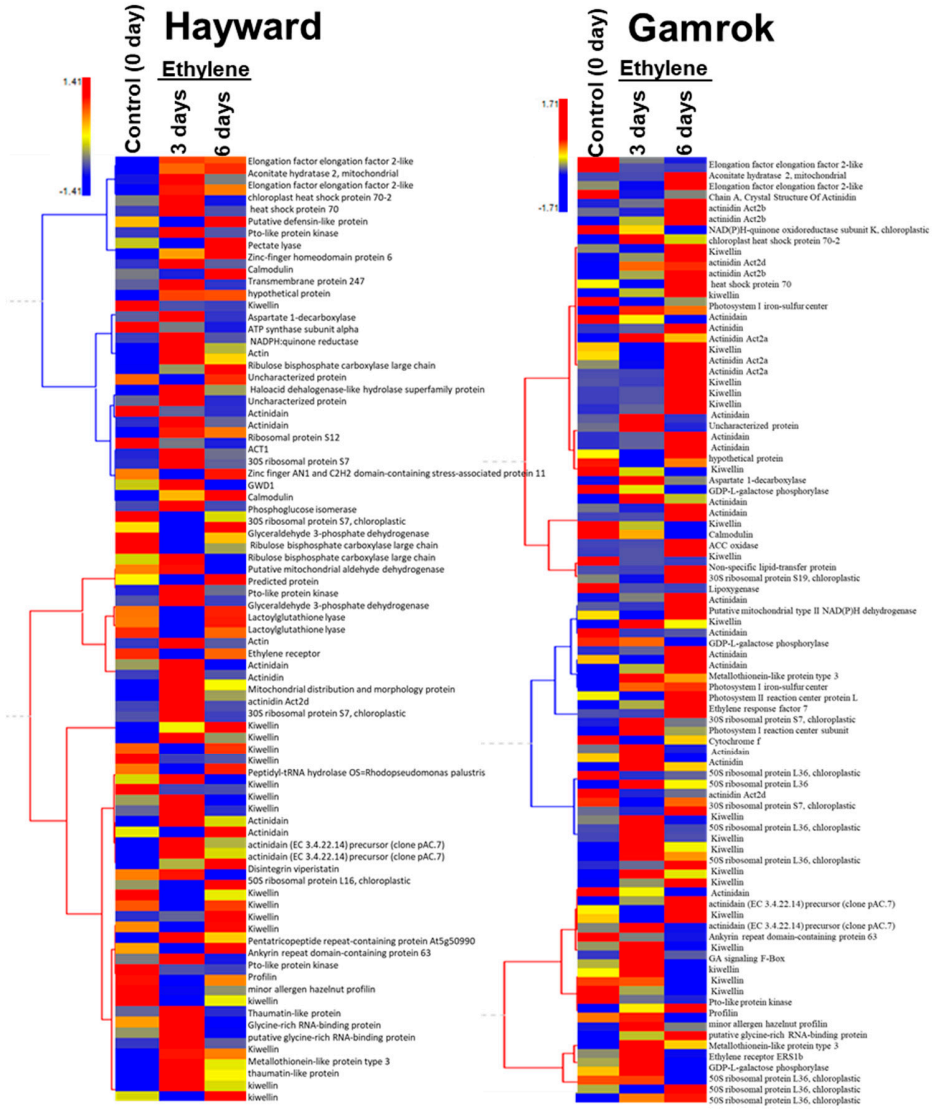

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Figure 5. Hierarchical clustering analysis of the protein spots that resulted to change their relative volume from the control (0 day) and ethylene treated (3 and 6 day) 'Hayward' and 'Gamrok' kiwifruit cultivars. Mean values of three independent determination for each treatment were expressed as ratios between control (0 days) and ethylene treated (3 and 6 days) using hierarchical clustering explorer (HCE 3.5 interactive power analysis) software. Relative quantification for each protein spot is provided in Supplementary Table 1 and Table 2.

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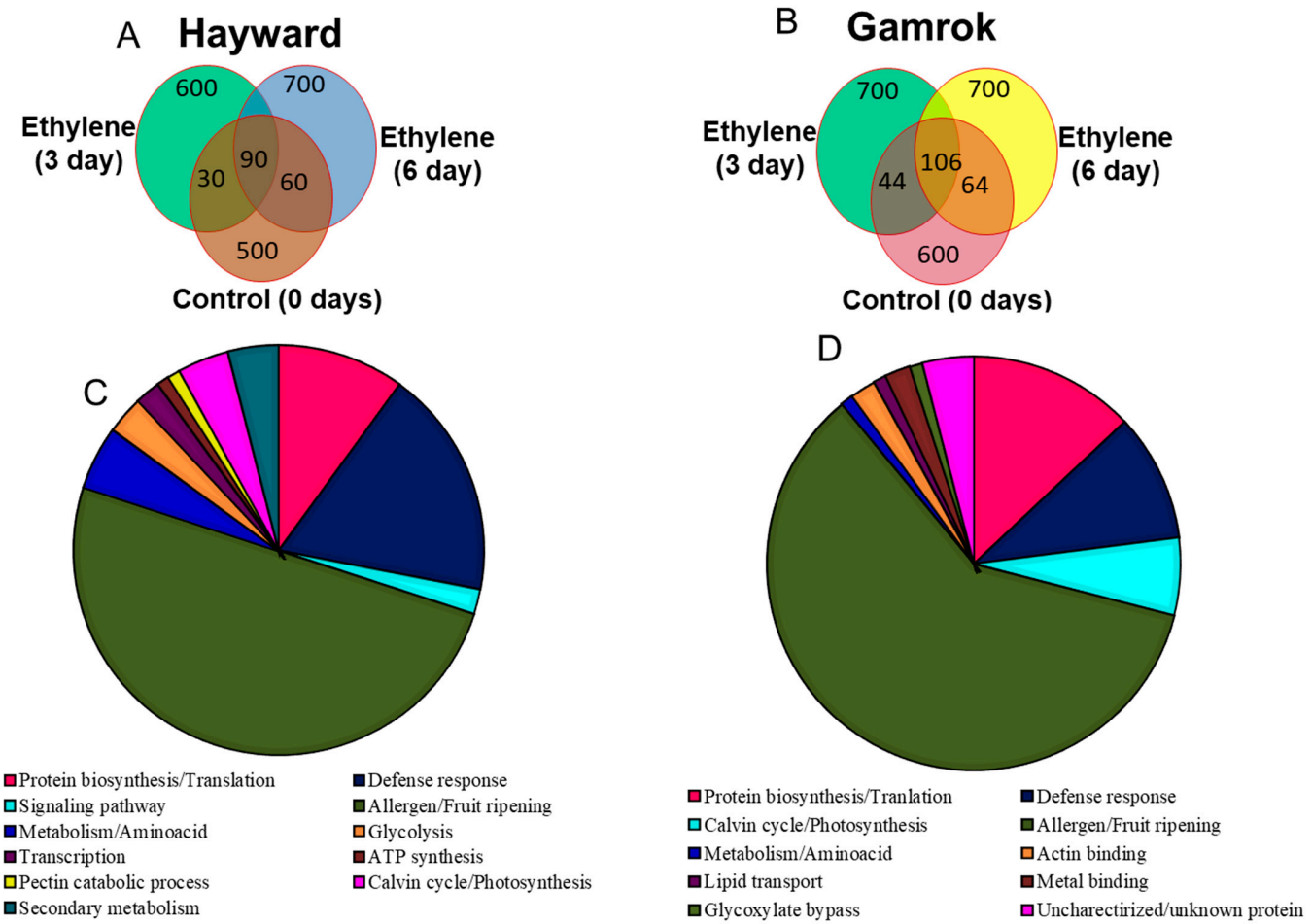

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2 **Figure 6.** (A-B) Comparative analysis and (C-D) Functional classification of identified proteins of kiwifruit cultivars. Comparative analysis was
3 created from three standard replicates of control (0 day) and ethylene treated (3 and 6 day) 'Hayward' and 'Gamrok' kiwifruit cultivars using
4 Progenesis SameSpots TotalLab (New Castle, UK). The functional annotations were created using panther gene analysis.

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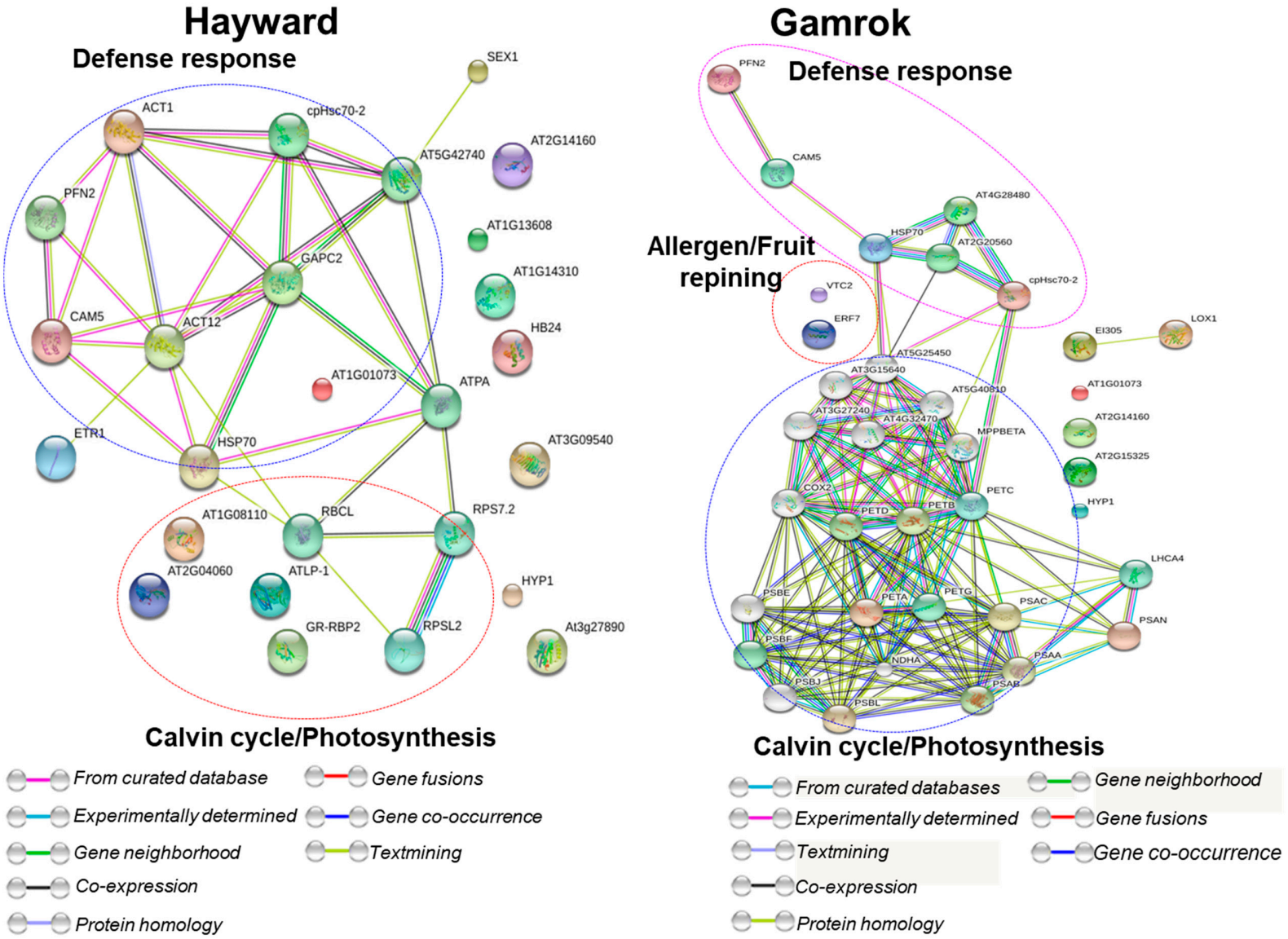

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Figure 7. Analysis of protein interaction network by STRING 9.1. TAIR homologous proteins from identified proteins were mapped by searching the STRING 9.1 software with a confidence of 0.4. using *Arabidopsis thaliana*. Colored lines between the proteins indicate the various types of interaction evidence. The clusters of highly interacting protein nodes are marked with oval dotted lines and include proteins involved in defense response, Calvin cycle/photosynthesis, and allergen/fruit ripening

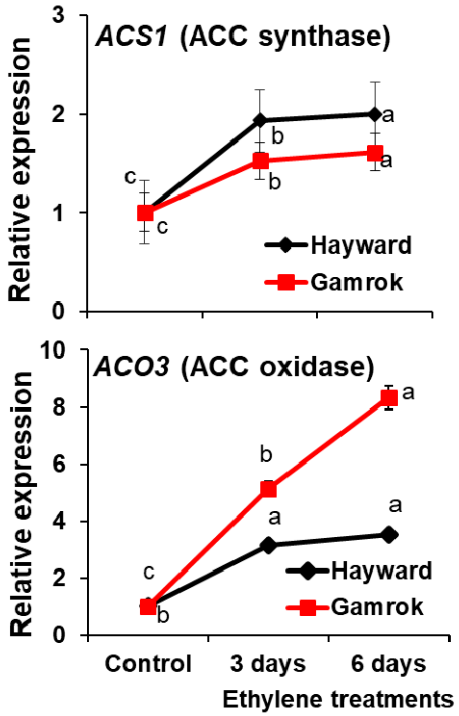

Figure 8. Relative transcript level of ACC synthase (*ACS1*), and ACC oxidase (*ACO3*) in kiwifruit (cv. 'Hayward' and 'Gamrok') under 0 day (control), 3 and 6-day ethylene treatments. The results were normalized to a housekeeping gene Actin. Red and black lines indicate Mean±SE for n=3. Bars denoted by the different letter are significantly different at $P \leq 0.05$ according to the Tukey's studentized range test bestowing to SAS analysis (statistical analysis software).

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