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Posted Date: 28 July 2023

doi: [10.20944/preprints2023071967v1](https://doi.org/10.20944/preprints2023071967v1)

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Article

# A Genome-Wide Analysis of Pentatricopeptide Repeat Protein Gene Family in Two Kiwifruit Species with an Emphasis on Roles of RNA Editing in Pathogens Stress

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**Abstract:** Kiwifruit is a perennial horticultural crop species with high nutritional and economic value. However, various pathogens stress brings a serious threat to kiwifruit. Pentatricopeptide repeat proteins (PPRs) are characterized by tandem repetitions of 35 amino acid motifs, and have been found to function in plant RNA editing. However, the roles of PPRs in plant development and disease resistance remain unclear. In this study, we performed a genome-wide identification and characterization of PPR gene family in two kiwifruit species (*Actinidia chinensis* (*Ach*), and *Actinidia eriantha* (*Ace*)) with markedly different disease resistance. A total of 497 and 499 PPRs were identified in *Ach* and *Ace*, respectively. All the kiwifruit PPRs could be phylogenetically divided into four subfamilies. There were about 40.68% PPRs predicted to be localized to mitochondria or chloroplasts. Synteny analysis showed that the expansion of kiwifruit PPRs mainly came from segmental duplication. Based on RNA-seq data from the fruit during 12 periods of development and maturity, weighted correlation network analysis suggested that two PPRs *Actinidia20495.t1* and *Actinidia15159.t1* were involved in the fruit development and maturation. In addition, we observed different gene expressions of PPRs and chloroplast RNA editing profiles between resistant and susceptible kiwifruits after pathogen infection, indicating the roles of PPRs in stress response by modulating the editing extend of mRNA. The differentially expressed upstream transcription factors of PPRs were further identified, they may regulate resistance adaption by modulating the PPRs expression. These results provide a solid foundation for further analyses of the functions and molecular evolution of PPRs, in particular, for clarifying the resistance mechanisms in kiwifruits and breeding new cultivars with high resistance.

**Keywords:** kiwifruit; pentatricopeptide repeat protein; pathogens stress; RNA editing

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## 1. Introduction

Kiwifruit (*Actinidia chinensis*, *Ach*), also called the Chinese gooseberry, is a perennial horticultural crop species, it is rich in vitamin C, minerals, dietary fiber, and other nutrients for health benefits, so it has enormous nutritional and economic value [1]. But biotic and abiotic stresses have adversely affected its growth and yield. The bacterial canker of kiwifruit is caused by the bacterial pathogen *Pseudomonas syringae* pv. *actinidiae* (*Psa*), is a severe threat to kiwifruit production [2]. This disease was first reported on *Actinidiae chinensis* var. *deliciosa* in Shizuoka, Japan in 1984, *Psa* can degrade lignins and phenols, which are destructive to its growth, it has been reported in the main kiwifruit-producing countries, including China, Chile, and European countries so far [3-5]. The kiwifruit cultivars are generally sensitive to *Psa*, such as the current master cultivars in New Zealand and China, 'Hort 16A' and 'HongYang', respectively, however, some kiwifruits, such as *Actinidia*

*eriantha* (*Ace*) cv. "HuTe", has been proven to be resistant to Psa in contrast to *Actinidia chinensis* cv. 'HongYang'. Psa can be colonized on the surface of kiwifruit, such as leaves, flowers, and stems, and enters the vine through natural stomata and wounds followed by systemic infection. The infection with Psa in kiwifruit is usually in a short time. Pathogen stress is destructive and economically damaging for kiwifruit, so it is of great significance to study the stress resistance mechanism in Kiwifruit.

As a typical post-transcriptional modification, RNA editing is mainly manifested as nucleotide insertion/deletion or conversion [2]. Plant RNA editing occurs mainly in plastids and mitochondria, and plays a key role in post-transcriptional regulation [6-8]. In flowering plants, RNA editing usually converts 400-500 C-to-U (Cytosine-to-Uracil) in mitochondria's transcripts and 30-40 C-to-U in plastids' transcripts [9]. RNA editing plays important roles in various plant developmental processes, such as organelle biogenesis, adaptation to environmental changes, and signal transduction [10,11]. RNA editing is an important process to maintain essential functions of encoded proteins at the RNA level, for example, pigment deficiency in tobacco cybrids is caused by the editing failure of the plastid ATP synthase alpha-subunit (atpA) mRNA [12]. Dynamic response of plant RNA editing to environmental factors was detected in previous studies [13-15]. In several recent studies, RNA editing was proven to be responsive to pathogen stress and affect disease resistance [16-18].

Plant RNA editing is mainly mediated by RNA editing complexes, including the pentatricopeptide repeat proteins (PPRs), organelle RNA recognition motif-containing proteins (ORRMs), protoporphyrinogen IX oxidases (PPOs), and RNA editing factor interacting proteins (RIPs)/multiple organellar RNA editing factors (MORFs) [10,19,20]. PPRs directly interact with mRNA to determine the specificity of RNA editing, and a PPR protein specifically recognizes one or several editing sites [21]. PPRs are characterized by the tandem repeats of a degenerate 35 amino acid sequence. The tandem repeats often range from 2 to 27 copies in the recently recognized eukaryotic PPR gene family. Based on domain assembling structure, the PPR gene family is classified into two subfamilies, the P subfamily, and the PLS subfamily. In general, the P-type PPRs function in most aspects of organelle gene expression, while the PLS-type PPRs mainly function in RNA editing [22]. The P-type subfamily is characterized by PPR motifs adjacent to each other without gaps. Most of the reported P-type PPRs play roles in organelle RNA stabilization and splicing, whereas P-type PPRs involved in both RNA splicing and editing have rarely been reported. However, the PLS subfamily consists of PPR motifs interspaced by two PPR-like motifs, the short PPR-like motif (PPR-like S) composed of 31 amino acids and the long PPR-like motif (PPR-like L) composed of 35-36 amino acids. Based on the motifs identified in the C terminal, PLS-type PPRs are divided into three subclasses, PPR-E, PPR-E+, and PPR-DYW, and function as critical factors in the C-to-U editing of mRNAs. The DYW domain in PPR-DYW proteins provide the cytidine deaminase activity for RNA C-to-U editing and PPR-E+ proteins recruit an atypical PPR-DYW protein to function in RNA editing [23]. Approximately 200 PLS-type PPRs are involved in RNA editing in plastids and mitochondria in *Arabidopsis thaliana* [21].

PPRs are extensively distributed across plant lineages, containing more than 400 family members, and have been extensively identified in many different plants, with 450, 477, and 486 members of PPRs predicted in diploid *Arabidopsis*, rice, and foxtail genomes, respectively [24]. PPRs function as organellar-specific RNA-binding proteins and play an essential role in multiple organelle functions and biological processes. Besides male fertility restoration, PPRs are involved in mediating many aspects of gene expression, RNA exons splicing, editing, stability, and translation of RNAs [25-27]. Defects in their functions often lead to organelle dysfunction, growth defects, embryo development defects, and biotic and abiotic stress sensitivity [23,28-32]. OsPPR11 was proven to be essential for chloroplast development and function by affecting group II intron splicing in rice [33]. Maize PPR-E proteins mediate RNA C-to-U editing in mitochondria by recruiting the Trans deaminase PCW1 [23]. In addition to the plant developmental process, PPRs have been reported to be involved in the responses to biotic and abiotic stresses. For instance, in *Arabidopsis*, several PPRs such as SOAR1, PGN, SLG1, and PPR96, have been shown to participate in responses to abiotic stresses. Engineering PPRs can be potentially applicable for synthetic and RNA biology and

harnessed for the manipulation of intron splicing and/or stabilization of organellar RNA molecules, and these redesigned PPRs will be a potential means to improve plants' fitness to developmental and environmental cues.

However, the function of PPRs in kiwifruit, especially their roles in disease resistance remains unclear, and the underlying response mechanisms particularly regarding the roles of RNA editing events are not fully understood. Accordingly, we studied PPRs in the kiwifruit genome based on public transcriptome data. In this study, we performed a meta-analysis to evaluate the expression pattern of PPRs and RNA editing profiles under pathogens infection. We observed apparent responses of RNA editing extent and PPR gene expression to infection and identified candidate upstream transcription factors that may regulate plant immunity by modulating the PPRs gene expression. Our results provide insight into the fascinating properties and biological functions of PPRs in response to pathogens stress in kiwifruit and will help elucidate the roles of RNA editing in plant immunity.

## 2. Results

### 2.1. Identification and Synteny Analysis of PPRs Members in Two Kiwifruit Species

We searched the *Ach* genome with known 450 *Arabidopsis* PPRs as queries, 644 and 497 putative *Ach* proteins were identified from HMM [34] and BLASTP searches, respectively, and 497 overlapping PPRs that were both identified were kept as PPRs of *Ach*. Similarly, 499 putative *Ace* PPRs were identified. Based on their phylogenetic relationship from full-length amino acid sequences (Figure 1), all the PPRs were classified into four clades, including P-, and PLS- types (E-type, E+-type, and DYW-type), with 251, 91, 95, and 60 in *Ach*, and 247, 74, 102, 76 in *Ace*, respectively. P-type PPRs were the most and DYW-type PPRs were the least in *Ach*, while P-type PPRs were the most and E class were the least in *Actinidia eriantha* (Table 1). A combination of TargetP2.0 [35] and Predotar4.0 [36] was used to predict the subcellular localization of PPRs. The results showed that 40.64% were localized in mitochondria or chloroplasts in *Ach*, with 28.37% of the PPRs located in mitochondria, and 12.27% in chloroplasts. For *Ace*, 43.88% were located in *Ace* mitochondria or chloroplasts, with 32.06% of the PPRs located in mitochondria, and 11.82% were located in chloroplasts. The number of amino acids in *Ach* ranged from 91 to 1871. The number of amino acids in *Ace* ranged from 111 to 1738 (Table S1 and S2). Chromosomal localization results revealed that kiwifruit PPRs were widely distributed on all of the chromosomes (Figure S1).

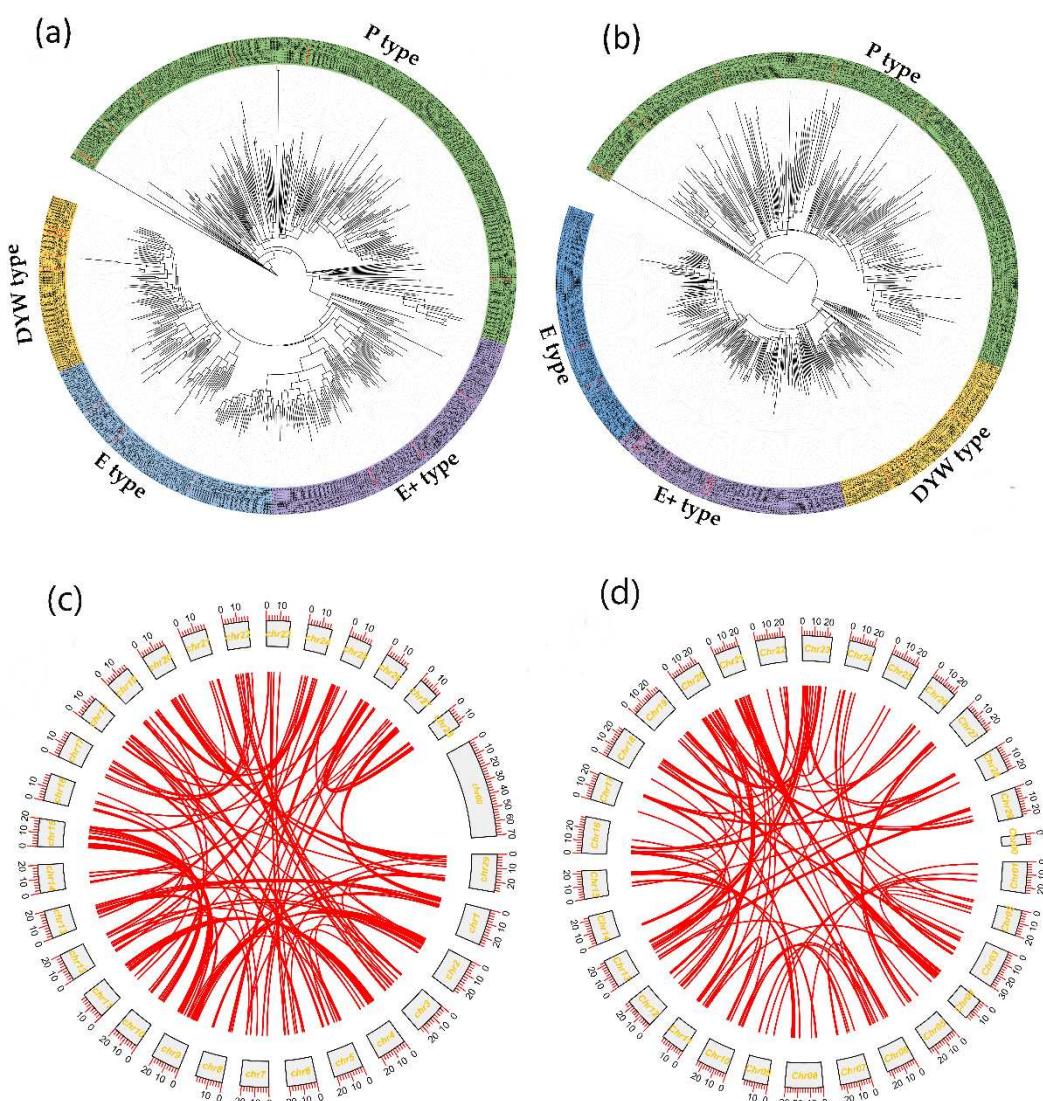
Synteny analysis within the kiwifruit genome using MCScanX software was further conducted to determine their duplication events and possible collinear blocks between genomes, as shown in Figure 1. The collinearity analysis of the PPR protein of *Ach* and *Arabidopsis thaliana* showed that 166 *Ach* PPRs showed syntenic relationships with 156 PPRs of *Arabidopsis thaliana*, while 136 *Ace* PPRs showed syntenic relationships with 134 PPRs in *Arabidopsis thaliana*, involving all the four types of PPRs, indicating the evolution conservation of PPRs. Synteny analysis within the genomes showed that kiwifruit PPRs shared a high homologous conservation. The origins of duplicated genes in the kiwifruit genome were further classified into five classes, including whole genome/segmental (match genes in synteny blocks), tandem (continuous repeat), proximal (in the nearby chromosomal region but not adjacent) or dispersed (other modes than segmental, tandem and proximal) duplications. The results showed that the origins of duplicated PPRs in *Ach* were classified into 11 singletons, 24 dispersed, 8 proximal, 9 tandem, and 445 segmental (Table S1). PPRs in *Ace* shared the same distribution, which indicated that the expansion of kiwifruit PPRs mainly resulted from segmental duplication that was accompanied by whole genome duplication.

The Ka/Ks values were calculated for the paralogous PPRs to explore the selection pressure during the evolution, about 60 pairs of paralogous PPRs were obtained from MCScanX [37] for each kiwifruit species, and all the Ka/Ks values of these gene paralogs were less than one, suggesting that these genes evolved under purifying selection. In addition, we obtained the orthologous PPRs between *Ach* and *Ace*, a total of 306 pairs were obtained from MCScanX, and only two pairs exhibited Ka/Ks with more than one, including pairs *Actinidia00869-DTZ79\_13g12210*, and *Actinidia08538-*

*DTZ79\_28g04010*, the former belongs to P-type, and the latter belongs to E+ type PPRs. This observation indicated that PPRs evolved slowly after the speciation of *Ach* and *Ace*.

**Table 1.** Distribution of different types of PPRs in two kiwifruit species.

	<i>Actinidia chinensis</i> (Ach)		<i>Ace</i> ( <i>Ace</i> )	
	Number	Percent	Number	Percent
P- type	251	50.5%	247	49.50%
E- type	91	18.31%	74	14.83%
E+ type	95	19.11%	102	20.44%
DYW type	60	12.07%	76	15.23%
All	497	/	499	/



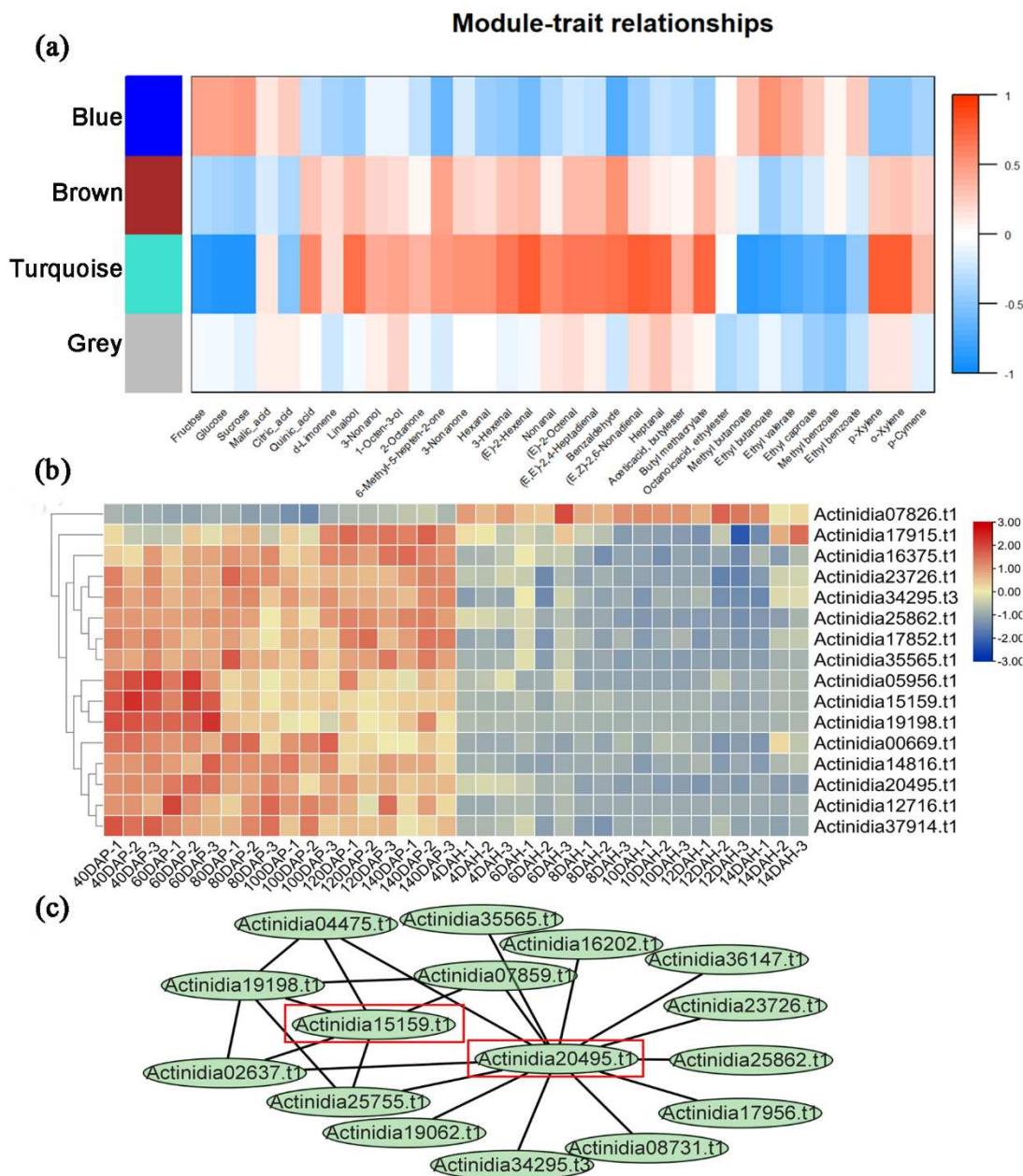
**Figure 1. The phylogenetic relationships and self-collinearity of PPRs in *Actinidia chinensis* (Ach) and *Actinidia eriantha* (Ace).** A total of 497 and 499 PPRs in Ach and Ace were used for phylogenetic tree construction by the maximum likelihood (ML) method [38], respectively. All the PPRs were classified into four groups; different groups are shaded by different colors, green, blue, purple, and yellow areas indicating P-, E-, E+-, and DYW- types of PPRs, respectively. (a) The phylogenetic relationships of PPRs in Ach. (b) The phylogenetic relationships of PPRs in Ace. (c) Interchromosomal

relationships of PPRs in *Ach*. (d) Interchromosomal relationships of PPRs in *Ace*. The red lines highlight the syntenic PPRs pairs.

## 2.2. PPRs Play Roles in Fruit Development and Ripening

Based on RNA-seq data [39] during the fruit development and maturation of *Ach*, we examined the gene expression profiles of PPRs in kiwifruit during different periods. After preliminary screening, a total of 260 PPRs showed expression during fruit development and maturation for *Ach*. Most PPRs were highly expressed in the development stage and lowly expressed after harvest, and a small part of PPRs were highly expressed in the maturity stage. 16 PPRs exhibited differentially expression at different time points (Figure 2). In terms of subfamily classification of PPRs, the expression of PLS-type PPRs was higher in the development stage, while the expression of P-type PPRs was highly expressed in the maturity stage. Module-feature clustering was conducted based on the expression of PPRs during fruit development and maturation of kiwifruit, based on weighted gene co-expression network analysis (WGCNA) results, PPRs were divided into four modules (turquoise, blue, brown, and grey), consisting of 188, 7, 5, and 60 PPRs, respectively (Table S3). The results showed that the turquoise module gene expression was mostly associated with traits such as glucose, quinic acid, and linalool, gene expression of PPRs in this module negatively correlated with the contents of sugars (such as glucose) and esters (such as ethyl butyrate), and positively correlated with the contents of alcohols (such as linalool) and acids (such as quinic acid) (Figure 2). In other words, during the fruit development and maturation stage, PPRs in the turquoise module negatively regulated the synthesis of the content of glucose, other sugars and ethyl butyrate, and other esters, but positively regulated the synthesis of quinic acid, linalool, etc. In terms of the relationships between different modules, the turquoise module was positively correlated with the brown module gene, and negatively correlated with the blue module gene, while the gray module was not associated with any other modules.

Furthermore, we used the turquoise module to screen hub PPRs that own a high degree in the module, which can better represent the overall expression level of this module gene and be more correlated with characteristics (Figure 2). The PPRs with module member closeness greater than 0.8 were selected as the hub genes. After screening, there were 72, 4, 7, and 11 hub genes related to glucose, quinic acid, linalool, and ethyl butanoate, respectively. Some hub PPRs are hub genes associated with multiple biomarkers' characteristic metabolites. For example, *Actinidia20495.t1* was a hub gene in regulating the synthesis of glucose, linalool, and ethyl butanoate. *Actinidia15159.t1* was a hub gene in regulating the synthesis of glucose, quinic acid, and linalool. *Actinidia14816.t1* and the other three genes were hub genes in regulating the synthesis of glucose and linalool. *Actinidia07826.t1* and the other eight genes were hub genes of glucose and ethyl butanoate. *Actinidia19198.t1* is a hub gene in regulating the synthesis of quinic acid and linalool. *Actinidia05956.t1* is a hub gene in regulating the synthesis of glucose and quinic acid. The PPRs which regulated glucose synthesis were closely related to themselves. Module membership-gene characteristics associated with glucose, quinic acid, linalool, and ethyl butyrate in kiwifruit were shown in Figure S2. In conclusion, we hypothesized that *Actinidia20495.t1* and *Actinidia15159.t1* were involved in the fruit development and maturation of kiwifruit, they may play roles in the synthesis of sugar and acid compounds, as well as other changes of content of alcohols, such as linalool and esters, ethyl butyrate in kiwifruit during development and ripening.



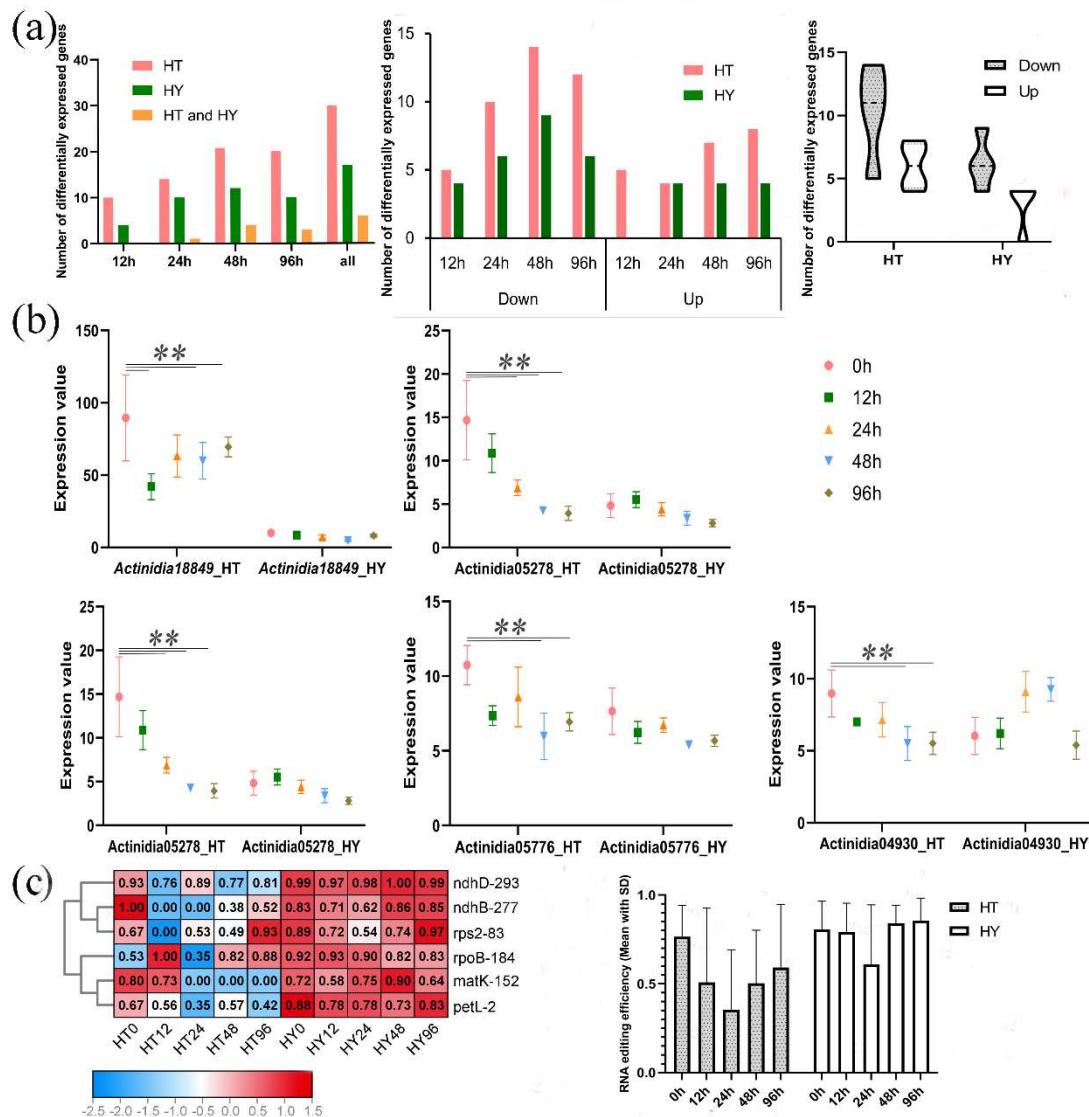
**Figure 2. Expression analysis of PPRs during fruit development and ripening in *Actinidia chinensis* (*Ach*).** (a) Module-feature clustering based on the expression of PPRs during fruit development and maturation of *Ach*. The color gradient of the heatmap indicates the level of correlation, with red indicating positive correlation and blue indicating negative correlation. (b) Gene expression heatmap of PPRs during fruit development and maturation of *Ach*. The x-axis represents six developmental periods (40, 60, 80, 100, 120, and 140 days after pollination (DAP), and six maturity periods (4, 6, 8, 10, 12, and 14 days after harvest of ripe fruit (DAH)). (c) Co-expression network of PPRs during fruit development and maturation of *Ach*, two hub PPRs were highlighted by red rectangles.

### 2.3. Different PPRs Expression and RNA Editing Patterns in Response to *Psa* Infection in Two Kiwifruit Species

Based on RNA-seq data [40] in resistant ('HuaTe', *Ace*) and susceptible ('HongYang', *Ach*) kiwifruits during early infection of *Pseudomonas syringae* pv. *Actinidiae* (*Psa*), we examined the expression of PPRs between two different kiwifruit species (Figure 3). After *Psa* infection, tens of PPRs were differentially expressed in both 'HongYang' and 'HuaTe'. A total of 45 differentially expressed PPRs were identified in two kiwifruits, there were 8 differentially expressed PPRs shared

in both kiwifruits, 26 specific to 'HuaTe' and 10 specific to 'HongYang'. The shared 8 differentially expressed PPRs were *Actinidia05776*, *Actinidia08478*, *Actinidia18849*, *Actinidia19198*, *Actinidia24301*, *Actinidia25020*, *Actinidia28434*, *Actinidia29423*, which were labeled in Table S4. 18 out of 45 differentially expressed PPRs were identified in 'HongYang', there were 4, 10, 13 and 10 differentially expressed PPRs at 12 hours after infection (hai), 24 hai, 48 hai and 96 hai, respectively. While there were more differentially expressed PPRs, with 34 in 'HuaTe' (Table S4), 10, 14, 21, and 20 differentially expressed genes were detected at 12 hai, 24 hai, 48 hai, and 96 hai respectively, furthermore, the fold changes of differentially expressed PPRs in 'HuaTe' were larger than that of 'HongYang'. For both kiwifruits, the number of differentially expressed PPRs was the most at 48 hai after infection, indicating the critical immune response occurred at this time point. In terms of the up-down-regulation trend of genes, more differentially down-regulated PPRs were found in both kiwifruits than differentially up-regulated PPRs especially in 'HuaTe'. For 'HuaTe', the number of differentially down-regulated PPRs were 5, 10, 14, and 12 at 12 hai, 24 hai, 48 hai, and 96 hai, respectively, while the up-regulated PPRs in 'HuaTe' were 5, 4, 7, 8 at 12 hai, 24 hai, 48 hai and 96 hai, respectively. For 'HongYang', the number of differentially down-regulated PPRs were 4, 6, 9, 6 at 12 hai, 24 hai, 48 hai, and 96 hai, respectively, while the up-regulated PPRs in 'HuaTe' were 0, 4, 4, 4 at 12 hai, 24 hai, 48 hai and 96 hai, respectively. These observations indicated the down-regulation expression is the overall tendency in response to Psa infection. We took five of the differentially expressed PPRs as illustrations, they were all down-regulated in 'HuaTe' after Psa infection but expressed stably in 'HongYang' (Figure 3b). For example, *Actinidia18966.t1* and *Actinidia05278.t1* were differentially expressed at 24 hai, 48 hai, and 96 hai only in 'HuaTe'. Thus, in comparison with 'HongYang', 'HuaTe' demonstrated more differentially expressed PPRs, especially down-regulated tendencies. Therefore, we speculated that the down-regulation of PPRs may be related to the resistance of 'HuaTe' to Psa.

Given the different expression patterns of PPRs between two kiwifruits in response to Psa infection, we further examined their corresponding RNA editing events in the chloroplast (Table S5), as shown in Figure 3c. A total of 61 RNA editing sites occurred in 29 genes were detected in this study. We observed that a wide reduction or loss of editing efficiency was detected in samples after Psa infection, especially in 'HuaTe'. We selected six RNA editing sites with obviously reduced editing efficiency to illustrate this point, such as *ndhB-277*, *rps2-83*, *matK-152*, *ndhD-293*, *petL-2*, and *rpoB-184*, editing in site *ndhD-293*, *petL-2* was reduced at 12 hai, whereas editing in sites of *ndhB-277*, *rps2-83* was completely lost at 12 hai, however, in 'HongYang', these notable reductions of editing were not detected. In addition, the differentially expressed PPRs were mostly located in chloroplast, which might thereby affect these chloroplast RNA editing. Taken together, the above results showed that PPRs were responsive to Psa infection, and exhibited significantly different expression levels between resistant and susceptible kiwifruit, suggesting the roles of PPRs in plant immunity. Compared with 'HongYang', resistant kiwifruit 'HuaTe' demonstrated a more dramatic response to Psa infection in not only RNA editing level but also gene expression of PPRs. Under pathogen attack, similar to MORF genes, PPRs were also prone to be down-regulated, thereby reducing the RNA editing level to trigger a series of defense responses.

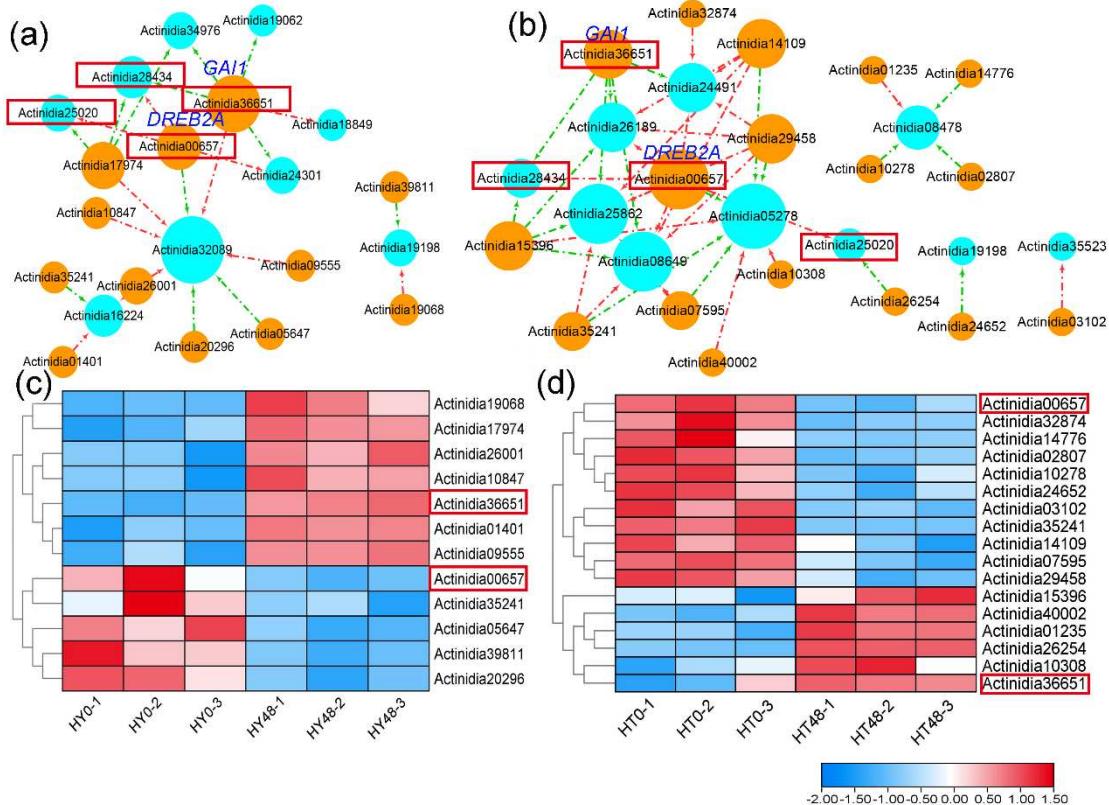


**Figure 3.** Different responses of PPRs expression and RNA editing to Psa infection in *Actinidia chinensis* (Ach) and *Actinidia eriantha* (Ace). (a) The number of differentially expressed PPRs and up-down-regulated differentially expressed PPRs after Psa infection in two kiwifruit species. (b) Gene expression of PPRs between resistant and susceptible kiwifruits after Psa infection. The x-axis represents hours after Psa infection (0, 12, 24, 48, and 96 hai), and the y-axis represents PPRs. Asterisks denote significant differences: \*\* p-value < 0.01. (c) RNA editing profiles in two kiwifruit species. The x-axis represents hours after Psa infection, the y-axis represents RNA editing frequency. 'HongYang' (HY) and 'HuaTe' (HT) represent susceptible and resistant kiwifruits, respectively.

#### 2.4. Upstream Transcription Factors Associated with PPRs in Kiwifruit

To investigate the underlying pathway that may regulate the PPRs expression in kiwifruit, we selected the differentially expressed PPRs at 48 hai and obtained their upstream transcription factors (TFs) from the PlantRegMap database, and only kept ones that demonstrated differential expression at 48 hai. Finally, the regulatory network of PPRs and upstream TFs was constructed, consisting of 9 differentially expressed PPRs and 12 upstream transcription factors in 'HongYang', 10 differentially expressed PPRs and 17 upstream transcription factors in 'HuaTe', respectively, as shown in Figure 4. These upstream TFs were up or down-regulated at 48 hai, and positively or negatively regulated the gene expression of PPRs in 'HongYang' and 'HuaTe'. There were two shared upstream TFs (*Actinidia00657*, *Actinidia36651*) that regulated the expression of differentially expressed PPRs (*Actinidia25020*, *Actinidia28434*) in both 'HongYang' and 'HuaTe', indicating their common regulatory function. One TF gene *Actinidia00657*, encoding dehydration-responsive element-binding

protein 2C (DREB2A), was down-regulated in both 'HongYang' and 'HuaTe', and negatively regulated the expression of the PPR gene *Actinidia25020* at 48 hai, another upstream TF gene *Actinidia36651*, encoding DELLA protein (GAI1) that acts as a repressor of the gibberellin (GA) signaling pathway, was up-regulated in both 'HongYang' and 'HuaTe' and positively regulated the expression of PPR gene *Actinidia28434*. Those differentially expressed transcription factors may participate in the upstream regulation of PPR gene expression and RNA editing in response to pathogen stress.



**Figure 4. Upstream transcription factors associated with PPRs in kiwifruit.** The regulatory network between differentially expressed PPRs and transcription factors at 48 hai in 'HongYang' and 'HuaTe' are shown in (a) and (b), respectively. The nodes of PPRs and transcription factors are denoted by cyan-blue and orange circles, respectively. The shared upstream TFs and PPRs were highlighted by red boxes, and the gene names of TFs were labeled. The differential expression patterns of TFs at 48 hai in 'HongYang' and 'HuaTe' are shown in (c) and (d), respectively. The shared upstream TFs and PPRs were highlighted by red boxes.

### 3. Discussion

Kiwifruit is a perennial horticultural crop species with high nutritional and economic value. However, various diseases bring a serious threat to kiwifruit. RNA C-to-U editing is widespread in vascular plants and plays an essential role in organellar gene expression and plant growth and development. PPRs are extensively distributed across plant lineages, containing more than 400 family members, and function as organellar-specific RNA-binding proteins [41], they are also important subunits of RNA editsome and play a crucial role in RNA editing regulation. Some PPRs in *Arabidopsis thaliana*, such as SOAR1 [42] and PPR96 [43], are involved in response to stress. However, the function of PPRs and their response to stress in kiwifruit have been rarely reported. In this study, we studied the structure, classification, and expression of the PPRs family in two representative kiwifruit species (*Ach*, and *Ace*) with different disease resistance. A total of 497 and 499 PPRs were identified in 'HongYang' and 'HuaTe' respectively. The results showed that the expansion of kiwifruit PPRs mainly resulted from segmental duplication. The comparable family numbers in two kiwifruits indicated the expansion of the PPR gene family occurred before the species differentiation.

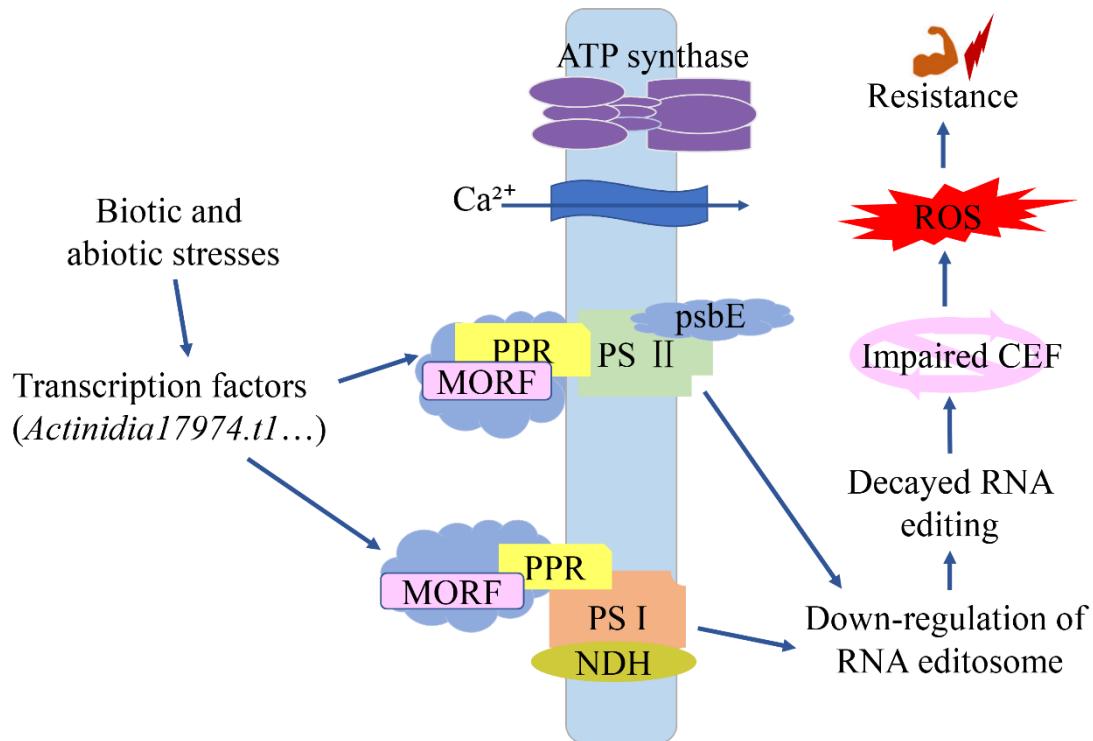
The vast majority of kiwifruit PPRs are predicted to be localized to mitochondria or chloroplasts, which is consistent with the studies of PPRs in other plants.

Based on RNA-Seq data during the fruit development and maturation of kiwifruit, we found that the PPRs were differentially expressed at different time points, indicating the roles of PPRs in fruit development. In addition, further transcriptome analysis was conducted based on RNA-seq data in resistant ('HuaTe', *Ace*) and susceptible ('HongYang', *Ach*) kiwifruits during early infection of *Psa*, we examined the expression of PPRs between two different kiwifruits. For both kiwifruits, the number of differentially expressed PPRs was the most at 48 hai after infection, indicating the critical immune response occurred at this time point. The results showed that *PPRs* genes were also responsive to *Psa* infection, indicating their roles in plant immunity. Further comparison of differentially expressed genes of PPRs revealed that more differentially down-regulated PPRs were found than differentially up-regulated PPRs in both kiwifruits, especially in 'HuaTe'. In addition, more differentially expressed PPRs were found in 'HuaTe' than in 'HongYang', the fold change of differentially expressed PPRs in 'HuaTe' was also larger than that of 'HongYang'. We further examined their corresponding RNA editing events in chloroplast and found that a wide reduction or loss of editing efficiency was detected in samples after *Psa* infection, especially in 'HuaTe'. From the results, we hypothesized that PPRs may play roles in regulating RNA editing and disease resistance response in kiwifruit. Several transcription factors that regulate the expression of kiwifruit PPRs were also identified in our study, such as *DREB2A*, and *GAI1*. Those differentially expressed transcription factors may participate in the upstream regulation of PPR gene expression and RNA editing in response to both development and stresses.

In our previous studies [17], we observed that *MORF* genes in 'HuaTe' and 'HongYang' were differentially expressed after pathogen infection, *MORF2.1*, *MORF9.1*, and *MORF7* in 'HuaTe' were significantly down-regulated, which is similar to the expression of PPRs analyzed in this study. We further identified upstream TFs of *PPR* (Figure 3b) and *MORF* genes (*MORF2.1*, *MORF9.1*, and *MORF7*) in kiwifruit (Figure S3), the regulatory network consists of 49 edges and 24 nodes, among which 9 TFs were differentially expressed, and 6 out of them were significantly up-regulated. Transcription factor *Actinidia17974.t1* co-regulated *PPR* and *MORF* genes, *Actinidia17974.t1* encodes *Transcription factor IIIA*, which regulates 5S rRNA levels during development. Other TFs belong to the C2H2 zinc finger gene family or BCR-BPC gene family, and function in transcription factor activity, sequence-specific DNA binding activity, 5SrRNA binding activity, developmental process, and metal ion binding function. Interestingly, most of the differentially expressed TFs only occurred in 'HuaTe', such as *Actinidia10847.t1*, *Actinidia26001.t1*, *Actinidia17974.t1*, and *Actinidia39948.t1*, these four transcription factors only respond to pathogen infection in 'HuaTe' while do not in 'HongYang'. We speculated that these transcription factors may play roles in pathogen resistance. Both *PPR* and *MORF* genes tended to be down-regulated in response to *Psa* infection and resulted in the loss and reduction of RNA editing especially in 'HuaTe' with higher resistance to *Psa*. Taken together, the expression response of *PPR* and *MORF* genes to pathogen and the difference between resistant and susceptible kiwifruit suggested the roles of RNA factors in plant immunity. Compared with 'HongYang', resistant kiwifruit demonstrated a more dramatic response to *Psa* infection in not only RNA editing level but also gene expression. Therefore, we speculated that *PPRs* and *MORF* genes play a role together in the process of pathogen infection. Under pathogen attack, similar to *MORF* genes, PPRs were also prone to be down-regulated, thereby reducing the RNA editing level to trigger a series of defense responses. Our results provide insight into the molecular evolution of PPR and their roles in pathogens stress in kiwifruit.

Mitochondria and chloroplasts, as intracellular energy conversion sites, play a key role in plant-pathogen interactions. They are also important sources of Reactive Oxygen Species (ROS). Reactive oxygen species act as a key defense molecule in plant immune response [44]. However, how mitochondrial and chloroplast proteins regulate the plant immune system remains unclear. Increasing molecular evidence suggested that many PPRs were involved in responses to a variety of biological and abiotic stresses. In *Arabidopsis thaliana*, *PPR40* is known to provide signaling connections between mitochondrial electron transport elements, and knockout of the *PPR40* gene

leads to the accumulation of reactive oxygen species (ROS), increased lipid peroxidation, and superoxide dismutase activities [45]. In our study, the comparison of resistant and susceptible kiwifruit also confirmed this hypothesis. After pathogen infection, down-regulated expression of PPRs and MORF genes (*MORF9.1*, *MORF7*, and *MORF2.1*) and decreased RNA editing were detected in resistant kiwifruit. The affected genes (such as *ndhB*, *ndhD*, and *cemA*) are mainly in the photosynthetic system and play a role in DNA-RNA transcription and RNA splicing. *ndhB* encodes the B subunit of the chloroplast NADH dehydrogenase-like complex (NDH), which is involved in the cyclic electron flow (CEF) of photosystem I. Therefore, we speculated that the reduced editing efficiency of these genes may trigger impaired CEF, leading to the activation of reactive oxygen-mediated retrograde signaling and significantly enhanced disease resistance to pathogens (Figure 5). PPR and MORF genes may regulate plant-pathogen interactions by controlling the degree of RNA editing, particularly the composition of the NDH complex.



**Figure 5. Schematic model for the roles of PPRs in kiwifruit.** PPRs regulated by transcription factors participate in the RNA editing of chloroplast photosystem genes and subsequently affect the cyclic electron flow activities. Pathogen infection leads to down-regulation of PPRs and MORF genes and reduced RNA editing efficiency, thereby impairing CEF, and up-regulating ROS levels, which enhances the immunity to pathogens. CEF: cyclic electron flow; PS I: photosystem I complex; PS II: photosystem II complex.

#### 4. Methods and Materials

##### 4.1. Genome-Wide Identification of PPRs in *Ach* and *Ace*

The genome and annotation files of *Ach* and *Ace* were downloaded from NCBI (<https://www.ncbi.nlm.nih.gov/>), with version 'ASM966300v1' and 'White\_v1.0', respectively. Using the previously identified 30 PPRs in *Arabidopsis* as a reference [46], including PP438\_ARATH, PP264\_ARATH, etc. of the P-type and PP320\_ARATH and PP207\_ARATH, etc. of the PLS-type. We used two search strategies to obtain kiwifruit PPRs. First, we implemented BLASTP searches of the complete genome with an E-value cut-off of 0.00001 to reduce false positives. Second, Hidden Markov Model (HMM) profiles of PPRs in *Arabidopsis* were constructed and used to search against the two kiwifruit protein databases by using HMMER software with an E-value cut-off of 0.001 [34].

Subsequently, we verified all sequences by checking the existence of PFAM (<http://pfam.sanger.ac.uk/>) domains PF01535, sequences containing less than 2 P motifs were excluded.

#### 4.2. Subcellular Localization and Physical Localization

TargetP [47] and Predotar [36] were used to predict the putative subcellular localization of kiwifruit PPRs. In addition, the physical localization information of the PPRs on the corresponding chromosome was obtained according to the annotation documents, and a sketch map of the gene's physical location was drawn by using TBtools [48].

#### 4.3. Synteny Analysis and Detection of Tandemly/segmentally Duplicated PPRs

We performed self-blast by comparing protein-coding genes against their genome using BLASTP with an E-value cut-off of 0.00001, all BLASTP hits were used as inputs for the software MCScanX [37] (Multiple Collinearity Scan toolkit) to identify possible collinear blocks within genomes of *Ach* and *Ace*. Based on the self-blast results, the tandemly/segmentally duplicated PPRs for each species were detected. The command of 'Duplicate\_gene\_classifier' was used to classify origins of the duplicate genes of ONE genome into whole genome/segmental (match genes in synteny blocks), tandem (continuous repeat), proximal (in the nearby chromosomal region but not adjacent) or dispersed (other modes than segmental, tandem and proximal) duplications. All intra/inter-genomic synteny relationships were visualized with Tbtools [48].

#### 4.4. Expression Analysis of PPR Genes in *Ach* and *Ace*

To examine the expression profiles of kiwifruit PPRs, we performed transcriptome analysis based on two sets of RNAseq data. For *Ach*, RNA-seq data of fruit samples were retrieved from the NGDC database (<https://ngdc.cncb.ac.cn/>) under the accession number PRJCA003268 [39]. Six developmental periods (40, 60, 80, 100, 120, and 140 days after pollination) and six maturity periods (4, 6, 8, 10, 12, and 14 days after harvest of ripe fruit) were selected for 'HongYang', each time point consists of three replicates. For *Ach* and *Ace*, RNA-seq data of leaves in response to *Pseudomonas syringae* pv. *Actinidiae* (Psa) during early infection was also retrieved with accession number PRJNA514180 [40]. Transcriptome analysis was implemented by the protocol in a previous study [17]. Gene expression levels were measured by FPKM (fragments per kilobase of transcript per million mapped reads). EdgeR was used to determine the differentially expressed genes. Heatmaps with all samples were plotted using the 'pheatmap' function in R.

#### 4.5. Gene Co-Expression Network Analysis

Based on RNA-seq data of fruit samples from *Ach*, WGCNA [49] was used to find the co-expression modules and key PPRs related to fruit development and maturation, the soft threshold  $\beta$  was set 16. First, the adjacency matrix of PPR expression was transformed into a topological overlap matrix (TOM). The characteristic genes were calculated and hierarchical clustering (mergeCutHeight value is 0.25) was used to identify the key modules. Module signature genes (ME) and module members (MM) were used to distinguish important modules associated with fruit development and maturation. ME shows the first main component in a module and describes the module's representation pattern. MM represents the relationship between genes and module-characteristic genes and refers to the reliability of genes as part of the module.

#### 4.6. Identification of RNA Editing Sites

First, the RNAseq data were mapped against the chloroplast genome reference using the HISAT2 software with default parameters [50]. The 'bcftools' tool was used to identify variants/SNPs and generate VCF files [51]. Thus RNA editing sites were filtered out based on the results of the variant. Based on the SNP-calling results (in "VCF" format) and genome annotation files (in "tbl" format), RNA editing sites were identified by using REDO tool [52]. As a comprehensive application

tool, REDO can accurately identify plant RNA editing sites based on variant results from RNA-seq data. A series of comprehensive rule-dependent and statistical filters were implemented in the REDO tool to reduce the false positives. We manually examined all mismatches to further minimize false positive sites. For each site, RNA editing efficiency was quantified by the proportion of edited transcripts in total covered transcripts.

#### 4.7. Identification of Upstream Regulatory Transcription Factors

PlantRegMap database was used to retrieve the transcriptional regulatory map of kiwifruit [53]. The transcriptional regulations in PlantRegMap were identified from the literature and ChIP-seq data, or inferred by combining transcript factors (TF) binding motifs and regulatory elements data, this tool was used to infer potential regulatory interactions between TF and target genes, found the TFs which possess overrepresented targets in the input gene set. Finally, the regulatory transcription factors were annotated based on blast results against reference proteins in Uniprot, the regulatory interactions were further drawn using Cytoscape [54].

### 5. Conclusions

In conclusion, this study analyzed the chromosomal positions, phylogenetic relationships, and evolution of PPRs in *Ach* and *Ace*, and provided their expression patterns at different stages of fruit development and under pathogen stress. The results showed that PPRs were differentially expressed at different stages of fruit development and maturation, indicating the role of PPRs in the fruit development and maturation of kiwifruit. The difference in expression and RNA editing profiles of PPRs between resistant and susceptible kiwifruit were also observed after pathogen infection, indicating the roles of the PPRs in stress response. Similar to *MORF* genes, the PPRs were also associated with resistance and affected RNA editing sites that occurred in chloroplasts. It is suggested that RNA editing involving *PPR* and *MORF* genes may be related to chloroplast-mediated immunity. The results of this study will provide a reference for further elucidation of the molecular mechanism of plant immunity and resistance breeding of kiwifruit.

**Supplementary Materials:** Additional file 1 (XLSX): Table S1. Detailed information for PPRs in the *Ach* genome. Additional file 1: Table S2. Detailed information for PPRs in the *Ace* genome. Table S3: Detailed information of PPRs modules during the fruit development and maturation of *Ach*. Table S4: Differentially expressed PPRs in response to *Psa* infection. Table S5: Detailed information of identified RNA editing sites in the plasmid of kiwifruit. Additional file 2 (DOCX): Figure S1. Chromosomal locations of PPR genes in kiwifruit. Figure S2. Module membership-gene characteristics associated with glucose, quinic acid, linalool, and ethyl butyrate in kiwifruit. Figure S3. Expression and interaction network of upstream transcription factors that regulated kiwifruit *PPR* genes and the *MORF* genes.

**Author Contributions:** Conceptualization, Y.X. and A.Z.; methodology, Y.X.; software, Y.X.; validation, A.Z.; writing—original draft preparation, Y.X., and A.Z.; writing—review and editing, Y.X., F.L. and A.Z.; visualization, X.Z.; supervision, A.Z.; project administration, X.Z.; funding acquisition, X.Z. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Natural Science Foundation of China, grant number 32070682, the National Science & Technology Innovation Zone Project, grant numbers 1716315XJ00200303 and 1816315XJ00100216, and CAS Pioneer Hundred Talents Program.

**Acknowledgments:** We would like to thank the members of the Bioinformatics Group of Wuhan Botanical Garden, Chinese Academy of Sciences, China for the discussion and suggestion to improve the manuscript.

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

### Abbreviation

PPRs: Pentatrico peptide repeat proteins; MORF: Multiple organelle RNA editing factors; *Psa*: *Pseudomonas syringae* pv. *Actinidiae*; C-to-U: cytidines substituting uridines; RNA-seq: RNA sequencing; SNPs: single nucleotide polymorphisms; WGS: whole-genome re-sequencing; *cox1*: cytochrome oxidase subunit 1; *nad*: NADH dehydrogenase; *rps*: ribosomal protein gene; *rps14*: 30S ribosomal

gene; *ccmB*: cytochrome c maturation gene; *hai*: hour after inoculation; HMM: Hidden Markov Model; CEF: cyclic electron flow; PS I: photosystem I complex; PS II: photosystem II complex; *Ach*: *Actinidia chinensis*, *Ace*: *Actinidia eriantha*. TFs: transcript factors.

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