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Full Length Article

Identification and Characterization of MicroRNAs and Their Targets in Peach (*Prunus persica*)

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Abstract

Through homology-based computational approach, 159 highly conserved microRNAs were identified in peach genomic sequence. These miRNAs were predicted to target 395 mRNAs, covering 481 gene ontology items. Majority of these targets were demonstrated to participate in a variety of biological processes. Through mapping to Kyoto Encyclopedia of Genes and Genomes database, it was found that the targets play roles in 93 metabolism pathways including the pathway K04075 that involves in plant hormone signal transduction and contains the most abundant genes. According to Gene Ontology prediction, peach microRNAs were found to regulate biological process relevant to agronomic traits such as fruit repining and flower development. In addition, microRNAs responding to abiotic and biotic stress were uncovered and the putative functions of their important targets related to stress resistance were discussed. In summary, the results have significantly advanced our knowledge of microRNA population in peach, provided a better understanding of microRNA-mediated gene regulation and allowed a better application of this knowledge for improving peach yields and qualities.

Keywords: microRNA; *Prunus persica*; Gene ontology; KEGG

Introduction

MicroRNAs (miRNAs) are a class of small RNAs that range from 16-26 nt in length, and regulate gene expression at the post-transcriptional level by mRNA cleavage or translation inhibition (Sun, 2012). In plants, miRNAs are coded by miRNA genes and transcribed by RNA polymerase II to generate the primary transcripts (pri-miRNA). Subsequently, pri-miRNAs are processed and transported into the cytoplasm by HYL1 (HYPONASTIC LEAVES1), DCL1 and SE (SERRATE) to form pre-miRNA with imperfect stem-loop structures (Voinnet, 2009). Finally, the hairpins were cut at the stem regions and the resulting miRNA-miRNA* duplexes undergo 2'-O-methylation. One strand is preferentially integrated into an RNA-induced silencing complex (RISC) as biological active miRNA which mediates cognate mRNAs recognition with perfect complementarity. As a result, it blocks the target's expression by inhibiting translation or by targeting the mRNA for degradation or deadenylation (Carthew and Sontheimer, 2009).

Increasing evidences revealed that miRNAs participate in regulation of diverse biological processes including development, proliferation, stress response, and substrates metabolism (Jung *et al.*, 2009), which highlighted its biological significance on plant growth and stress response (Colaiacovo *et al.*, 2012). Comparison of miRNAs across plant species showed that miRNAs have been highly conserved throughout evolution, thus enabling identification of thousands of miRNAs through bioinformatical approaches in a wide range of plant species (Sunkar and Jagadeeswaran, 2008; Xie *et al.,* 2011).

Peach (*Prunus persica* Batch) is one of the most important fruit crops worldwide. Several studies have been conducted to identify miRNAs in peach by next generation sequencing or computational prediction (Barakat *et al.*, 2012; Colaiacovo *et al.*, 2012; Zhang *et al.*, 2012). However, compared with model or well-studied plant species, a large number of miRNA remain unknown in peach. It was estimated that miRNA genes comprise about 1% of all genes in high plant species.

In this research, the highly conserved miRNAs were identified and characterized from peach genomic sequence using the well-defined computational approach (Vignesh *et al.*, 2011). Furthermore, the functional annotation of miRNA targets based on the GO database and KEGG pathway database was conducted to provide additional information regarding the biological functions of peach miRNAs.

Materials and Methods

All currently known plant miRNAs were downloaded from the miRBase database V18.0 (Griffiths-Jones *et al.,* 2008) to form a reference miRNA set for predicting conserved miRNAs. There were a total of 4677 known entries derived from 53 species. Peach genomic sequences were downloaded from genome database for Rosaceae (http://www.rosaceae.org).

The prediction of conserved miRNAs was conducted

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by miRPI software (Vignesh *et al.*, 2011). It identifies highly conserved mature miRNA sequences without any mismatches against the peach genomic sequences. Then, matched genomic sequences with flanking sequences were extracted for automatic identification of pre-miRNA based on GC contents, and were adjusted for minimum folding free energy (AMFE) and minimum free energy index (MEFI) (Zhang *et al.,* 2006). Sequence alignment was performed by Clustalw software, and structural alignment was conducted by R-coffee software (Moretti *et al*., 2008). Putative targets were predicted by psRNATarget web server using peach DFCI gene index (Release 2) as target datasets (http://plantgrn.noble.org/psRNATarget/) (Dai and Zhao, 2011). Sequences of targets were mapped to GO and KEGG databases using blast2go software for functional annotation (Conesa *et al.*, 2005).

Results and Discussion

A total of 2,324 putative miRNAs was identified, which represented 159 unique mature sequences. The length of mature sequences were varied from 17-24nt, and 21-nt was the predominant length (77/159) followed by 20-nt (30/159). Given the -S setting at 0.95, blastclust analysis showed that Ppe-miR319, 172, 167, 166, 171, 157 and miR396 were the top six abundant groups, while other 61 groups each had only one miRNA. Similar to what had been identified by deep sequencing, many peach miRNAs were assigned into modifications of the same sequences, or termed isomer. For example, ppe-miR166a m1 and 166a were assigned to a pair of miRNAs that were highly homology to ath-miR166a, except with a base addition at the 3' terminus (Table 1). However, the composition of targets of modified miRNAs was different. For ppe-miR172a, 8 mRNAs were recognized as targets in nearly perfect complement manner, while 7 were for ppe-miR172a m2 and 0 was for ppe-miR172a m1. Therefore, the miRNAs with sequence modifications within mature regions were listed as different peach miRNAs.

Similar to miRNAs in other plant species, the secondary structures of peach miRNAs were typical hairpin forms and the mature sequences were located in stem regions (Fig. 1A). Furthermore, the 2nd structure alignment also indicated a highly conservative structure, especially at the mature region (Fig. 1B), which is well agree with the conclusion that miRNAs are strongly conservative in primary sequence, and rarely lost secondarily once integrated into a gene regulatory network (Guo and Lu, 2010).

Recently, some miRNA star (miRNA*) sequences were reported as guide miRNAs with abundant expression and biological functions (Guo and Lu, 2010). In this study, total 13 miRNA* were identified, of which 2 miRNA*s were identified with their miRNAs simultaneously. More evidence suggested that the miRNAs and miRNA*s expressed of either similar or different abundance or miRNA/miRNA* ratios might vary dramatically among different developmental stages (Guo and Lu, 2010). Therefore, the exact expressional pattern of miRNAs and miRNA*s should be investigated further to elucidate their functions in the physiological context in peach.

The fact that plant miRNAs bind to their target mRNAs near-perfectly allows searching targets by bioinformatics approaches (Dai and Zhao, 2011; Vignesh *et al.*, 2011). The target-prediction results showed 395 peach mRNAs as putative targets of 34 miRNAs with a specificity value of 0.85 and signal to noise ratio of 0.91. Notably, the potential targets availably are inadequate since the mRNA sequences of peach are not fully cloned yet. Therefore, a large number of targets remain unidentified. Furthermore, the results indicated that several mRNAs were binded by different miRNAs to perform cooperative regulation as being observed on other plant miRNAs (Sunkar *et al.*, 2005). For example, TC8809 was targeted by Ppe-miR156a, miR156h, miR156g, miR156k, miR414a and miR414b, and these 6 miRNAs were categorized to 2 families.

Functional annotation of targets suggested that peach miRNAs participated in regulating a wide range of physiological processes including plant growth, development, as well as stress resistance (Fig. 2). In addition, KEGG analysis showed the functions of these targets related to 93 biological pathways including substance metabolism and six signal transduction pathways, of which plant hormone signal transduction was the pathway containing the most abundant genes. These results highlighted the biological significance of peach miRNAs.

It has been well established that phytohormones are one of key regulators controlling biological process in plants. The genes participating in metabolism, transport and signal transduction of several types of hormones were intensively targeted by peach miRNAs. Based on GO annotation, total 30 targets were assigned to this function category (Supplement 1), and some of which were related to economic traits. For example, MRNA TC17685, encoding 1-aminocyclopropane-1-carboxylate oxidase, was targeted by Ppe-miR-160a with perfectly sequence complementary. This enzyme catalyzes the last step of ethylene biosynthesis and has been proved to participate in fruit ripening process in apple and tomato plants (Moxon *et al.*, 2008). Since it has been well established that the plant miRNAs regulate genes involved in hormone signaling (Chen, 2005), the results provided here suggested that peach miRNAs likely control gene expression partly through hormone pathway as a common mechanism conserved in higher plants.

It is well known that one of the most important functions of plant miRNAs are response to abiotic stress. Several peach miRNAs give the potential targets regarding resistance to a wide range of abiotic stresses such as drought, UV radiation, cold, salt stress, hypoxia, water deprivation, nutrition deficiency and heavy metal, which are the most harmful factors for growth and productivity of crops worldwide (Gao *et al*., 2007; Sunkar and Jagadeeswaran, 2008; Jung *et al.*, 2009) (Supplement 1).

Table 1: Sequence modifications between similar mature sequences

miRNA ID	Sequence	Modification type [a]
ppe-miR172a m1	AGAAUC**C**UGAUGAUGCUGCA**G**	Base replacement;
ppe-miR172a	AGAAUC**U**UGAUGAUGCUGCA	3' End modification;
ppe-miR172a m2	AGAAUC**U**UGAUGAUGCUGCA**U**	
ppe-miR166a	UCGGACCAGGCUUCAUUCCCC	3' End modification;
ppe-miR166a m1	UCGGACCAGGCUUCAUUCCCC**C**	
ppe-miR166e*	GGAAUGUUGUCUGGC**A**CGAGG	Base replacement;
ppe-miR166e*	GGAAUGUUGUCUGGC**U**CGAGG	

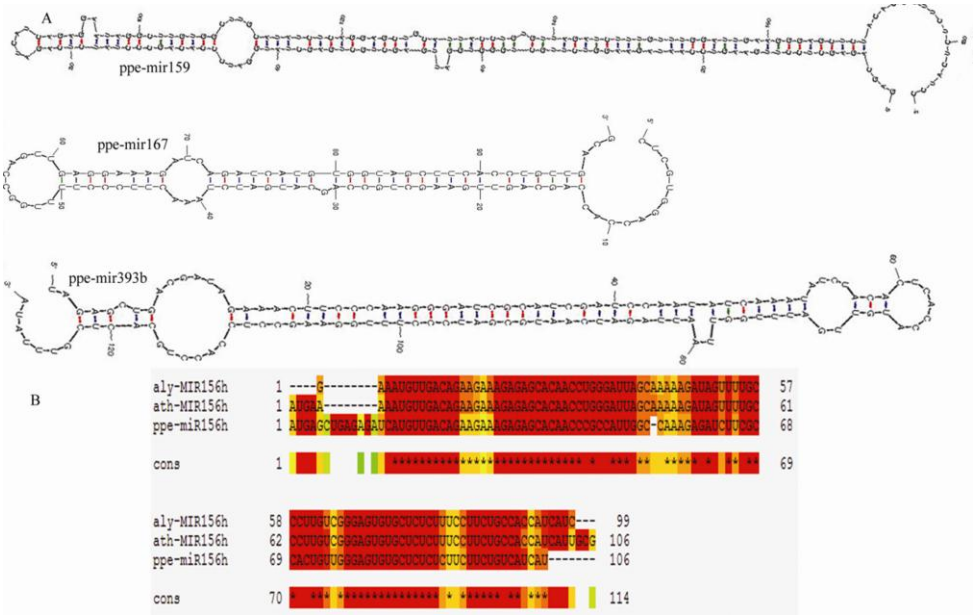

Fig. 1: Secondary structure prediction and alignment of some peach miRNAs

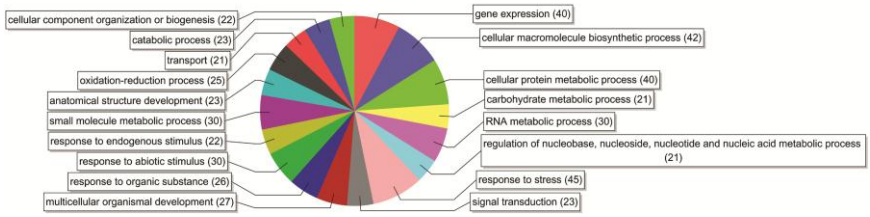

Fig. 2: Functional annotations of targets in category of biological process
This illustration was drawn based on blast2go analysis; The cutoff was set as 20

Particularly, six genes annotated to encounter oxidative stress were potential targets of Ppe-miR156b, miR164a, miR5021 and miR466i. Among them, AJ822400 encodes a protein with high homology to methionine sulfoxide reductase, a gene that plays vital roles against oxidative stress inspired by many types of environmental stress in both plants and animals. Therefore, putative controlling of Ppe-miR156b on methionine sulfoxide reductase in stress response needs further analysis.

In addition, several genes with functions related to biotic stress were identified as targets of peach miRNAs. For instance, TC10691 encodes a protein characterized by an AIG1 domain (so termed aig1 domain-containing protein), and this gene was predicted to be a target of Ppe-miR5083. The aig1 domain-containing protein was of special interest because it has been shown with higher levels of transcription in tissues infected by pathogenic bacterial and is critical regulators involved in plant resistance to bacteria (Wang and Li, 2009). This result will facilitate the research about bacterial infection in peach. Beside, 3 miRNAs were predicted to regulate transcripts with functions related to virus and nematode attacking (Supplement 1), thus it is promising that the peach miRNAs could be potential targets for improving the resistance.

To date, over 4600 plant miRNA genes across 53 plant species were identified and annotated in miRBase v18. The number of miRNA genes is expected to increase

to 500-1000 per species (Bartel, 2004). Thus, hundreds of peach miRNAs could be identify by computational prediction. This study predicted and characterized 159 peach miRNAs, and provided additional information in terms of conservative and regulatory functions on biological processes. On the other hand, the miRNAs identified here were highly conservative to known ones with no mismatch for mature sequences. Thus, the procedure inevitably missed many diverse and novel miRNAs that were remained to be identified using *de novo* approach.

Recently it has been shown that plant miRNAs negatively regulate a wide range of developmental processes through binding to their cognate mRNAs (Xie *et al.*, 2011). Leaf senescence, flower development and lateral root growth were all interesting biological process relevant to agronomic traits of peach in addition to fruit ripening process. Except for Ppe-miR-160a and TC 17685, Ppe-miR393 was found to target TC11240 which encodes a protein termed transport inhibitor response 1. This protein is a critical factor for lateral root development to allow pericycle cells to overcome G2 arrest prior to emergency of root primordium. Collectively, the results provide a clue on how miRNAs control the development of peach, and promise the molecular breeding in which miRNAs are used to improve agronomic traits of peach (Liu and Chen, 2012).

Environmental stresses, including biotic and abiotic stress, are important factors limiting plant growth and development resulted in reduced crop yield and quality. It has been well known that plant miRNAs are key regulators in response and resistance to biotic and abiotic stimuli (Sun, 2012). A total of 53 targets fell into this functional category, covering response to water deprivation, viral infection, salt stress, oxidative stress, nematode attacking, heavy metal stress and cold stress. In addition, the identified miRNAs were likely cope with different stress, such as miR165, miR167, miR168 and miR393, and were highly conservative across the plant kingdom. This is consistent with the conclusion that the regulatory mechanism in which miRNA mediated is evolutionarily conservative (Sun, 2012). However, the roles of peach miRNAs in the response to stress need further experimental validation.

In crux, the identified miRNAs in this study would significantly advance our knowledge of miRNA population in peach, provide a better understanding of miRNA-mediated gene regulation and allow a better application of this knowledge in improving peach yields and qualities.

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