MicroRNA156 and microRNA529 in Land Plants

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The sequences of miR156 and miR529 family members are highly similar, and both target SQUAMOSA promoter binding protein-like (SPL or SBP box) genes, which encode transcription factors that share a common SBP domain. Many studies have focused on the functions of the highly conserved miR156 family, whereas there have been relatively few studies of miR529 . miR156-SPL is a broadly investigated regulatory module in plants. By regulating SPL expression, miR156 is involved in regulating many biological processes, including flowering time, branching/tillering, and environmental stress responses. Considering the high degree of sequence similarity between miR529 and miR156, it would be interesting to determine whether miR529 functions independently, redundantly, or synergistically with respect to miR156.

Keywords: miR156 ; miR529 ; SQUAMOSA promoter binding protein-like genes ; evolution ; land plants

1. Introduction

miR156 is preferentially expressed in rice seedlings, whereas miR529 is mainly expressed in panicles, and both miRNAs confer complex spatiotemporal regulation of their target gene *ideal plant architecture1* (*IPA1*), which has pleiotropic effects on rice plant architecture [1]. A study exploring the evolutionary different of *SPL* genes targeted by miR156 and miR529 in three plant species (moss (*Physcomitrella patens*), rice (*Oryza sativa*), and maize (*Zea mays*)) revealed that miR529 targets comprise a subset of miR156 targets and that the targets regulated by miR156 alone and by both miR156 and miR529 are under different levels of selection pressure [2]. A few studies have sought to dissect the differences between the two miRNA families. miR529 precursors, and especially mature miR529 sequences, evolve more rapidly than miR156 precursors, and *MIR529* genes have a higher average loss rate than *MIR156* genes after identical duplication events [3]. Interestingly, miR529 can be detected in bryophyta and monocots [4][5][6][7] but is absent in intermediate species (some lycophytes and coniferophyte) and eudicots [6][7]. Interestingly, despite the loss of miR529s, some specific eudicot *SPL* family genes have retained miR529 target sites [8]. Transgenic *Arabidopsis thaliana* plants overexpressing a miR529 precursor from a monocot that naturally lacks miR529 displayed phenotypes similar to that of an *spl* mutant, indicating that the retained target sites of miR529 were still functional [8]. However, no method is currently available to effectively distinguish certain *MIR529* and *MIR156* genes.

Some annotated pre-miR156s (such as maize Zma-miR156j) also contain a 21-nt sequence that can be annotated as miR529 in the stem region just behind the mature miR156 sequence. Perhaps these annotated pre-miR156s can also generate miR529 by alternative processing. In some cases, different miRNA family members are indeed formed by alternative processing, such as the rice miR444 family members miR444a.1 and miR444a.2 [9] and *A. thaliana* miR161 family members miR161.1 and miR161.2 [10]. Some ancient and highly conserved *MIRNAs* were also found to undergo alternative processing, such as *MIR319C* in melon (*Cucumis melo* L.) [11]. Small RNA sequencing data showed that miR319c precursor could not only generate miR319c but also produce an alternative #miR319c with very different sequences under cold stress [11]. It is important to determine whether the annotated pre-miR156s that include miR529 sequences can generate miR529s. Significant numbers of divergent and convergent gene pairs have been found in many plant species [12] and whether miR156 and miR529 underwent divergent or convergent processes remains elusive. The relationship between miR156 and miR529 required further investigation to better understand their functions in plants.

2. The Distinction between miR529 and miR156

miR529 is an ancient miRNA family that was first reported in rice [13] and whose members were subsequently discovered in many other plant species, such as *S. bicolor*, *P. patens*, and *M. polymorpha* [14][15][16]. Despite more than ten years of research, the biological functions of miR529s remain poorly understood. This is partly attributable to the existence of miR156, a miRNA family with high sequence similarity to miR529. miR156 and miR529 share common targets, i.e., *SPL* genes. Thus, most studies to date have focused on the miR156-*SPL* module, while the miR529-*SPL* regulatory relationship has to some extent been ignored. In the current study, we discovered that miR156 and miR529 exhibit

different expression patterns in different tissues and developmental stages, indicating that the miR529-*SPL* module has a unique biological function. It is therefore important to distinguish between these two miRNA families and clarify their evolutionary relationship.

The MS region is an important marker of differentiating miRNAs in plants. Notably, we determined that the MS regions of miR156 and miR529 are not identical: their sequences differ by three nucleotides, GAC, located at positions 2–4. However, this difference cannot be used to distinguish miR529s from miR156s because some pre-miR156s contain miR529-like sequences; thus, we investigated whether such miR156s and miR529s are alternatively processed from a single pre-miRNA. We collected available data for miR156s and miR529s from plant species in which miRNAs were identified by sRNA-seq and found no evidence of miR529-like sequences being produced from pre-miR156s. This finding implies that miR529s are not the result of alternative processing of miR156 precursor sequences. Consistent with this notion, no obvious evolutionary relationship was identified between pre-miR529s and pre-miR156s. Recently, another miRNA-miR535 was found to have sequence similarity to miR156 and miR529, and it has been reported to target *SPL* genes as well [17]. However, the MS regions of miR535s are very different compared to those of miR156s and miR529s, making it easier to distinguish them from miR156s and miR529s [17].

By analyzing the 40 bp-miR529-5p/loop/miR529-3p-40 bp sequences, we found that UGAC/CGAC, the first four nucleotides of miR156, never appear in miR529s, and therefore, this characteristic can be used to rapidly distinguish miR156s from miR529s. The first four nucleotides of Aqc-miR529 in miRbase are UGAC, and the Aqc-*MIR529* sequence has greater similarity to *MIR156* than to *MIR529* genes, suggesting that it was mistakenly annotated as miR529 in miRbase.

3. Comparative Analysis of the Origins and Continuity of miR156 and miR529 in Land Plants

Our analyses showed that miR529 has frequently been lost in plant species during evolution. We traced the origin of miR529 to early land plants before the emergence of *M. polymorpha*, one of the most ancient land plants. Among green algae, we were not able to determine whether miR529 is present in Zygnematophyta and Coleochaetophyta, which are considered sister groups to land plants, due to the lack of reference genomes for these clades. However, miR529 is absent in other green algae, including *Chlamydomonas reinhardtii*, *Dunaliella salina*, *Volvox carteri*, *Coccomyxa subellipsoidea*, *Micromonas* sp. RCC299, and *Ostreococcus lucimarinus*. We could not determine with certainty whether miR529 appeared in green algae, but we suspect that miR529 was important in the early history of land plants.

4. miR156 and miR529 Exhibit Different Expression Patterns and Function Cooperatively or Independently by Regulating Common or Specific Targets

The high degree of similarity between miR156 and miR529 raises the issue of whether they participate in common or diversified regulatory pathways. We detected different expression patterns for miR529 compared to miR156 in maize. miR529 levels are high in tassels and relatively low in other tissues, while miR156 is mainly present during the vegetative stage. As *SPL* genes are common targets of miR156 and miR529, they could be regulated by the two miRNAs during different developmental stages or in different tissues. Indeed, some *SPL* genes are targeted by miR156 in shoots and miR529 in tassels.

However, not all *SPL* genes are targeted by both miR156 and miR529: some are miR156-specific or miR529-specific targets. Some *SPL* genes (*SPL3*, *SPL4*, *SPL11*, *SPL12*, *SPL13*) in rice are thought to contain only miR156 target sites [18]. The *SPL13* orthologs Zm00001d006451 and Zm00001d021573 in maize are cleaved only by miR156, as revealed in our PARE analysis. We also detected miR529-specific targets in maize, but these targets are not conserved in rice. PARE analysis revealed that *MYB* genes are targets of miR529 in *M. polymorpha* [19] but are not targeted by miR529 in other plant species. These results suggest that during evolution, in addition to the conserved miR156/miR529-*SPL* module, the two miRNAs might have evolved specific targets and undergone functional diversification to better coordinate plant growth and development.

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