Review of: "Identification and regulatory network analysis of SPL family transcription factors in Populus euphratica Oliv. heteromorphic leaves"

Rajiv K. Tripathi[1], Jaswinder Singh[1]

1 McGill University

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Plant morphogenesis is a complex process involving differential growth of tissues and organs. Leaves are specialized photosynthetic organs which vary widely within and among species in respect to size and shape. The leaves can be simple, compound, smooth, serrated, flat, and with curvatures. Efforts are being made to understand this complex phenomenon by constructing gene regulatory network (Ichihashi et al., 2014), investigating the transcriptional regulation (Tu et al., 2020) and by studying endogenous small RNAs (Zhou et al., 2021). The current study by Qin et al. (2022) identified *SQUAMOSA Promoter binding-like* (*SPL*) gene family and their regulatory network during heteromorphic leaf development in *Populus euphratica* Oliv. Authors utilized the competing endogenous RNA (ceRNA) hypothesis to investigate the multilayered regulation of *SPL* family members through microRNA 156 (miR156), circular RNAs (circRNAs) and long noncoding RNAs (lncRNAs) and their roles during linear (Li), lanceolate (La), Ovate (Ov), and broad-ovate (Bo) leaf shapes morphogenesis. Authors retrieved 78 *SPL* associated sequences, out of which 45 were found to be redundant and generated from alternative splicing of *SPL* genes. After careful analysis, 33 true *SPL* genes were identified in the *P. euphratica* genome. Alternative splicing event in *SPL* genes have also been reported recently on other species including barley and brachypodium (Tripathi et al., 2018; Tripathi et al., 2020). Interestingly, in these studies, the splice variants have been implicated for their distinctive roles during vegetative to reproductive phase change due to presence / absence of microRNA recognition element (MRE) sites. However, in this study, authors have not reflected on such relationship in *P. euphratica*.

Authors grouped the SPLs into two subgroups, class I and class II, based on their homology with SPL proteins from other plant species. Phylogeny analysis and the motif distribution in SPL protein sequences differentiated the sequences into specific sub-groups. Diverse exon/intron structure and motif distribution has been noticed among SPLs from other species and thus suggested their functional divergence during evolution.

Gene ontology (GO) based functional annotation of SPLs revealed their multifaceted roles in DNA binding, flower development, growth phase change, and leaf development. Protein-protein interaction network has predicted that majority of SPLs interact with each other for their actions. The RNA-seq based expression profiling data exhibited higher expression of *SPLs* in Bo shape leaves as compared to Li shape leaves. How *SPLs* are modulating these phenotypes remain to be seen.

Authors utilized MRE to investigate the regulatory relationship among protein coding mRNAs, circRNA, lncRNA, and miRNA during Li, La, Ov, and Bo developmental stages of leaves. The constructed regulatory network based on

circRNA/lncRNA-miRNA156-SPL interactions identified 33 lncRNA and 14 circRNA as a novel regulator of *SPL* genes during these leaf developmental stages. Authors observed an interesting antagonistic relationship between certain RNAs (circRNAs and lncRNAs) and miR156 family members leading to the differential expression of several *SPL* family members during leaf development in *P. euphratica*. The study highlights the novel ceRNA-mediated regulatory paradigm of *SPLs* and their key roles in *P. euphratica* leaf morphology.

Overall, this is very interesting study about the *SPL* gene family in *P. euphratica* and their potential role in leaf architecture. The study requires further experiments to validate the bioinformatic and expression data. We believe, following points could be considered in future studies:

1. *SPL* genes could be categorized by grouping into miR156 targeted and miR156 non-targeted and their association with leaf shape morphology in *P. euphratica*.
2. It is not clear if the transcripts of *SPL* genes from *P. euphratica* also generated lncRNA and circRNA. As, 45 out of 78 *SPLs* identified initially in *P. euphratica* were generated from alternative splicing, authors could investigate if these splice variants also exhibited differential expression patterns and underwent back splicing to produce *SPL*-specific circ-RNA.
3. Validation of the miR156 mediated cleavage of lncRNA and circRNA through 5'-RNA ligase mediated rapid amplification of cDNA ends (5'-RLM-RACE) could provide proof if these circ-RNAs act as miRNA sponges.
4. Authors did not reflect upon the potential SPL-targets for leaf morphology. Do the promoters of gene targets possess SPL binding motifs? Potential motifs discerned through bioinformatics, certainly requires biological validation to avoid misrepresentation of gene targets (Tripathi et al., 2021).
5. Fig. 3: Authors found that expression of miR156 was higher at Li stage and lower at Bo stage of leaf development while certain *SPLs* exhibited antagonistic expression pattern when compared to miR156. It is not clear if these *SPLs* are modulated by miR156 or not. In addition, authors have not explained the clear antagonistic expression patterns of a subset of *SPL* genes in Li and Ov leaf shapes.
6. Fig. 7: Authors report the upregulation of SPLs modulated the expression of its downstream genes in the result and discussion sections, and mentioned in Figures 7 & 9, However, the list of these downstream genes is missing in the manuscript.
7. Fig. 8: Authors state that expression pattern of selected *SPLs* and ceRNAs was similar in their RNA-sequencing and qPCR results. However, it seems this is not true in every scenario, therefore requires some explanation.

Taken together, this is a nice work and provides useful information about *SPL* gene family and its association with leaf development which will be a basis for future functional validation of the roles of *SPLs* and ceRNAs and their interaction in *P. euphratica* leaf morphogenesis.

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