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IN BRIEF

A New Tool for Investigating Small RNA Function

Small noncoding RNAs are important regulatory molecules in numerous developmental and stress response pathways in eukaryotes. Hundreds of microRNAs (miRNAs) and hundreds of thousands of endogenous small interfering RNAs have been identified from dozens of plant species, but regulatory functions are known for only a small number of families. In addition, improvements in high-throughput sequencing and the completion of a growing number of whole-genome sequences are greatly expanding the potential for small RNA discovery. Thus, there is an increasing need for improved methods in functional analysis of small RNAs. **Yan et al. (pages 415–427)** present an effective tool for in vivo targeted knockout of small RNA function based on the expression of a small tandem target mimic (STTM), which targets endogenous small RNAs for degradation, thus blocking their function. Importantly, STTM targets all related members of a small RNA family, allowing for simultaneous derepression of all target genes regulated by a small RNA family. This provides a much-needed complementary approach to the widely used method of exploring small RNA function in transgenic lines expressing miRNA-resistant target genes containing silent mutations.

The authors designed and tested STTM against the miR165/166 miRNA family, as this family has a well-defined set of target mRNAs and disruption of miR165/166 function results in a severe phenotype characterized by loss of apical dominance and changes in leaf symmetry (McConnell and Barton, 1998). The STTM construct harbored two copies of imperfect miR165/166 target binding sites, one corresponding to each miRNA, linked together by a 48-nucleotide RNA spacer. The construct was further designed to form a cleavage-preventive bulge between the STTM construct and miR165/166, such that binding would not lead to cleavage of the STTM construct itself. The STTM construct was expressed in *Arabidopsis thaliana* under the control of a modified cauliflower mosaic virus 2× 35S promoter. Transgenic plants exhibited severe loss of apical dominance and loss of leaf symmetry phenotypes, which were stably transmitted to subsequent generations, and showed upregulation of the known miR165/166 target genes (*PHB*, *PHV*, *REV*, *ATHB8*, and *ATHB15*). The effect of STTM165/166 was similar to that seen in the *phb-1d* mutant (which carries a dominant mutation that disrupts the miR165/166 binding site in the *PHB* gene) and was much more severe than that of transgenic plants expressing MIM165, another type of miRNA target mimicry construct (see figure).

The effect of STTM165/166 was shown to be caused by a reduction in miR165/166 levels, instead of formation of an inactive STTM-miRNA complex. The authors further show that STTM construct likely triggers degradation of the target miRNAs by small RNA degrading nucleases (SDNs), as expression of STTM165/166 in *sdn1-1 sdn2-1* double mutant plants (which lack SDN activity but show no obvious phenotype) did not display the STTM phenotype found in the wild-type background.

Further experiments were conducted to determine the most effective arrangement of miRNA binding sites and the optimal length and secondary structure of the RNA spacer between the two miRNA binding sites, and general rules for STTM design are presented. The STTM approach was tested and shown to be successful in targeting two additional miRNA families, miR156/157 and miR160, and one family of endogenous transacting small interfering RNAs, D7(+) and D8(+), in *Arabidopsis*. The approach is predicted to be widely applicable in plants, and even species recalcitrant to transformation might be amenable to transient expression of STTM constructs. In summary, STTM expression is shown to be a highly effective and versatile tool for functional analysis of small RNA families through targeted degradation.

Phenotypes of 3-week-old STTM165/166 transformants compared with transformants carrying a vector control or a MIM165 construct (another type of miRNA target mimicry) and *phb-1d* mutant plants. Bars = 1.0 cm.

Nancy A. Eckardt
Senior Features Editor
neckardt@aspb.org

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