

Systematically Assigning Gene Functions in Soybean Employing RNAi Technology

Submitted by Zhanyuan Zhang, University of Missouri

Zhang: zhangzh@missouri.edu

IMBA Project 2007-4

Project period: 10/1/06-9/30/08

Progress Report (covers period of 10/1/06-9/30/07)

NOTE: Greater detail can be obtained from the Principal Investigator by signing a Confidentiality Agreement.

Backgrounds and Rationales

Construction and optimization of a high-throughput transformation vector anticipates a comprehensive analysis of methods to maximize cloning efficiency and to achieve high and sustainable transgene expression with effective knock-down of target genes. Our project is aimed at the construction of different sets of transformation vectors for RNA interference and a comparison of their silencing efficiency.

General design of a transgene is based on a pioneer finding of Waterhouse et al. that a transgene-produced intron-spliced double-stranded RNA (ihpRNA) is an efficient substrate for Dicer and RISC complexes and can trigger a degradation of a homologous endogenous mRNAs *in vivo*. For high-throughput applications, specific *att* sequences are incorporated into the transgene to allow a recombination-mediated cloning of a target sequences.

Vectors of such a design have been previously reported to effectively silence target genes in Arabidopsis, rice and barley. However, no data on high-throughput gene silencing in soybean is available yet. Though efficient RNA silencing was observed with targets in a broad size range (90-900 bp), no optimization of target size relatively to spacer region has been performed. Therefore, there are two parameters in our constructs to be tested: the size/structure of the region between the inverted target sequences (I) and the size/part of the gene to be targeted (II).

Construction of pMUGate vectors

As a basic vector for generating transgenic lines, plasmid pFGC5941 was used. This vector contains two origins of replication (one for *E. coli* and one for *Agrobacterium tumefaciens*), *aadA* antibiotic resistance marker for bacteria, *bar* gene as a selectable marker for plants, and 35S promoter and OCS terminator for RNAi expression cassette within T-DNA region. The vector was modified for large-scale applications using Gateway technology (Invitrogen): specific attR-sequences were incorporated to allow a two-step recombination-mediated cloning of target

sequences instead of a conventional cloning. As a source of a recombination cassette, a commercially available plasmid pDONR201 (Invitrogen) was used.

Analysis of transformants and comparison of transformation efficiency

In total, 21 expression vectors were obtained for 7 targets and mobilized into soybean, genotypes Williams 82 and Jack, through an *Agrobacterium*-dependent transformation. Of 304 plants recovered after herbicide glufosinate selection, 86 (28%) contained transgene cassette. As of now, a T1 progeny of a very few transgenic plants is available, and first results indicate that transmission efficiency is within 10%-50% range.

Some of the targets chosen have very distinct knock-out phenotypes which were characterized in *Arabidopsis* and in rice. For instance, loss-of-function mutations in *pid* gene for PINOID kinase, a major regulator of polar auxin transport, lead to retarded root development and often result in dwarfism because of lack of an apical meristem. Using our constructs to silence *pinoid* in soybean, we observed very similar phenotypes in transgenics.

The transformants are being analyzed in two independent ways: by PCR (for the presence of inverted copies of target sequence) and by leaf painting (bar gene screening). For a massive preparation of micro-samples of DNA and PCR we use a Sigma Extract-N-Amp plant PCR kit ; it allows analysis of the plantlets before putting them into a soil and significantly reduces screening costs. Our data indicate that PCR monitoring is a robust method to identify transgenic events as the results are identical to those of Southern analysis. Comparison of different expression cassettes showed that size of the target sequence has no significant impact though increase in target length from 100 bp to 300 bp can improve transformation efficiency; and structure/size of spacer region has a profound effect on transgene stability/transformation efficiency.

Publication related to this project:

Flores T, Karpova O, Su X, Zeng P, Bilyeu K, Sleper D, Nguyen H, Zhang Z. 2008. Silencing of GmFAD3 gene by siRNA leads to low α -linolenic acids (18:3) of *fad3*-mutant phenotype in soybean [*Glycine max* (L.) Merr.] *Transgenic Research* (online first: DOI 10.1007/s11248-008-9167-6).