Computational Identification of MicroRNA-targeted Nucleotide-Binding Site-Leucine-Rich Repeat Genes in Plants

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[Abstract] Plant genomes harbor dozens to hundreds of nucleotide-binding site-leucine-rich repeat (NBS-LRR, NBS for short) type disease resistance genes (Shao *et al.*, 2014; Zhang *et al.*, 2015). Proper regulation of these genes is important for normal growth of plants by reducing unnecessary fitness costs in the absence of pathogen infection. Recent studies have revealed that microRNAs are involved in regulation of NBS genes in plants (Zhai *et al.*, 2011; Shivaprasad *et al.*, 2012). This protocol describes computational methods for the genome-wide identification of plant NBS genes potentially regulated by microRNAs.

Equipment

1. Personal computer (an internet connection is needed) (Intel Core i5-2300 CPU, 8 GB RAM)

Sequence data and software

1. Sequence data compilation
   The coding sequence (CDS) and protein sequences of interested genomes should be downloaded from relevant databases. A recommended database containing a relatively large number of sequenced plant genomes is Phytozome (http://www.phytozome.org/) (Goodstein, Shu *et al.*, 2012). MicroRNAs of interested genomes can be retrieved from the miRBase (http://www.mirbase.org/) (Kozomara and Griffiths-Jones, 2014) or from in-house sequenced data.
2. Required software and online tools
   The following software should be locally installed in your computer:
   a. Hmmer 3.0 (http://hmmer.janelia.org/) (Johnson, Eddy *et al.*, 2010), for perform local hidden Markov models (HMM) search of NBS homologous proteins.
   b. NCBI BLAST[+] or NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), for perform local blastp search of NBS homologous proteins.
   c. ActivePerl 5.14.2 (http://www.activestate.com/activeperl/downloads), for running

scripts written in perl language.

Manuals for installation/operation of these software could be downloaded in the referred websites.

The online tools to be used are:

d. COILS (http://www.ch.embnet.org/software/COILS_form.html) (Lupas, Van Dyke *et al.*, 1991), a program for identification of coiled-coils (CC) domain in protein sequences.

e. Pfam (http://pfam. sanger.ac.uk/) (Finn, Bateman *et al.*, 2014), a database for identification of protein domains.

f. psRNATarget (http://plantgrn.noble.org/psRNATarget/) (Dai and Zhao, 2011), a website designed for prediction microRNA targets in plants.

Procedure

A. Preparation of the query file and local database

For a given plant genome of interest, do the following:

1. Download all CDS and protein sequences of all protein-coding genes from a relevant database such as phytozome (http://www.phytozome.org/) (Goodstein *et al.*, 2012). Make sure that each gene has the same name in both CDS and protein sequence files.
2. Download the HMM profile and the extended amino acid sequence for NB-ARC domain (Pfam no. PF00931) from the Pfam database (http://pfam.sanger.ac.uk/) (Finn *et al.*, 2014).
3. Download all microRNA sequences from a relevant database such as miRBase (http://www.mirbase.org/) (Kozomara and Griffiths-Jones, 2014) or prepare a fasta file including all the microRNA sequences obtained in in house experiments.
4. Create a local database of your downloaded protein sequences for blast search using the formatdb program of the blast software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

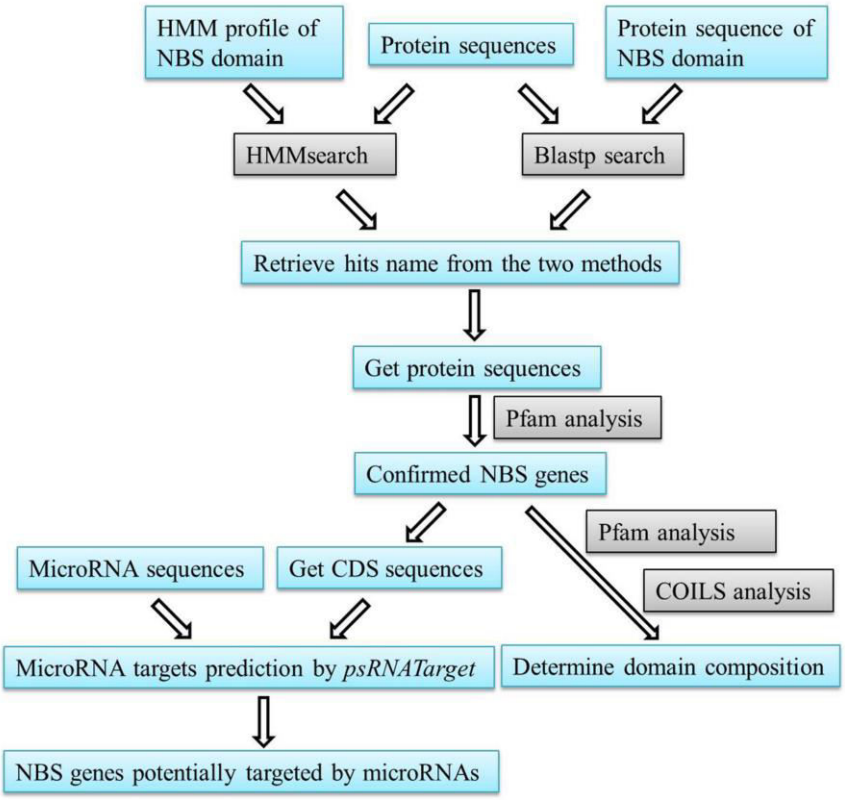


Figure 1. A flow chart of the steps described in our procedure

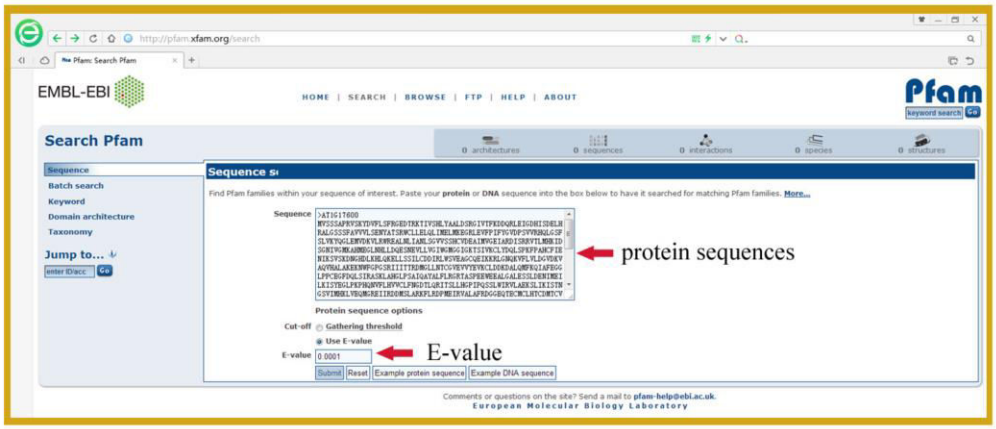
B. Computational identification of NBS genes

1. Perform the HMM search against the fasta file that contains protein sequences you downloaded using the hmmsearch.exe program in the hmmer 3.0 package (Johnson, *et al*., 2010) with the HMM profile of NB-ARC domain as a query in default settings.
2. Run a local BLASTp search against the protein database that was created in the procedure step A4 using the amino acid sequence of the NB-ARC domain as a query. The threshold expectation value was set to 1.0 as used in previous studies (Li *et al*., 2010; Shao *et al*., 2014).
3. Retrieve the gene name of potential NBS genes from the results of HMM search and BLAST search and combined them together to obtain the maximal number of hits.
4. These gene names are used to retrieve the protein sequences from protein dataset downloaded in procedure step A1. This step could be achieved manually if only a few NBS genes are found in the genome. For large datasets, we recommend the researchers writing a Perl script for this step (a script is also available from the authors upon request).
5. The obtained sequences are further subjected to the online Pfam analysis to verify whether they indeed possess the NBS domain, with the E-value setting to 10^{-4} (Figure 2A). Sequences that do not have a detectable NB-ARC domain are discarded. The

remaining sequences represent all NBS proteins of the dataset.

6. The Pfam analysis is also important to detect whether these proteins have an N-terminal toll/interleukin-1 receptor (TIR) domain or RESISTANCE TO POWDERY MILDEW8 (RPW8) domain or a C-terminal LRR domain (Meyers *et al.*, 2003). Protein sequences that do not have a detectable N-terminal domain by Pfam are further analyzed using the COILS Server (http://www.ch.embnet.org/software/COILS_form.html) (Lupas *et al.*, 1991) in default settings to detect whether they have a coiled-coils domain at the N-terminal.

A



B

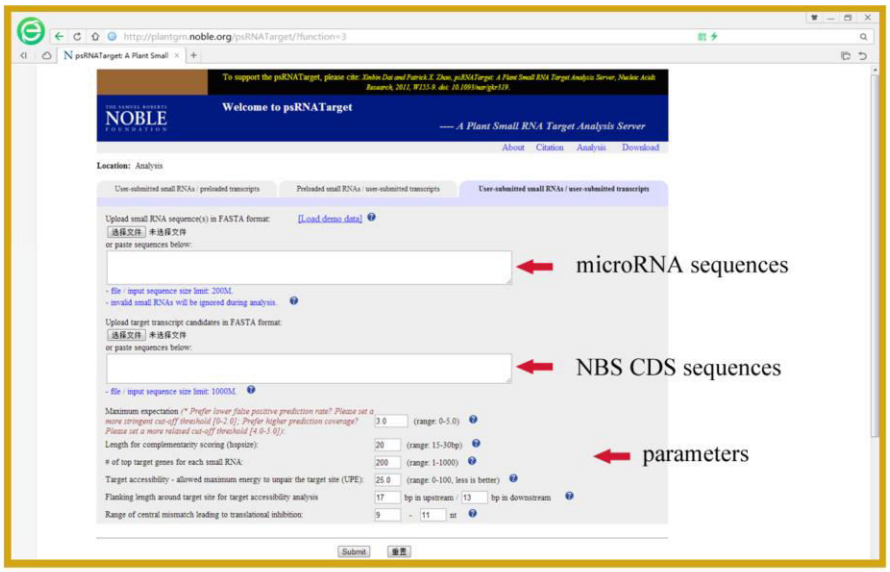


Figure 2. Screen shots for steps A) B5, and B) C2

C. Identification of NBS genes potentially targeted by microRNAs

1. To predict NBS genes targeted by microRNAs, retrieve the CDS sequences of identified NBS genes by searching gene names from the downloaded CDS dataset.
2. Submit the sequences corresponding to CDS of identified NBS genes and to

microRNAs to the psRNATarget Server (http://plantgrn.noble.org/psRNATarget/) (Dai and Zhao, 2011) in fasta format for microRNA target prediction (Figure 2B).

Note: At this step, researchers can change the parameter settings to restrict or expand the number of predicted targets. For example, one can set the Maximum expectation (transformed from mismatch penalty) to 3 to obtain fewer hits with lower false positive prediction rate; or set the Maximum expectation to 5 to maximize the number of potential targets at a higher risk of false positive prediction rate.

3. Download the prediction results from the psRNATarget Server and retrieve those NBS genes predicted to be targeted by microRNAs.
4. The predicted regulation of NBS genes by microRNAs could be experimentally validated by co-expression of them in tobacco leaves as described in several studies (Liu *et al.*, 2014; Yu and Pilot, 2014).

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