Functional analysis of the 1143 bp upstream region of the *Nicotiana benthamiana* wound-responsive *phytaspase 1* gene

Abdullina Mariia*, *Shevchenko Tais
Student
Trusova Svetlana
Supervisor
Shenzhen MSU-BIT University
Faculty of Biology, Shenzhen, China
E–mail: maria.lenpack@gmail.com

Phytaspases are plant subtilisin-like serine proteases with aspartic specificity that play a crucial role in stress responses. Specifically, for *Nicotiana benthamiana* phytaspase 1, the *NbPhyt1* gene has been shown to be induced by mechanical wounding [1]. These findings led us to the objective of the current study: to identify the functional *cis*-regulatory elements governing this transcriptional response.

To investigate this transcriptional regulation *in vivo*, we analyzed the 5'-flanking region using the PlantPAN 4.0 database, which predicted several stress-responsive transcription factor binding sites (TFBS) within the first 1.1 kb upstream of the *NbPhyt1* gene. Based on these *in silico* data, a 1143 bp upstream regulatory sequence (*NbPhyt1-Prom*) was PCR-amplified from wild-type *N. benthamiana* and cloned into the pCAMBIA1301 vector to drive the expression of the β-glucuronidase (GUS) reporter gene. Sequencing confirmed the identity of the cloned fragment, with only minor single-nucleotide polymorphisms (SNPs) identified. For *in planta* assessment, *Agrobacterium*-mediated transient expression was optimized by vacuum-assisted infiltration of *N. benthamiana* leaves. Reporter activity was subsequently quantified via a fluorometric MUG assay. Interestingly, the cloned *NbPhyt1-Prom* demonstrated strong constitutive activity (~500 RFU/h), which was nearly comparable to the highly active viral 35S promoter control (~700 RFU/h).

To evaluate stress inducibility, infiltrated plants were subjected to mechanical wounding. While this treatment successfully induced endogenous phytaspase activity, the GUS reporter activity driven by *NbPhyt1-Prom* remained unaltered at its high initial level. This unexpected uncoupling reveals a significant regulatory paradox: while *NbPhyt1-Prom* acts as a robust constitutive promoter, it lacks the necessary *cis*-regulatory elements required for wound-induced expression. The discrepancy between the high activity of the reporter and the low basal expression of the endogenous gene suggests that distal silencer elements may be required to repress the promoter under non-stressed conditions. These missing stress-responsive components may reside further upstream, within introns, or downstream of the coding sequence. Ultimately, this study identifies a 1143 bp region sufficient for high-level expression, providing a robust constitutive promoter for biotechnology and a critical baseline molecular tool for mapping precise stress-responsive elements in future functional genomics studies.

References

1. Long-term phytaspase responses in *Nicotiana benthamiana*: sustained activation by mechanical wounding, but not by drought, heat, cold, or salinity stress. Abdullina M.A., Li J., Liu F., Luo X., Barsukova A.I., Trusova S.V. *IJMS*, 2025, 26, 7170.