NISR Research GRANT
2010 Young Investigator Research Grant

Analysis of Plant Secondary Metabolite Transport and Application to High Production Using Plant Cultured Cells

Nobukazu SHITAN

Laboratory of Natural Medicinal Chemistry, Kobe Pharmaceutical University

Research aims

Plants produce numerous secondary metabolites which show various biological activities[1]. Some of these are used as medicines, such as anticancer drugs. To effectively harvest these valuable compounds from plant cells, biosynthetic enzymes and cell culturing techniques have been studied for many decades[2]. However, it is difficult to achieve high production of the bioactive secondary metabolites with plant cells. Our recent studies showed that not only genes involved in biosynthesis but also genes relevant to the transport of these metabolites are important for achieving high production of these compounds[3]. Hence, knowledge of membrane transport mechanisms is necessary for systemic metabolic engineering. Recently, we were able to isolate the novel vacuolar-type alkaloid transporter, Nt-JAT1 (*Nicotiana tabacum* jasmonate-inducible alkaloid transporter1) by analyzing the vacuolar nicotine transport in tobacco cultured cells (BY-2 cells)[4]. In this study, we sought to identify tobacco proteins that regulate the transport activity of Nt-JAT1 using the yeast two-hybrid screening. We also attempted to overexpress the alkaloid transporter in order to investigate its effect on alkaloid biosynthesis in BY-2 cells.

Methods

To identify regulatory protein factors that control the transport activity of Nt-JAT1, yeast two-hybrid assays were performed. Cultured tobacco cells (BY-2 cells) were treated with 50 μM methyl jasmonate to induce the expression of Nt-JAT1. An *N. tabacum* DNA library was constructed from the induced BY-2 cells using pJG4-5 prey vector. Then, an Nt-JAT1 C-terminal bait was used to screen 120,000 clones of the tobacco cDNA library with the LexA-based GFP two-hybrid system (Grow'n'Glow system; MoBiTec). Positive clones were selected for leucine prototrophy and green fluorescent protein expression in sequential steps of selection on plates. Colony PCRs were performed on potential positive clones, and amplified DNAs were sequenced.

To investigate the effect of alkaloid transporter overexpression on alkaloid biosynthesis, we transformed BY-2 cells with GFP-chimera constructs of Nt-JAT1 and Nt-JAT2, the homologue of Nt-JAT1, using an *Agrobacterium*-mediated transformation method. Nt-JAT2-GFP-expression in BY-2 cells was observed under a Zeiss LSM700 confocal microscope.

Results

Our yeast two-hybrid screen of approximately 120,000 yeast clones from tobacco cDNA library resulted in isolation of 4 candidate clones for regulators of Nt-JAT1 (No. 326, 759, 778, 870) (Fig. 1). Although we were not able to amplify the cDNA of No. 870, cDNAs of other clones could be amplified well and were sequenced. The cDNA inserts in the clones No. 759 and No. 778 were ca. 700 bp and 1,400 bp, respectively. BLAST search analysis revealed that their gene products have homology with 'hypothetical proteins'. The cDNA insert in the clone No. 326 was ca. 1,500 bp and encoded a protein similar to SEC12P-LIKE 2 PROTEIN. This gene was expressed constitutively in BY-2 cells during the entire growth period. In the model plant *Arabidopsis thaliana,* this protein was reported to be localized to the ER and to regulate the expression of phosphate transporter[5].

Transformation of Nt-JAT1-GFP and Nt-JAT2-GFP constructs into BY-2 cells was performed to examine the effects of the alkaloid transporter overexpression on alkaloid

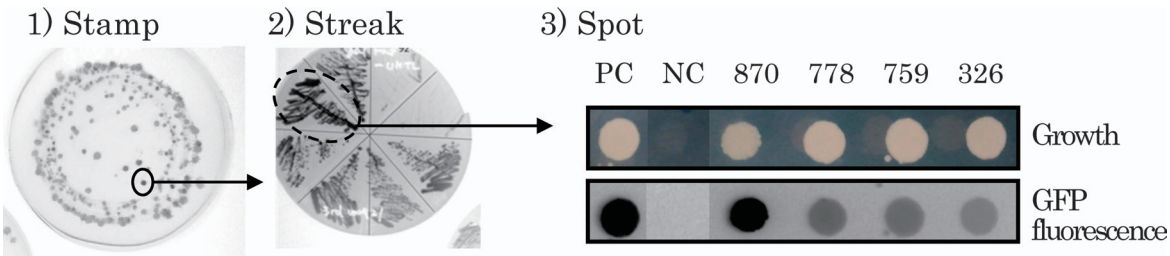

Fig. 1 Screening of candidates of Nt-JAT1 regulatory protein

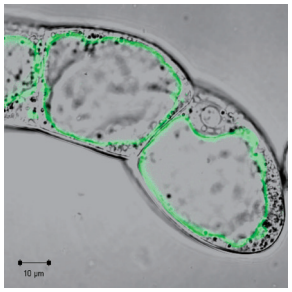

Fig. 2 Fluorescence of Nt-JAT2-GFP in BY-2 cells

biosynthesis. We successfully obtained two transgenic lines (OX1 and OX3) overexpressing Nt-JAT2-GFP. The fluorescence of Nt-JAT2-GFP suggests that this protein is localized to tonoplast (Fig. 2).

Conclusion

We isolated three candidate clones (No. 326, 759, 778) encoding proteins that regulate the function of Nt-JAT1, the nicotine transporter in tobacco. Particularly, clone No. 326 was identified as a strong candidate based on its homology to a protein involved in regulation of transporters. Furthermore, transgenic BY-2 cells which express Nt-JAT2-GFP at the tonoplast were established. Further investigation of alkaloid transport mechanisms using the clones and transgenic cells obtained in this study would provide important knowledge for transport engineering and enable us to achieve the high production of valuable secondary metabolites in cultured cells.

References

1) Croteau, R., Kutchan, T. M., and Lewis, N. G. (2000) *In Biochemistry & Molecular Biology of Plants*, Buchanan, B., Gruissem, W., and Jones, R., (eds.), American Society of Plant Physiologists, Maryland, pp. 1250–1318.
2) Yazaki, K. (2004) In *Handbook of Plant Biotechnology,* Vol. 2, Christou, P., and Klee, H. (eds.), John Wiley & Sons, pp. 811–857.
3) Shitan, N., and Yazaki, K. (2007) *Curr Pharm Biotechnol*. 8: 244–252.
4) Morita, M., Shitan, N., Sawada, K., Van Montagu, M. C. E., Inzé, D., Rischer, H., Goossens, A., Oksman-Caldentey, K.-M., Moriyama, Y., and Yazaki, K. (2009) *Proc Natl Acad Sci USA*. 106: 2447–2452.
5) González, E., Solano, R., Rubio, V., Leyva, A., and Paz-Ares, J. (2005) *Plant Cell* 17: 3500–3512.