Controlling Endophyte Colonization to Reduce TSNA in Tobacco Leaves

PI: Shengming Yang

Even it includes several kinetically distinct steps, conversion of nicotine to NNK is almost spontaneous and no plant-derived enzyme is involved at each step (Caldwell et al., 1993). As a result, it is challenging to regulate NNK synthesis by engineering tobacco plant. Majority of NNK in tobacco leaves is produced during curing by nitrosation of nicotine oxidation product. Nitrosamine required for nitrosation is mostly derived from nitrate assimilation by bacteria or fungi. Greenhouse-grown tobacco without colonization of endophytes produced a very small amount of TSNA, but spraying leaf with a soil suspension significantly increased TSNA formation, including NNK (Cui, 1998). Presumably, restriction of endosymbiosis of microbes with tobacco would lead to reduced NNK production due to suppression of releasing nitrite. However, no endophyte-free mutant or plant is available for studies in this field. Therefore, the endeavors to reduce tobacco TSNA via restriction of endophyte colonization will preserve and strengthen tobacco agriculture in Kentucky, when the FDA regulation on alkaloid content in tobacco products is released.

As an evolutionary technique, CRISPR/Cas9, with increased genetic editing efficiency, offers a fast and easy means to generate desired gene-knockout mutants. More importantly, the CRISPR/Cas9 technology is one of a number of new plant breeding techniques (NPBTs). It can be used to manipulate plant genomes without introducing any foreign DNA into plants. Therefore, the precise modifications introduced by CRISPR/Cas are indistinguishable from those created by conventional breeding or chemical or physical mutagenesis. Introduction of mutations using the CRISPR/Cas system is valuable for speeding up the process of plant breeding. Our objective is to develop endophyte-free mutants, in order to reduce TSNA, especially NNK. Below are the major progresses we have made so far.

1. To choose the target for mutagenesis

It has been revealed that the microbes on tobacco leaves during curing are predominantly bacteria. Thus, the bacterial endosymbionts will be our focus. The signaling pathway for bacterial endosymbiosis remains largely unknown. Numerous genetic studies have been conducted to explore the signaling pathway for both rhizobial and mycorrhizal symbiosis. Interestingly, mycorrhizal and rhizobial symbioses share a common signaling pathway (*Sym*) which is activated in plant cells upon perception of the rhizobial Nod factors and mycorrhizal Myc factors, respectively (Figure 1). Nod factors produced by rhizobia are lipo-chitoologosaccharides (LCO), which is structurally similar to Myc factors released by AM fungi (Madsen et al., 2003; Maillet et al., 2011). Both Nod factor receptors and Myc factor receptor are featured by conserved lysin-motif (Lysm) which also involving in perception of fungal elicitors to trigger plant defense.

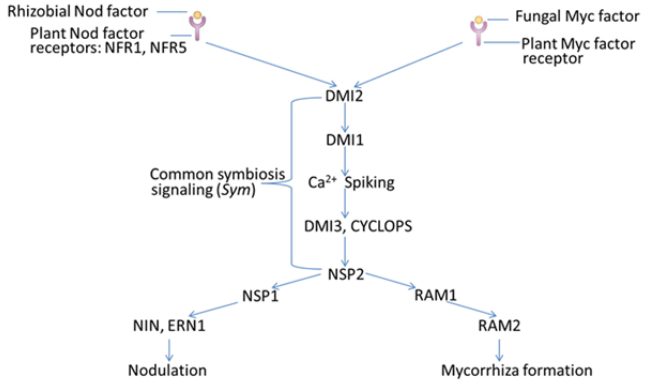


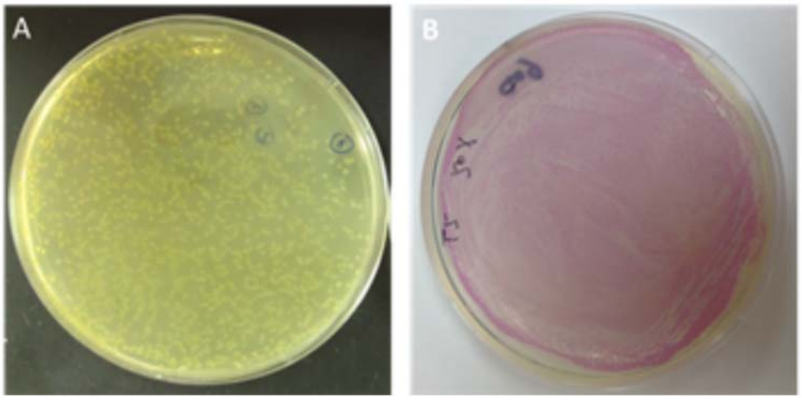
Figure 1. The signaling pathways for both rhizobial and AM symbioses.

Using BLAST analyses, we searched the existing set of sequenced tobacco genomes for genes homologous to *LjNFR1* and *OsCERK1*, encoding Lysm-containing receptors. LjNFR1 and OsCERK1 function to recognize chitooligosaccharide-based signal secreted by beneficial rhizobia, mycorrhizal fungi and pathogenic bacteria. We identified the *NtPERK1* gene, encoding a Lysm-containing receptor with highest similarity with LjNFR1 and OsCERK1. For developing the CRISPR/Cas9 gene knockout constructs, we used the pHSE401 vector described by Xing et al. (2014). The transgenic tobacco plant are available (Figure 2), and these plants will be potted very soon. DNA sequencing will be performed to check the resulting mutation, and the homozygous mutants without transgene will be obtained through selfing in the next generation.

Figure 2. Transgenic tobacco plants with potential mutation at the *NtPERK1* locus.

2. Establishment of an assay for tobacco-bacteria endosymbiosis

To establish a convenient assay for screening endosymbiosis between tobacco and bacteria, we isolated one endophytic bacterium strain from surface-disinfected leaves of TN90 growing in field, and tagged with this strain with mCherry fluorescence (Figure 3). This strain was determined taxonomically by 16S rRNA sequencing as species of *Pseudomonas*, a dominating genus among bacterial endosymbionts in tobacco. Hereafter it is referred to as *Pseudo-A*. We inoculated either leaves or roots of TN90 with *Endo-A*, and both ways led to effective endophytic colonization at the inoculation sites and spread throughout the plant three weeks later (Figure 3). Thus, this well-established tobacco-*Endo-A* endophytic association will be greatly facilitate us to screen endophyte-free mutants.



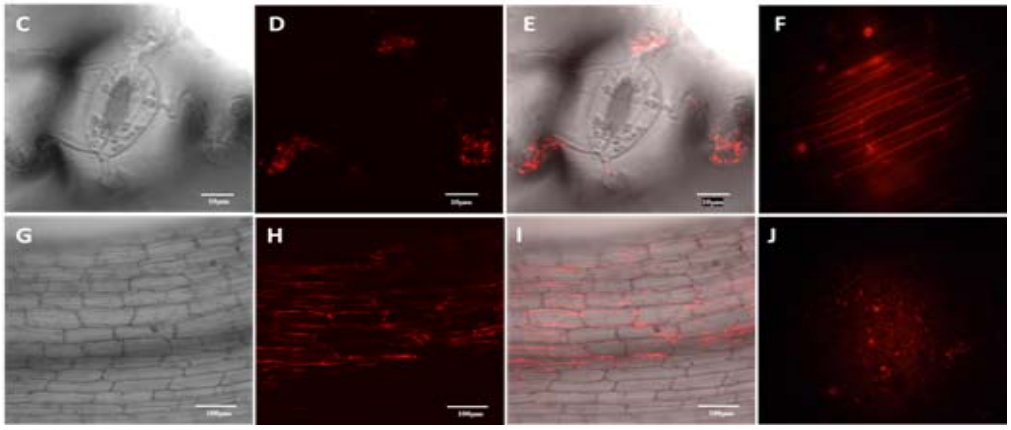


Figure 1. Inoculation of tobacco leaf (C-F) and root (G-J) with *Pseduo-A* (A-B). Endophytic bacteria were extracted from surface-sterilized leaves of TN90 growing in field, and the colony marked with "6" was *Pseduo-A* (A). mCherry-tagged *Pseduo-A* can be visualized to be pink (B). Stomate-entry of mCherry-tagged *Pseduo-A* to colonize in leaves was checked with confocal microscope under bright-(C), red- (D), and merged-field (E) at 1 DAI (days after inoculation); and the bacteria were transduced to roots from inoculation sites of leaves at 20 DAI (F). Root inoculation by mCherry-tagged *Endo-A* was also checked with confocal microscope under bright-(G), red- (H), and merged-field (I) at 1 DAI; also the bacteria were transduced to leaves from inoculated roots at 20 DAI.

3. What's the next

We will collect the seeds for each transgenic tobacco plant and select the homozygous mutants at the next generation. By using the established assay for tobacco-*Pseduo-A* endosymbiosis, we will measure NNK production with GC-MS if we identify some endophyte-free mutants.

We are also creating tobacco mutants with mutated DMI3 function. Inoculation of *Medicago truncatula* by a bacterial endosymbiont, *Pseudomonas fluorescens*, triggered DMI3-dependent activation of genes related to a putative signal transduction pathway with unknown function yet. Therefore, we assume that DMI3 mutation may suppress or affect colonization of endophytes in tobacco. The transgenic plants will be obtained in 3 months for DMI3 mutagenesis.

Reference

Caldwell, W. S., Greene, J. M., Dobson, G. P., and deBethizy, J. D. (1993). Intragastric nitrosation of nicotine is not a significant contributor to nitrosamine exposure. *Ann NY Acad Sci* 686, 213-27; discussion 227-8.

Cui, M. W. (1998). The source and the regulation of nitrogen oxide production for tobacco specific nitrosamine formation during air-curing tobacco. *Ph.D. dissertation*, University of Kentucky, 178 pages.

Madsen, E. B., Madsen, L. H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczyglowski, K., Sato, S., Kaneko, T., Tabata, S., Sandal, N., and Stougaard, J. (2003). A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. *Nature* 425, 637-40.

Maillet, F., Poinsot, V., Andre, O., Puech-Pages, V., Haouy, A., Gueunier, M., Cromer, L., Giraudet, D., Formey, D., Niebel, A., Martinez, E. A., Driguez, H., Becard, G., and Denarie, J. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature* 469, 58-63.

Xing, H.L., Dong, L., Wang, Z.P., Zhang, H.Y., Han, C.Y., Liu, B., Wang, X.C. and Chen, Q.J. (2014). A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biol* 14: 327