Reverse genetics of influenza virus and its application

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Influenza A virus is a highly infectious pathogen of birds and mammals, including humans. This virus belongs to the family *Orthomyxoviridae* and contains eight segments of single-stranded, negative-sense vRNA. Recently, an efficient plasmid-driven system for the generation of infectious influenza virus-like particles (VLPs) and influenza A viruses entirely from cDNAs has been established. This ability to genetically manipulate the influenza viral genome can contribute greatly to our understanding of this pathogen and hence, to improved methods of disease control. In my study, the author exploited this new system to determine the role of the M 2 ion channel protein in the life cycle of influenza A virus, to create a live-attenuated influenza vaccine, and to generate a replication-incompetent influenza VLP vaccine.

Influenza A virus has the M 2 protein, which has proton ion channel activity that is thought to play an important role *in viral* replication. Because direct support for this function is lacking, we attempted to generate viruses with defective M 2 ion channel activity. Unexpectedly, mutants with apparent loss of M 2 ion channel activity by an *in vitro* assay replicated as efficiently as the wild-type virus in cell culture, but were attenuated in mice. The author also generated a mutant lacking both the transmembrane and cytoplasmic domains of M 2 protein. This mutant grew poorly in cell culture and showed no growth in mice. Thus, influenza A virus can undergo multiple cycles of replication without the M 2 transmembrane domain responsible for ion channel activity, although this activity promotes efficient viral replication.

Next, the author proposes a rational approach to the design of live virus vaccines against influenza infection by alteration of the influenza A virus M 2 proteins, which are responsible for ion channel activity. The author demonstrated that a mutant WSN influenza virus with defective M 2 ion channel activity did not show appreciable growth defects in cell culture, although its growth was attenuated in mice. Here, the author shows that this M 2 ion channel-defective mutant virus protected mice against challenge with lethal doses of influenza virus, indicating the potential of incorporating this M 2 alteration in a live influenza vaccine as one of the attenuating mutations.

Although live influenza virus vaccines have shown considerable promise in ongoing clinical trials, they have a concern about the emergence of virulent revertants, warranting efforts to develop alternative vaccines. Therefore, the author describes here the immunogenicity and protective capacity of replication-incompetent influenza VLPs, which were generated entirely from cDNAs and lacked the NS 2 gene. When challenged with lethal doses of an antigenically homologous mouse-adapted influenza virus, 94% of mice vaccinated with the NS 2 -knockout VLPs survived. These results demonstrate the potential of replication-incompetent NS 2 -knockout VLPs as novel influenza vaccines and perhaps also as vectors to express genes from entirely unrelated pathogens.

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Augmentation of natural immune response by orally administered cytokines expressed in transgenic plants

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Cytokines play a major role in the homeostatic maintenance of the mucosal immune system where foreign antigens including several infectious agents are encountered for initiation of immune response. It is therefore considered that administration of cytokines onto the mucosal sites should be effective to prevent infectious diseases because these cytokines would mimic the natural immune response against pathogens invading via the mucosal sites.

At present, cytokines with potential pharmaceutical values are produced as recombinant proteins in *Escherichia coli*, insect or mammalian cell culture. However, plant expression systems have large advantages over other *in vitro* expression systems in terms of low production costs and low risk of contamination of animal pathogens such as viruses and bacterial endotoxins. In addition, recombinant products expressed in edible plant tissues can be orally administered to human and animals.

In this study, genes encoding cytokines were introduced into plants, and the usefulness and applicability of obtained transformants to enhance natural immune response are discussed using *Listeria monocytogenes* infection in mice as a model.

At first, human interferon (HuIFN) -α cDNA was introduced into potato plant (*Solanum tuberosum*) using *Agrobacterium tumefacience*-mediated transformation. Successful expression of biologically active HuIFN-α in transgenic potatoes {560 international unit (IU) /g plant tissue} revealed that biologically active cytokines with potential pharmaceutical value could be expressed in transgenic potato plants.

Although bioactive HuIFN-α was expressed in plants, it was not detected by western blotting analysis due to low expression levels (6 ng/g plant tissue). Therefore, to enhance translation efficiency of mammalian genes and accumulation of recombinant proteins in plant cells, HuIFN-α fused with a signal sequence of seed storage protein and an endoplasmic reticurum retention signal was expressed in tobacco plants (*Nicotiana tabacum*) under the control of the e35S promoter {cauliflower mosaic virus (CaMV) 35 S core promoter with the CaMV enhancer sequence and tobacco mosaic virus (TMV) Ω sequence}. The expression level of the fused HuIFN-α in tobacco plant reached 1.2μg/g plant tissue.

In order to further determine whether the translation efficiency is improved in the fused gene construct, HuTNF-α and the fused HuTNF-α gene construct were introduced into potato plants. HuTNF-α expressed in po-