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REVIEW

RESEARCH AND DEVELOPMENT OF GENETIC ENGINEERING IN MEDICINE AND AGRICULTURE IN THE UNITED STATES OF AMERICA

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SUMMARY

The status of research, development and application of genetic technology in the US has been reflected through efforts and accomplishments in numerous fields including research, medicine, industrial biotechnology and agriculture in the past decades. In the area of medicine, the field of therapeutic purposes on human is the pioneer, in which gene therapy is attempted to carry out in various clinical trials. Diagnostic applications of human diseases which focus primarily on infectious diseases, cancer, pharmacogenomics and screening for inherited diseases by using molecular techniques related to PCR, next generation sequencing are followed. In addition, preparatory studies on human cells utilizing CRISPR/Cas9 genome editing technology have been undertaken in hopes of finding new treatments for cancer and rare form of eye disorder. In the field of agriculture, many large companies in the US have been developing varieties of genetically modified crops with traits of herbicide tolerance, insect resistance, drought resistance and nutrition enhancement. Among the biotech crops, proportion of planted acres of genetically engineered soybean, corn and cotton were increased rapidly and forecasted to expand in the coming years. Studies on generating genetically modified animals and fisheries have also been concentrated in order to not only resist diseases, enhance nutrition, but also provide pharmaceutical compounds. Application of new gene editing techniques such as CRISPR/Cas9 on plants and animals help biotech products have more opportunities to be approved for commercial sale in the US market. In general, although the research and application of genetic engineering in the US has outstripped worldwide, numerous obstacles are still encountered due to serious ethical regulations and controversy regarding to human health and environment. The US government continues to establish suitable policies and invest in science and technology to improve the quality of human life.

Keywords: *Genetic technology, PCR, next generation sequencing, CRISPR/Cas9 genome editing, gene therapy, genetically modified crops*

INTRODUCTION

The United States of America is a leading country in biotechnology research and application in the world. In 2016, revenue from commercial activities of public companies in the US biotech sector reached US$ 112.2 billion. Research and development (R&D) expenses jumped 14% over the year 2015 and accounted for about US$ 38.8 billion. Large biotech companies such as Amgen, Biogen, Celgene, Regeneron Pharmaceuticals, along with Gilead represented for nearly three quarters of the US biotech revenue and more than half of total biotech revenue worldwide (http://www.ey.com). Therapeutic purposes on human is the pioneer and the largest major area in biotechnology. With new developed genetic technologies, the diagnostic applications of human diseases are expected to grow quickly, stand in the second place after therapeutic field, and focus primarily on infectious diseases, cancer, pharmacogenomics and screening for inherited diseases. The agricultural sector has made a rapid progress with lots of practical applications although it comes behind the two sectors outlined above. Genetically modified (GM) maize acres continue to grow rapidly in the US, while GM soybean acres are anticipated to expand in the coming years. Studies using genetic technology on

animals and fisheries have also been concentrated in order to increase biotech products and to solve problems of environmental pollution. This review summarizes the current status of research, development and application of genetic engineering in the field of medicine and agriculture in the US, thereby assessing the level of technology that the US has achieved over the past few years.

RESEARCH AND APPLICATION OF GENETIC ENGINEERING IN THE FIELD OF MEDICINE

Genetic technology in basic medical research related to human genome

The Human Genome Project was an international scientific research project that formally launched in 1990 by the US Department of Energy and the National Institutes of Health (NIH) and was headed by James D. Watson. The technique used to conduct the study was primarily hierarchical shotgun sequencing method which shears DNA randomly into numerous large chunks and clones into a bacterial artificial chromosomes host (International Human Genome Sequencing Consortium, 2001; Venter *et al*., 2001). The results of whole genome sequencing revealed that only 1.1% of the genome is spanned by exons, whereas 24% is in introns, with 75% of the genome being intergenic DNA (Venter *et al*., 2001). Although it was obviously restricted in applying complicated technology, the accomplishment of this project have opened a new era for developing genetic engineering in order to improve DNA identification and analysis methods. In addition, significant medical benefits have been contributed including the discovery of 1800 disease genes, over 2000 genetic tests for identifying risks of human health problems, and many relevant biotech products applied in clinical trials. In 2002, the International HapMap Project which developed a haplotype map (HapMap) of the human genome aimed to map and understand the common patterns of human genetic variation. Thenceforth, the project could accelerate an elicitation of genetic variants affecting health, disease and individual responses to pharmacological agents (Thorisson *et al*., 2005). In 2010, the Phase III of the HapMap project was claimed with approximate 1.6 million single nucleotide polymorphism (SNPs) were genotyped from 1184 individuals of 11 global ancestry groups, and ten 100-kilobase regions of 692 individuals were sequenced (International HapMap 3 Consortium, 2010). The database of this project has been the largest survey of human genetic variant and contributed to find SNPs in any region of interest and their allele frequencies, or to identify genes related to common human diseases. Nowadays, since the next generation sequencing (NGS) technology has been improved and developed rapidly, whole genome sequencing (WGS) of an individual is no longer a difficult challenge for scientific research. NGS technology uses parallel analyses to sequence multiple genes of interest, whole exome sequencing (WES) or WGS of variants in a variety of rare and complex disorders. In addition, due to a sharp reduction in the cost of WES or WGS, recent studies of comparative genomics identified the causes of rare diseases such as Kabuki and Miller syndromes. In comparison with the WGS method, WES was verified to be a quick and accurate approach for some of the Mendelian disorder (Worthey *et al*., 2011). It is explained that WES successfully captured 95% of the coding regions with a minimal coverage of 20X, in which 85% mutations of Mendelian disorder and SNPs across the genome were detected (Rabbani *et al*., 2014). Furthermore, WES is improved to analyze more efficiently by sequencing whole exome of patient and his parents (trio sequencing) or other family members, allowing to detect *de novo* mutations which are the cause of many severe early-onset disease (Katsanis, Katsanis, 2013).

A new ambitious initiative, The Cancer Genome Atlas (TCGA), was suggested with a comprehensive and coordinated effort to accelerate understanding of the molecular basis of cancer. The mission of TCGA project is to identify and to catalogue all the genetic abnormalities found in 50 different types of cancer. The project applies high-throughput genome analysis techniques and bioinformatics to generate publicly available data source, to improve diagnostic methods, treatment standards, and to develop strategies for cancer prevention (Chin *et al*., 2011). TCGA completed genomic characterization of 33 cancer types that have poor prognosis and affect public health, including 10 rare cancers. The targeted types of cancer for this study were comprised of breast, central nervous system, endocrine, gastrointestinal, gynecologic, head and neck, hematologic, skin, soft tissue, thoracic and urologic cancers (https://cancergenome.nih.gov/cancersselected). Researchers believed the project's accomplishment would expand the comprehension of molecular cancer, characterize the genetic traits of tumors in

order to become therapeutic or drug targets. These basic genetic studies will be the foundation for personalized analyses based on individual genome in precision medicine to provide appropriate treatment for genetic diseases and cancer. With the goal of improving personalized medicine, the largest cohort study for President Obama's Precision Medicine Initiative (PMI) has been launched since 2015. Thenceforth, one million volunteers were recruited and sequenced their whole genomes. The result of this project will be a revolutionary approach for studying a large number of diseases, providing predictions of risk disease better, and improving the diagnosis, prevention and treatment that takes into account individual differences in lifestyle, environment, and biology. Through advances in research, technology, and policies that empower patients, the PMI will enable a new era of medicine in which researchers, health care providers, and patients work together to develop individualized care.

Recent advances in the development of gene editing technologies based on programmable nuclease enzymes have significantly ameliorated the implementation of accurate modifications in eukaryotic genomes. These techniques which include meganuclease and its derivatives, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9 open the potential for genome editing therapy in treating disease cells and tissues, removing or modifying harmful mutations, introducing protectable mutations, supplementing therapeutic genes, or disrupting the viral DNA. In the US, many studies using CRISPR/Cas9 technology can alter gain-of-function mutations (such as the *SOD1* G93A mutation in amyotrophic lateral sclerosis, and point mutation p.A673T of *APP* gene in Alzheimers disease) or loss-of-function mutations (mutations in Tay-Sachs disease, for instance) to restore normal function (Cox *et al*., 2015). In addition, this genome editing technique was successfully demonstrated in treating tyrosinemia disease due to *Fah* mutations in hepatocytes (Yin *et al*., 2014). Experiments on mouse models of human genetic disease generated permanent alteration which was able to disrupt the PCSK9 gene *in vivo* with high efficiency (> 50%), decreased plasma PCSK9 levels, increased hepatic low-density lipoprotein receptor levels and reduced plasma cholesterol levels (by 35 - 40%), leading in preventing cardiovascular disease (Ding *et al*., 2014). However, the CRISPR/Cas9 system has a disadvantage in limiting the precise target site that usually causes unwanted genomic modifications. Numerous studies evaluating the specificity of this type of genetic modification system in many cell lineages indicated that the sequences which are highly homologous with target sites are also mutated considerably. Furthermore, as DNA repair systems may not integrate DNA fragment into the genome, target alleles are possible to carry additional variants such as deletions, partial or multiple integrations of the targeting vector, and even duplications (Li *et al*., 2015; Pavlovic *et al*., 2016). To reduce the ratio of off-target mutagenic effects, several research groups proposed solutions to improve the specificity of Cas9. One of them was to create a mutation in one of two Cas9's nuclease regions to form the Cas9 nickase (nCas9) that can only break single-stranded DNA (Mali *et al*., 2013). Therefore, it is capable of generating a double-stranded DNA break by producing two separate single-stranded DNA breaks on both complementary DNA target strands using two different guide RNAs. Additionally, this manner was relevant to enhance specificity and decrease the formation of indels at off-target sites (Ran *et al*., 2013; Shen *et al*., 2014). The other methods in which guide RNA fragment is shorter than 20 nucleotides (Fu *et al*., 2014) or RNA-guided FokI nuclease is based on a combination of inactive FokI and Cas9 nuclease regions (Cas9 mutated in both nuclease regions) (Guilinger *et al*., 2014; Tsai *et al*., 2014) were demonstrated to improve considerably efficiencies of on-target genome editing. Another approach involving in manipulations of *Streptococcus pyogenes* Cas9 (SpCas9) to obtain the SpCas9-HF1 variant was also performed the accurate interaction with target genes in multiple human cell lines with more than 85% single-guide RNAs (sgRNAs) (Kleinstiver *et al*., 2016). The application of CRISPR/Cas9 technology to human cell trials has been approved by the NIH in June 2016. In 2017, the study based on the first human-based trial using the CRISPR/Cas9 technique by Chinese scientists was carried out by scientists from the University of Pennsylvania. Specifically, T cells were obtained from 18 patients with advanced stages of myeloma, sarcoma and melanoma. CRISPR was then used to remove the gene encoding PD-1 protein, which functions to regulate the immune response of T cells to prevent it from attacking healthy cells, and the two genes that encode T cell receptors which direct T cells to target on tumors instead of exotic DNA or viruses. Furthermore, these T cells were also inserted

the NY-ESO-1 receptor-encoding gene which is capable of detecting NY-ESO-1 protein in certain tumors via viral vectors. Ultimately, these edited T cells were cloned and infused into the patient's blood in the hope that they can attack and eliminate cancer (Reardon, 2016). The US researchers assume that the combination of the two technologies can help cancer treatment more effectively. On the other hand, this first CRISPR clinical trial implemented also aimed to demonstrate that the technique is safe for human since there are many concerns about the accuracy of breakage site in target gene. The generation of cancer causing mutations is hypothesized to turn T cells into cancerous cells, however, no abnormalities have been observed during modified T cells have been cultured. If this test is safe, the US will apply CRISPR in a clinical trial for a rare form of eye disorder.

Application of genetic technology in medical diagnosis

The US is expected to be the largest molecular diagnostics market with a growth is projected to reach US$ 4.2 billion by 2023. At the present, the molecular diagnostics forms a small segment in a global market but it is determined as the fastest-growing market. The major factors driving this market are an augment in the incidence of chronic disorders, aging population and a trend toward personalized medicine. Therefore, molecular diagnostic tests have become a powerful tool for detecting rapidly and identifying disease-associated DNA or RNA sequences precisely. Current clinical trial applications concentrate on the screening and detecting infectious diseases, genetic disorders and cancer at the early stage. Based on technology, PCR and its advanced variants are expected to command the largest share, accounting for more than 75%. NGS, microarray and fluorescence in situ hybridization (FISH) methods that are applicable in many cases are following.

For viral infectious diseases, the LAMP and NASBA assays yielded 100% sensitivity for detecting influenza A virus subtypes H1N1 and H3N2 (Poon *et al*., 2005), and H5N1 (Moore *et al*., 2004), respectively, while influenza B virus could be detected with a sensitivity up to 97.9% by SAMBA technique (Wu *et al*., 2010). There are currently 21 tests which have been approved by the US Food and Drug Administration (FDA) for influenza diagnosis (Vemula *et al*., 2016). Besides, issues about determination of HIV-1 infection and assessment of HIV/AIDS progression has also been solved. Specifically, the US clinical microbiology researchers combined viral RNA quantitative assay with serological testing. HIV-1 infection usually results in prolonged survival of the virus. Thus, HIV-1 RNA is commonly determined by RT-PCR, NASBA or branched chain DNA (bDNA). Several companies released tests approved by FDA to monitor HIV-1-infected patients. The typical COBAS AmpliPrep/COBAS TaqMan HIV-1 (Roche Diagnostics, Indianapolis, IN, USA) test is proved to be capable of quantitating HIV-1 viral load with limited detection in the range of 50-1,000,000 copies/ml (Scott *et al*., 2009). The quantification of HIV-1 RNA also contributed to assessing HIV-1-transmitted drug resistance (TDR) (Shafer, 2002). Currently, two commercial assays are available for HIV-1 genotyping: (i) the TruGene HIV-1 Genotyping Kit and OpenGene DNA Sequencing System (Siemens Healthcare Diagnostics, Tarrytown, NY, USA); and (ii) the ViroSeq HIV-1 Genotyping System (Abbott Molecular). Both systems work well for the HIV-1 B subtype circulating in North America (Tang, Ou, 2012). Virus quantification tests are also used to screen and measure the drug response of patients with hepatitis B virus (HBV) or hepatitis C virus (HCV) infection. Nowadays, there are a lots of commercial HBV DNA quantification kits available with high sensitivity in blood or blood products. Two archetypal kits are VERSANT HBV DNA 3.0 (Bayer Healthcare LLC, NY, USA) based on bDNA and COBAS AmPliprep (Roche Diagnostics, NJ, USA) based on real-time PCR with the limit detection threshold 2×10^3 copies/ml (Yao *et al*., 2004) and 6 IU/ml (Ronsin *et al*., 2006), respectively. For bacterial infectious diseases, high-sensitivity PCR method have replaced conventional methods such as direct fluorescent-antibody and culture for detecting *Chlamydia trachomatis* and *Neisseria gonorrheae* in vaginal specimens (Cook *et al*., 2005). Application of multiplex-PCR allowed to identify *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* type B which accounts for 90% cases of bacterial meningitis (Tzanakaki *et al*., 2005). Besides the burden of infectious disease, microbial resistance is a serious problem. Therefore, rapid detection and report of antibiotic-resistant strains such as Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant enterococci (VRE), multi-drug-resistant tuberculosis (MDR-

TB) are a challenge for clinical microbiology laboratory. In the US, although doctors have coped with MDR-TB for many years (especially in New York, Miami, and Los Angeles), some MDR-TB strains became resistant to the second-line agents such as aminoglycosides, polypeptides, fluorquinolones, thioamides, cycloserine and para-aminosalicylic acid. Nevertheless, thanks to the development of technology, pyrosequencing technique enabled to evaluate mycobacteria species, their drugs resistance, and SNP sites to distinguish the genotypes of *Mycobacterium tuberculosis* rapidly.

Based on database of the NIH Genetic Testing Registry, there are currently more than 5800 genetic diseases in which diagnostic tests have been developed and provided by hundreds of laboratories in the US (http://www.ncbi.nlm.nih.gov/gtr/). For detecting point mutations and small variants, bidirectional Sanger sequencing has been considered as the "gold standard" in clinical genetic testing for the past decade. Sequencing the gene *TCOF1* allowed to identify up to 90% of mutations in patients with Treacher Collins syndrome (Katsanis, Jabs, 2012), or focally sequencing only the *FGFR2* gene could confirm or rule out a diagnosis of Apert's syndrome with fairly low cost (Robin *et al*., 2011). The Sanger sequencing, however, is impossible to detect genomic structural variation. Thus, this method alone cannot diagnose some genetic disorders sufficiently. The DNA microarray technology hereby has become an effective tool for analyzing the expression of thousands of genes simultaneously. In the diagnosis of genetic disorders, using microarray can achieve results quickly and precisely through detection of chromosomal abnormality, investigation of mutation, screening and identification of SNP and post-translational variation. Xu and colleagues used microarray to analyze *CFTR*-regulated genes in cystic fibrosis (Xu *et al*., 2003). However, this approach is more advantageous in prenatal and cancer diagnosis. Chromosomal microarray analysis (CMA) was used extensively through a trial proceeded at 29 centers funded by NIH. The study demonstrated that the microarray successfully analyzed for 98.8% of embryonic samples, of which 87.9% of the samples were directly used without culturing. On the other hand, the CMA detected significant difference in 1.7% of pregnant cases with normal karyotype, and 6% of pregnancies who have structural abnormalities involved in genomic fragment deletion/duplication (Wapner *et al*., 2012; Hillman *et al*., 2013). In cancer screening, the microarray benefits researchers because it permits to test a large numbers of genetic samples, to identify SNPs and mutations, to classify tumors, to determine target genes of tumor suppressors, biomarkers of cancer, genes regarding to drug resistance, and to find out new specific drug simultaneously. An array-based comparative genomic hybridization (aCGH) technique was used to map abnormal genes in a variety of tumors including large B cell lymphoma (Alizadeh *et al*., 2000), breast cancer (West *et al*., 2001), bladder cancer (Veltman *et al*., 2003), fallopian tube carcinoma (Snijders *et al*., 2003), brain cancer (Mischel *et al*., 2004) ... In addition, the microarray is also utilized to analyze the CpG island methylation status in the promoter regions which are inactivated even in the presence of transcription factors, for instance in ovarian cancer (Wei *et al*., 2006).

Although NGS technology has been widely used in the field of cancer research, the application of NGS in clinical molecular diagnostics of cancer has been proceeded recently (Gagan, Van Allen, 2015; Corless, 2016). The database from large-scale projects of International Cancer Genome Consortium (ICGC) and TCGA which recruited and analyzed thousands of tumors facilitated to generate comprehensive catalogues of genomic abnormalities (somatic mutations, abnormal expression of genes, epigenetic modifications) from different cancer types and/or subtypes. In breast cancer, for instance, many studies indicated that NGS is suitable for detecting point mutations and indels in the *BRCA1/BRCA2* gene. In addition, when examining 25 genes that are associated with a genetic predisposition to breast cancer, mutations were identified in 16 genes with high frequency such as *BRCA1*, *BRCA2*, *CHEK2*, *ATM* and *PALB2* genes, of which 4.3% of cases mutated on non-*BRCA1/BRCA2* genes (Tung *et al*., 2015). This technology was also applied for clinical diagnosis of 310 colorectal cancer specimens. As the results, mutations were detected in the *KRAS* gene, of which 17% occurred in codons 12 and 13, and in the *PIK3CA* gene with 48% in codons 542, 545 and 1047. At the same time, the rate of formation of the resistant mutants for anti-EGFR therapy increased from 40% to 47%, 48%, 58% and 59% when examining mutations only in exon 2 of *KRAS* gene, in exons 2 - 4 of the *KRAS* gene, exon 2 - 4 of both *KRAS* and *NRAS* gene, additional codon 600 of the *BRAF* gene and exon 20 of the *PIK3CA* gene (Haley

et al., 2015). This suggested that NGS is a powerful tool for detecting mutations in clinical laboratories with high analytical sensitivity and a wide range of assessment which allows to identify numerous mutations simultaneously and quantify allele frequency of mutation in order to predict tumor heterogeneity and allelic imbalance.

Application of genetic technology in treatment of human diseases

Gene therapy has become the representative application of genetic technology in treatment. The US has been at the forefront of the gene therapy research and implement gene therapy on human to cure adenosine deaminase (ADA) deficiency due to a lack of the enzyme ADA, resulting in severe combined immunodeficiency (SCID). Basically, gene therapy is defined as a method that treats or reduces a disease by transferring gene, gene fragment, or oligonucleotide into patient cells, causing genetic modification in patient cells (Strachan, Read, 1999). Gene therapy can be performed in two manners *in vivo* or *ex vivo*. In the *in vivo* gene therapy, target cells are approached directly by microinjection or biopsy. Gene transfer can be accomplished by viral or non-viral vectors, in which recombinant viruses are manipulated to carry tissue-specific promoters. On the other hand, target cells are selected from the tumor, then cultured in suitable microenvironment in the *ex vivo* gene therapy. Afterward, cells are genetically modified by inserting a new gene into their genome and turned back to the patient's body.

For anticancer gene therapy, initial efforts to inactivate oncogenes and replace inactive tumor suppressor genes have been unsuccessful. Subsequently, new approaches have been developed to transfer genes directly into target cells to change temporarily or permanently their phenotypes (Miller, 1992). Target cells may be normal cells, cancer cells, immune cells or pluripotent stem cells. Once the gene is transferred to a cancer cell, it can support the process of apoptosis or recover the healthy cellular function. Meanwhile, for normal cells, transgene can protect them from drug-induced toxicity or activate immune cells to eliminate cancer cells (Weichselbaum, Kufe, 1997).

Hitherto, the US accounts for 62.9% of clinical trials of gene therapy in the world with 1550 trials (Deng *et al*., 2017). Two-thirds of these tests focused on cancer treatment. Trial reports presented that gene therapy is beneficial for many genetic disorders such as Alzheimer's disease, retinopathy due to mutation of *RPE65* gene, cystic fibrosis, hemophilia, HIV, Huntington's disease, muscular dystrophy, Parkinson's disease, SCID and many types of cancer (http://www.genetherapynet.com/clinicaltrialsgov.html). Some of the drugs were approved commercially for cancer gene therapy including ONYX-15 (Onyx Pharmaceuticals) to cure head and neck cancer (Chiocca *et al*., 2004), HPV vaccine (Gardasil) (Merck Sharp & Dohme) to prevent cervical cancer (Block *et al*., 2006) and modified dendritic cells known as sipuleucel-T (ProvengeTM, Dendreon Corporation, Seattle, WA, USA) to treat metastatic castrate-resistant prostate cancer (Kantoff *et al*., 2010; Pieczonka *et al*., 2015). Due to a dramatically high prevalence rate of cancer in the US, the gene therapy segment is anticipated to grow substantially in the cancer therapeutics market. According to economic experts, the US gene therapy industry contributed over 95% of the market share of the North American cancer therapeutics market in 2015 (around US$ 235 million), and is expected to grow to 20.9% in the next 7 years. Furthermore, government funding for cancer research programs and beneficial plans for cancer screening program is believed to generate profitable opportunities for the cancer gene therapy market and facilitate new gene therapies.

Application of genetic technology in disease prevention

Vaccine was initially developed on an experimental basis, primarily based on the reduction or inactivation of the pathogen. However, advances in immunology, molecular biology, biochemistry, genomics and proteomics provided new insights into immunization. With the rapid development of science and technology, the US has studied and applied a variety of modern genetic techniques in vaccine technology to produce numerous vaccines for specific immune responses to many new and urgent diseases. The usage of WGS of microorganisms and bioinformatics analysis for vaccine design is a relatively new approach in detecting antigen, inducing neutralization of humoral immune responses and generating T cell vaccines. This technology includes following steps: 1) Identification of subjects with broadly neutralizing antibodies in serum (Simek *et al*., 2009); 2)

Identification of broadly neutralizing monoclonal antibodies (bnAbs) from these subjects by single-cell technique of memory B cell with or without antigen selection and cloning heavy chain and light chain to the IgG vector; 3) Determination of the crystal structure of these bnAbs' binding sites by crystallization method (Scheid *et al*., 2011; Burton *et al*., 2012; Kwong, Mascola, 2012); and 4) Mimicking the binding sites of bnAbs on protein or vector which acts as the molecular basis for the immunogenicity to create the bnAbs (Burton *et al*., 2012; Kwong, Mascola, 2012). Indeed, pathogens with highly antigenic variation such as HIV (Burton, 2002), HCV (Law *et al*., 2008) and influenza (Ekiert *et al*., 2011) are suitable candidates for designing antigen by this reverse vaccinology. The first success was achieved on respiratory syncytial virus (RSV), in which immune genes were mimically designed as the binding site of an RSV-neutralizing monoclonal antibody and generate specific RSV-neutralizing antibody in monkey (Schief, 2012).

Recently, some of vaccines applied by chimeric antigen receptors (CARs) technology have been proved to be able to prevent many cancers. The researchers designed a lentiviral vector which expressed specific CAR for CD19 antigen of B cell, in combination with CD137 and CD3-zeta signaling region. It could proliferate and eliminate abnormal white blood cells from patients with acute lymphoid leukemia (Grupp *et al*., 2013) and chronic lymphoid leukemia (Porter *et al*., 2011). DNA vaccine studies have also been performed on animal models to enhance the humoral and cell-mediated immune responses against pathogens and tumor antigens. In comparison with other cancer vaccines, DNA vaccines are well tolerated, safe, low cost, easy to produce and preserve and have a high potential for stimulating immune system of the body. Besides, new strategies have been developed to increase the efficiency of transferring gene and improve the effectiveness of DNA vaccines. Many studies demonstrated that the simultaneous distribution of plasmids encoding cytokines, chemokines or costimulatory molecules could augment the immune response. Unlike traditional adjuvants that can trigger nonspecific inflammatory response, molecular adjuvants can regulate adaptive immune responses. For instance, co-distribution of interleukin (IL) 12 and IL-28B enhanced antigen-specific CD8+ T cell responses, and also increased cytotoxic T cells' ability to kill target cells (Morrow *et al*., 2010a, 2010b). Injection of plasmid DNA encoding Melan-A antigen (MART-1) and tyrosinase in stage IV melanoma patients detected immunogenicity of Melan-A/MART-1 (Weber *et al*., 2008). The NY-ESO-1 DNA vaccine was tested in prostate cancer patients and indicated that 93% of patients who were unrecognized any immune response previously responded to both antigen-specific CD8+ and CD4+ T cells (Gnjatic *et al*., 2009). A phase I clinical trial of a Mammaglobin-A (Mam-A) cDNA vaccination was shown the ability of inducing Mam-A-specific CD8+ T cell-mediated immune response in patients with metastatic breast cancer. Moreover, CD4+ T cells were also activated and such T-helper cells produced cytokines switching IL-10 to INF-γ and induced preferential lysis of human breast cancer cells expressing Mam-A protein. (Tiriveedhi *et al*., 2013). Even though a lots of studies aiming to improve the immunity and antitumor potential of DNA vaccines, DNA vaccines still need to be combined with other cancer therapy to control and eliminate tumors completely.

GENETIC ENGINEERING IN AGRICULTURE

Development of genetically modified crops

In the field of agricultural biotechnology, the US was the leader in commercializing biotech crops since 1996. Afterwards, the GM planted area has grown rapidly yearly and reached 72.92 million hectares by 2016 with many types of crops such as maize, soybean, cotton, rapeseed, alfalfa, papaya and squash (Table 1) (James, 2016). Among these GM crops, proportion of planted acres of biotech soybean, corn and cotton were over 90% (Figure 1). Notably, the costs of R&D in the seed industry have increased speedily, especially in the field of crop seed. New technologies based on modern biotechnology and changes in intellectual property rights enable companies to earn huge profits from developed seeds. Therefore, seed selection will continue to be the research direction which is primarily interested. A special section on new breeding technologies was added in 2016 to underline the advancements in plant biotechnology using cisgenesis, CRISPR/Cas9, zinc finger nuclease technology, synthetic genomics, and other techniques that overcome the limitations of conventional breeding and recombinant DNA technology.

According to the United States Department of Agriculture (USDA), the total biotech maize planted was 35.05 million hectares. The 92% adoption rate

was composed of 3% insect resistant (IR), 13% herbicide tolerant (HT), and 76% stacked IR/HT (James, 2016). *Bt* corn is a variant of maize that has been genetically altered to express one or more proteins from the bacterium *Bacillus thuringiensis*. In 1996, the first GM maize producing a *Bt* Cry protein was approved. Agrisure™ RW Rootworm-Protected Corn contains event MIR604, which produced a modified Cry3A (mCry3A) endotoxin recreated from *B. thuringiensis* subsp. *tenebrionis* have enhanced activity against larvae of the western corn rootworm and northern corn rootworm (USEPA 2006). SmartStax™ (Monsanto and Dow AgroSciences) was registered as another stacked hybrid containing events MON 89034, TC1507, MON 88017 and DAS-59122-7 expressing Cry1A.105 and Cry2Ab2; Cry1F; Cry3Bb1; and Cry34Ab1 and Cry35Ab1 endotoxins, respectively (USEPA 2009). It was supposed that *Bt* hybrids exhibit different levels of protection, depending on the type of genetic event and promoter used in developing a hybrid. Indeed, the genetic event, in addition to a promoter, affects the amount, type, and location of the production of the endotoxin in the plant. *Bt* hybrids with events Bt11 and MON810, for example, provided protection against first and second generation European corn borer larvae (Ostlie *et al*., 1997).

A corn variety resistant to glyphosate herbicides known as "Roundup Ready Corn" was first commercialized in 1996 by Monsanto. Afterward, Bayer CropScience developed "Liberty Link Corn" that is resistant to glufosinate. In 2000, Pioneer Hi-Bred generated maize which was able to resist to imidazolinone herbicides through targeted modification of endogenous genes using chimeric RNA/DNA oligonucleotides. The results demonstrated that oligonucleotide-mediated gene manipulation can be applied to crop improvement (Zhu *et al*., 2000). Since the new trait is obtained through modifying a gene within its normal chromosomal context, position effects, transgene silencing, or other concerns that arise as part of developing transgenic events are avoided. In 2016, MON 87419 with stacked herbicide tolerance (glufosinate and dicamba) and MZIR098 with glufosinate-resistance and stacked IR (multiple) were approved for food, feed and cultivation (ISAAA GM Approval Database, 2016). Although glyphosate-resistant crops have been very successful, the evolution of glyphosate-resistant weeds was faster and more widespread than expected. Therefore, the next wave of technologies will combine resistance to glyphosate and other herbicides to provide growers with more herbicide options with different mode of actions as well as the possibility of using herbicides with both foliar and soil residual activity (Green, Owen, 2011).

Besides that, due to the continued deterioration of drought conditions in the south and southeast of the US as dry conditions and above average temperatures, the total value lost hundreds of million dollars. Thus, the approval on December 21, 2011 by the USDA of the first generation drought tolerant trait for maize, MON87460 provided by the insertion of the gene for "cold shock protein B" (*cspB*) from the soil microbe *Bacillus subtilis* was a timely solution to the worsening drought in the US (Federal Register, 2011). The drought trait was developed by Monsanto in collaboration with BASF Plant Science, combining the drought tolerant traits and improved hydro efficiency to ensure conservation of soil moisture and reduces yield loss under drought conditions. DroughtGard™ maize hybrids were planted to 1173 million hectares in the US in 2016 - equivalent to 45% increase from 2015. As of November 2016, US regulators have approved 44 single maize events since 1996 with insect resistance, herbicide tolerance, drought tolerance and stacks thereof, for food, feed, and cultivation.

The majority of the soybeans grown in the US are from seeds that have been enhanced through biotechnology. The soybean RReady2Yield™ was a representative of the first new generation of GM crops and most successful herbicide tolerant soybean to be commercialized in the US since 1996 with 24 GM soybean events approved for food, feed, and cultivation by 2016. Roundup Ready® soybeans expressed a version of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from the *Agrobacterium tumefaciens* CP4 strain, which could survive in a glyphosate production facility. The expression is regulated by an enhanced 35S promoter (E35S) from cauliflower mosaic virus (CaMV), a chloroplast transit peptide (CTP4) coding sequence from Petunia hybrida, and a nopaline synthase (nos 3') transcriptional termination element from *A. tumefaciens* (Padgette *et al*., 1995). The plasmid with EPSPS and the other genetic elements mentioned above was inserted into soybean germplasm with a gene gun by scientists at Monsanto and Asgrow (Funke *et al*., 2006). After this accomplishment, additional

varieties with resistance to dicamba and 2,4-D were scheduled for release as regulatory approvals are obtained, and will form the backbone of weed management strategies in the US non-organic soybean production, thus helping prolong the effectiveness of the current system that mostly depends on using glyphosate with glyphosate-resistant varieties. Beyond herbicide resistance, forthcoming varieties will possess value-added traits to improve product functionality and health benefits. The observation that targeted down-regulation of *FAD2-1A* and *-1B* genes, and *SAD* genes via seed-specific expression of posttranscriptional gene-silencing elements could increase oleic and stearic soybean oils, respectively (Clemente, Cahoon, 2009). Another valuable trait of soybean was acquired in soybean seed with low phytic acid mutations that both improved human absorption of iron and zinc, and also improved animal feed that will reduce phosphorus pollution (Yuan *et al*., 2007).

Other crops approved for commercialization include varieties of flax, papaya, potatoes, radicchio, canola, rice, squash, alfalfa, sugar beets, and tomatoes. Some of these crops are not commercialized or not widely planted. In general, even though GM crops provide a number of economic and ecological benefits, there are still various concerns about their risks to human health. Very little of the US commodity crops are sold directly to consumers as food. Recent approvals of new biotech apples and potatoes have some biotechnology supporters hoping new products, with traits such as disease resistance or nutrition enhancement, will move more quickly through the regulatory pipeline. Typically, Yang and colleagues engineered the common white button (*Agaricus bisporus*) mushroom to resist browning. The effect was achieved by targeting the family of genes that encodes polyphenol oxidase (PPO) - an enzyme that causes browning. By using the gene-editing tool CRISPR/Cas9 to remove just a handful of base pairs in the mushroom's genome, he knocked out one of six *PPO* genes, leading to reduce the enzyme's activity by 30% (Waltz, 2016). The mushroom is one of about 30 genetically modified organisms (GMOs) to sidestep the USDA regulatory system in the past five year, making it the first CRISPR-edited organism to receive a green light from the US government. Not only mushroom, new varieties of corn, tomatoes, and cotton were also developed by this technique. Adoption of the CRISPR/Cas9 technology in plant research would enable the investigation of plant biology at an unprecedented depth and create innovative applications in precise crop breeding.

Table 1. Biotech crop hectarage in the US, 2016.

Crops	Total area (million ha)	Biotech area (million ha) (% of total biotech)					% of total area
		IR	HT	IR/HT	Other traits	Total	
Soybean	33.87	-	31.84 (100%)	-		31.84	94
Maize	38.10	1.14 (3%)	4.95 (13%)	28.96 (76%)		35.05	92
Cotton	3.98	0.16 (4%)	0.36 (9%)	3.18 (80%)		3.70	93
Canola	0.69	-	0.62 (100%)	-		0.62	90
Sugar beet	0.47	-	0.47 (100%)	-		0.47	100
Alfalfa	8.46	-	1.21 (98%)		0.02	1.23	14
Papaya	<0.01	-	-	-	<0.01	<0.01	<0.01
Squash	<0.01	-	-	-	<0.01	<0.01	<0.01
Potato	<0.01	-	-	-	<0.01	<0.01	<0.01
Total	**85.60**	**-**	**-**	**-**		**72.92**	**86**

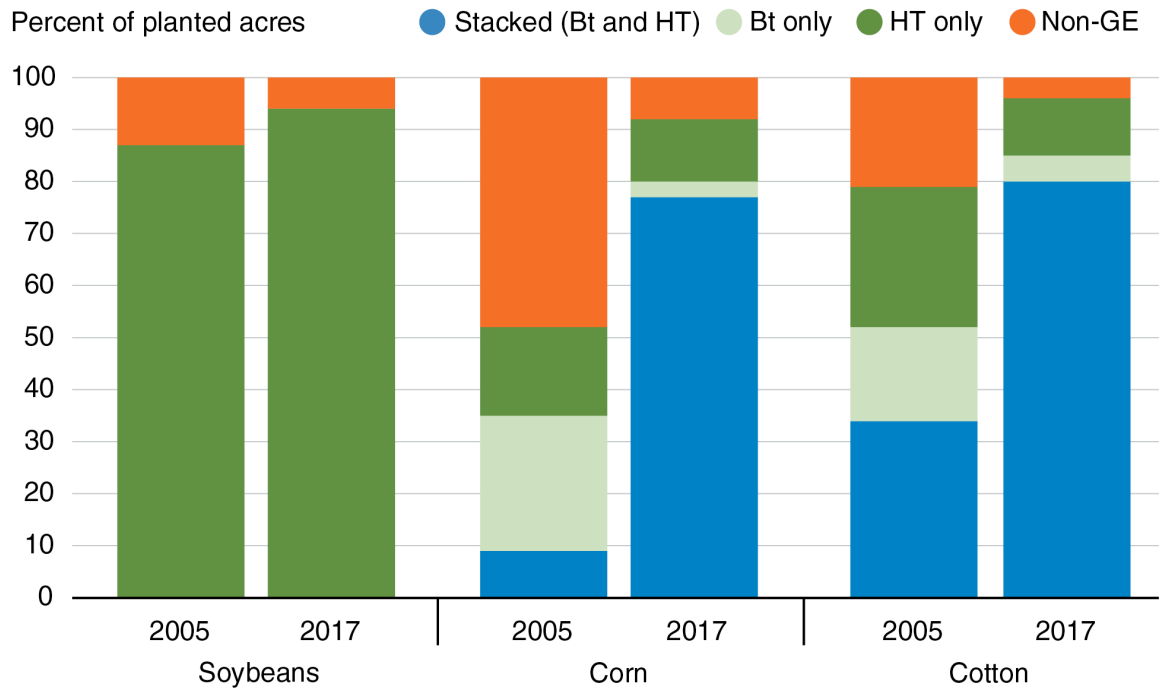

Figure 1. Adoption of genetically modified crops by seed trait in the US in 2005 and 2017 (USDA, Economic Research Service using data from the USDA, National Agricultural Statistic Service's June Agricultural Survey). Data for each crop include seed varieties with herbicide tolerance (HT), insect resistance (Bt), or both (Stacked) traits; soybean have only HT varieties.

Generation of transgenic animals

In the field of agriculture, genetic engineering is a potential power not only for generating GM crops with novel traits in order to resist diseases, increase yield and enhance nutrition, but also for developing GM animals and animal products with a goal of drug provision. Chymosin, a biotechnology-produced enzyme, is used widely in cheese production. Bovine somatotropin (BST, also known as "bovine growth hormone") is a naturally occurring protein that can be produced in greater quantities through genetic technology. The genetically engineered version of BST (recombinant BST) was first approved by FDA in 1993 (Cowan, 2015). In 2006, the US scientists generated cloned transgenic pigs which are rich in omega-3 fatty acids. By nuclear transferred a vector pCAGGS-h*fat*-1 containing a humanized *Caenorhabditis elegans* gene, fat-1, encoding an *n*-3 fatty acid desaturase into PCFF4-3/pST103 cells, hfat-1 transgenic pigs produced high levels of *n*-3 fatty acids from *n*-6 analogs, and their tissues reduced a ratio of *n*-6/*n*-3 fatty acids significantly (Lai *et al*., 2006). In 2009, FDA approved the first product from a transgenic goat, an anticlotting protein known as ATryn, for treatment of patients with hereditary antithrombin deficiency who are undergoing surgical or childbirth procedures. Through microinjection of human antithrombin genes into the cell nucleus of goats' embryos, a recombinant human antithrombin III protein was manufactured in their milk. On November 19, 2015, the FDA approved the first GM animal as human food, announced that the fast-growing AquAdvatage Atlantic Salmon produced by AquaBounty Technologies is as safe to eat and nutritious as non-GM Atlantic salmon. The GM salmon was inserted with a growth hormone gene from Chinook salmon under the control of a promoter from ocean eelpout that permits the salmon to grow at approximately twice the rate of a traditional Atlantic salmon (Cowan, 2015). After a rigorous evaluation on the safety and effectiveness of the GM salmon, the FDA concluded that the inserted genes remained stable over all generations of fish, therefore the modification is safe for the fish and the food derived

therefrom are safe for human and animal consumption (Wong, Chan, 2016).

The tendencies of future transgenic livestock in animal agriculture are to increase sow milk output in order to produce faster-growing piglets in swine, to develop genetically engineered cattle enabling to resist the bacterium that causes mastitis, and to yield pharmaceuticals and/or human organ and tissue replacements. Application of new gene editing techniques is allowing scientists to more easily perform cisgenic breeding - genetic manipulation without inserting the foreign genes into the host genome. Those products face lower regulatory hurdles when compared with transgenics, in which genes are moved into plants or animals from other species. Recently, pigs are capable of deadly porcine reproductive and respiratory syndrome virus (PRRSV)-resistance through manipulating *CD163* gene by CRISPR/Cas9 technology. The results demonstrated a practical means to eliminate PRRSV-associated reproductive disease, a major source of economic hardship to agriculture (Whitworth, Prather, 2017; Prather *et al*., 2017).

CONCLUSION

Research and development in modern biotechnology and gene technology in the US are always at the forefront in the world. There were lots of accomplishments in numerous fields including research, medicine, industrial biotechnology and agriculture in the past decades. New technologies have been permitted restrictedly to apply in clinical trials with expectation of curing genetic diseases and cancers. GM crops and animals with novel traits have also been developed and approved to commercialize in the US market. However, due to serious ethical regulations and controversy regarding to human health and environment, limitations of research subjects and applied techniques are still challenges for scientists in the US and worldwide. The US government has developed suitable policies and continues to invest in order to promote science and technology, and improve the quality of human life.

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