Activities of GMO analysis in China

Litao Yang
Shanghai Jiao Tong University
<table>
<thead>
<tr>
<th>Year Range</th>
<th>Name and Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1896 - 1904</td>
<td>Nan Yang Public School</td>
</tr>
<tr>
<td>1905 - 1906</td>
<td>Imperial Polytechnic College of the Commerce Ministry</td>
</tr>
<tr>
<td>1906 - 1911</td>
<td>Shanghai Industrial College of the Ministry of Posts and Telegraphs</td>
</tr>
<tr>
<td>1911 - 1912</td>
<td>Nan Yang College</td>
</tr>
<tr>
<td>1912 - 1921</td>
<td>Government Institute of Technology of the Communications Ministry</td>
</tr>
<tr>
<td>1921 - 1922</td>
<td>Nan Yang College of Chiao Tung</td>
</tr>
<tr>
<td>1922 - 1927</td>
<td>Nan Yang University of the Communications Ministry</td>
</tr>
<tr>
<td>1927 - 1928</td>
<td>First Chiao Tung University of the Communications Ministry</td>
</tr>
<tr>
<td>1928 - 1942</td>
<td>National Chiao Tung University (Main Campus in Shanghai)</td>
</tr>
<tr>
<td>1942 - 1946</td>
<td>National Chiao Tung University (Main Campus in Chong Qing)</td>
</tr>
<tr>
<td>1946 - 1949</td>
<td>National Chiao Tung University</td>
</tr>
<tr>
<td>1949 - 1957</td>
<td>Jiao Tong University</td>
</tr>
<tr>
<td>1957 - 1959</td>
<td>Jiao Tong University (Shanghai Campus)</td>
</tr>
<tr>
<td>1959 -</td>
<td>Shanghai Jiao Tong University</td>
</tr>
</tbody>
</table>
School of Life Science and Biotechnology

History

- jointly founded in 1997 by the Shanghai Jiao Tong University (SJTU) and Shanghai branch of the Chinese Academy of Sciences (CAS)
- developed from the former Department of Biological Science and Technology and Department of Biomedical Engineering
Zhang lab major research Interests:

I. Rice reproductive development and related functional genomics---Laboratory of Plant Development Biology

II. Biosafety and Food Safety---National Center for Molecular Characterization of Genetically Modified Organisms (GMOs)

http://zhanglab.sjtu.edu.cn/english/
Biosafety and Food Safety---National Center for Molecular Characterization of Genetically Modified Organisms (GMOs)

- Development of new methods and techniques for GMO research using omics approaches; Establishment of theories and technical systems for molecular characterization of GMOs.

- Establishment of technical standards and systems for GMO monitoring and preparation of certified reference materials (CRM).

- Providing GMO analysis and molecular characterization services, technical training and education of general public about GMOs.
Outline:

- The development of GMOs
- GMO labeling regulations
- GMO detection methods
- GMO certified reference materials
**Chinese commercialized GM plants**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Trait</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato (CMV)</td>
<td>(CMV) resistant</td>
</tr>
<tr>
<td>Tomato</td>
<td>shelf-life altered</td>
</tr>
<tr>
<td>Sweet Pepper</td>
<td>virus resistant</td>
</tr>
<tr>
<td>Cotton (2)</td>
<td>insect resistant</td>
</tr>
<tr>
<td>Petunia</td>
<td>color altered</td>
</tr>
<tr>
<td>papaya</td>
<td>virus resistant</td>
</tr>
</tbody>
</table>
GM plants (Peking University)

* Petunia hybrida, genetically modified with chalcone synthase-A(CHSA). Red flower is from non-GM plant, white and bi-colored flowers are from GM plants

* Virus-resistant tomato genetically modified with CMV-CP

* Virus-resistant sweet pepper genetically modified with CMV-CP
GM papaya

Huanong 1, GM papaya with replicase gene of papaya ringspot virus (PRSV), demonstrating high quality and virus resistance

Symptom in papaya showing PRSV infection

Field trials of GM papaya

Flow chart showing the procedures of propagating Huanong 1 in factory

Huanong 1, GM papaya, showing high quality and resistance
GM cotton for insect resistance
(Biotechnology Research Institute, CAAS)

GM cotton varieties for insect resistance with modified Cry1A and CpTI genes approved for commercial cultivation

Greenhouse testing

Bioassay

Commercial cultivation of GM cotton
GM rice and GM maize got the certificates since 2008

Bt63 and Huahui no.1 rice

GM phytase gene maize
GM fish

Lines of GM fish with gene(s) from another fish for fast growth have been approved for field testing, including a GM triploid carp line.

- GM triploid carp
- Testing and Monitoring Center for GM Aquatic Organisms, MOA
Transgenic cloning cattle and goat

Transgenic cattle have been obtained with either human lysozyme (hLY) gene or human fucosylated sugar transferase gene expression. Transgenic cloning cattle with human lactoferrin cDNA (hLF) gene expression have also succeeded.

- Transgenic cloning cattle “Jinwa” and “Panwa” with hLY gene
- Transgenic cloning cattle “Xiangwa” and “Tiewa” with hLF gene
- Mother and two transgenic cattle
Outline:

- The development of GMOs
- GMO labeling regulations
- GMO detection methods
- GMO certified reference materials
中华人民共和国国务院令

第 304 号

《农业转基因生物安全管理条例》已经 2001 年 5 月 9 日国务院第 38 次常务会议通过，现予公布，自公布之日起施行。

总理 朱镕基

二00一年五月二十三日

Legislation systems
A total of 17 kinds of GM products should be labeled.

<table>
<thead>
<tr>
<th>Product</th>
<th>Labeling Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean</td>
<td>seed, soybean, flour, oil, soy meal</td>
</tr>
<tr>
<td>Corn</td>
<td>seed, corn, flour, oil, corn meal</td>
</tr>
<tr>
<td>Rapeseed</td>
<td>seed, rapeseed, oil, rapeseed meal</td>
</tr>
<tr>
<td>Cotton</td>
<td>seed</td>
</tr>
<tr>
<td>Tomato</td>
<td>seed, fresh tomato, tomato sauce</td>
</tr>
</tbody>
</table>

Three labeling methods:

**GM raw materials:** seeds

**GM products:** containing detectable GM materials

**GM products:** containing non-detectable GM materials
Most of the oil productions were labeled with GM materials.
Detection labs of GMOs in China

Official:

Ministry of Agriculture (MOA) (42):
  Domestic Inspection and Detection

Entry-Exit Inspection and Quarantine Bureaus (26):
  Entry-Exit Inspection

The third parties:

Companies (Eurofins, SGS, etc)
Outline:

- The development of GMOs
- GMO labeling regulations
- GMO detection methods
- GMO certified reference materials
Currently used techniques in routine analysis

- PCR
- Real-time PCR
- DNA Arrays
- NGS
- ELISA
- LFD

Nucleic acids analysis
Protein analysis

GM crops

Exogenous DNA
Host genomic DNA
Recombinant DNA
1. Development, validation, and harmonization of Endogenous reference genes

The model for plant endogenous reference genes development and validation was established;

The for GM rice, tomato, wheat, canola, cotton, papaya were developed, such as SPS, LAT52, HMG I/Y, Sadl, and Chypapain, etc.
1. Development, validation, and harmonization of Endogenous reference genes

Some endogenous reference genes were validated by collaborative ring trial, and adapted by ISO standards.

- **LAT52**, used for GM tomato detection (8 countries/14 labs)
- **SPS**, used for GM rice detection (8 countries/14 labs)
- **Chypapain**, used for GM papaya detection (4 countries, 12 labs)
One crop, one endogenous reference gene.

**Harmonization of rice reference genes**

58 rice cultivars

*SPS* and *ppi-PPF* quantitative PCR systems are applicable for being used as rice endogenous reference assays.
Finally, the suitable endogenous reference genes for rice (SPS), maize (zSSIIb), and wheat (Waxy-1) were confirmed.
2. Development and validation of PCR and quantitative real-time PCR methods

- **Screening PCR method:**
  
  *CaMV 35S promoter, NOS terminator, FMV35s promoter, NptII gene, etc.*

- **Gene-specific PCR method:**

  *Cp4-epsps, Cry1Ac, Cry1Ab, Cry9c, bar/pat*

- **Construct-specific PCR method:**

  *Huafan No.1, GK19, SGK321, MON531, MON810, GA21 etc.*

- **Event-specific PCR method:**

  ✓ More than 6 GM soybean events (GTS40-3-2, MON89788, 5547, A2704, etc)
  ✓ More than 15 GM maize events (GA21, Bt11, Bt176, MON810, T25, Mon863, TC1507, NK603, CBH351, MIR162, etc)
  ✓ 7 GM canola events (Oxy235, T45, Ms1Rf1, Ms1Rf2, Ms8Rf3, GT73, etc)
  ✓ More than 6 GM cotton events (Bt-cotton531, RR-cotton 1445, MON88913)
  ✓ 4 GM rice events
  ✓ 1 GM tomato event and 1 GM papaya event.
In the routine analysis, the screen methods were primarily used, including the assays of universal elements, marker genes, and exogenous genes.

A Multiplex Degenerate PCR Analytical Approach Targeting to Eight Genes for Screening GMOs
Degenerate PCR assays for \textit{bar/pat} and \textit{cp4-epsps} have been adapted in national standards.
3. Standardization of PCR and quantitative real-time PCR methods

- **Method Establishment**
  - Target sequence
  - Primers design
  - PCR conditions
  - Specificity
  - Sensitivity
  - Practical samples

- **Standard Drafts**
  - 1st round
  - 2nd round

- **Method Validation**
  - Protocol design
  - Samples preparation
  - Validated ring trial
  - Data analysis
  - Reporting

- **Standard Application**
  - Final drafts
  - Defense
  - Approved
The standard systems of GMO analysis

**General**
- Principles and Definition, Sampling, DNA and protein Isolation

**Methodology**
- Conventional PCR, Real-time PCR, ELISA

**Case by case**
- Universal elements, such as 35s, NOS, FMV35s; (4)
- Gene-specific, such as bar, epsps, etc; (6)
- Event-specific, such as MON810, TT51-1, etc. (about 80)
3. Development of lateral flow device for field test

Expression and purification of exogenous proteins

Commercialized LFDs for cry1Ab/Ac, Cp4EPSPS
4. New methods for quick, high thoughput analysis

**DNA extraction:**
- Conventional methods (CTAB, SDS);
- Commercialized kits based on silica column or magnetic beads (Qiagen, Promega, etc);
- Automatic workstation (Tecan, Qiagen, etc);
- Simple DNA extraction device without specific equipment

**Target DNA fragment enrichment:**
- Target specific amplification (Conventional PCR, **Multiplex PCR**, Isothermal DNA amplification)
- Whole genome amplification
- Droplet PCR with individual primer pairs

**Enriched DNA fragments identification:**
- Agarose Gel electrophoresis
- Capillary electrophoresis
- Melt-curve analysis
- Sequencing
- Re-sequencing
- MicroArray
An Integrative System Combining a rapid DNA Extraction Device and vLAMP for Rapid On-site Detection of GMOs

One Simple DNA Extraction Device and Its Combination with Modified Visual Loop-Mediated Isothermal Amplification for Rapid On-Field Detection of Genetically Modified Organisms

Miao Zhang,§ Yinan Liu,§ Lili Chen,† Sheng Quan,† Shimeng Jiang,† Dабing Zhang,†,‡ and Litao Yang∗,†
Disadvantages:
1: need precise instruments;
2: need long time (3–4h)

Solution:
Study the fast, easy and on-spot detection method without clumsy instruments.
Diagram of the developed DNA extraction device

10-15mins
Agarose gel electrophoresis analysis of extracted genomic DNA and their corresponding PCR amplicons of six crops.

Lanes 1-6 are the six crops used in the study (1=soybean, 2=rice, 3=canola, 4=cotton, 5=maize, and 6=wheat)

The results showed that the quality and integrity of gDNA extracted using our device were totally comparable to those extracted using the two commercial kits.
Sensitivity of event-specific assays using Genomic DNA extracted with DNA extraction device.

**MON89788**

**GTS 40-3-2**
Field test employing GM rice as examples

- **NOS terminator**
  - A

- **CaMV35s promoter**
  - B

- **bar gene**
  - C

- **Genotypes**: T1c-19, Ke6, TT51-1, Minhui63
MPIC: A high-throughput Analytical Method for Multiple DNA Targets

Droplet PCR (W/O emulsion PCR)

- Single or low copies molecule PCR amplification
- High throughput
- High efficiency
Principle of MPIC
Multiplex Microdroplet PCR Implemented CGE

(1) Target sequences were pre-amplified in multiplex PCR reaction

(2) Products were purified

(3) Universal microdroplet PCR amplification

(4) Microdroplets were gathered and PCR amplicons were purified

(5) CGE analysis

Detectable amplicons

PCR mixture as aqueous phase

Thermostable emulsion
Optimization of the MPIC System

Selection of DNA polymerase and Mg$^{2+}$ concentration.

Cleanup of the pre-amplified amplicons

Cycles of the pre-amplified PCR
The Flexibility of the MPIC Setup

Total of 24 targets can be clearly identified.
MACRO: A High-throughput and Flexible Platform for Monitoring of Genetically Modified Organisms
Principles

(a) Printing primers into microwells

(b) Multiplex PCR amplification on chip

(c) Co-amplification and labeling in tube

(d) Readout by oligo microarray

Results output tool (GDP)

Monitoring Report
Detected Species: XXXX
Detected events: XXXX...
Putative events: XXXX...

Initial PCR
Mineral oil
Amplicon mixture
2nd round PCR with universal primer pair

Amplicon
The high sensitivity of the MACRO system.
<table>
<thead>
<tr>
<th>Samples</th>
<th>Detected Events</th>
<th>Detected / Contained</th>
<th>FPR</th>
<th>FNR</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>BT176, BT11, 59122, 3272, GA21, MIR604, MON810</td>
<td>7 / 7</td>
<td>0</td>
<td>1.1%</td>
</tr>
<tr>
<td>S2</td>
<td>MON863, MON88017, MON89034, NK603, TC1507, BVLA430101</td>
<td>6 / 6</td>
<td>0.55%</td>
<td>0</td>
</tr>
<tr>
<td>S3</td>
<td>15985, 531, MON88913, LCotton25, <strong>MON89034</strong></td>
<td>5 / 4</td>
<td>0.55%</td>
<td>0</td>
</tr>
<tr>
<td>S4</td>
<td>RT73, OXY235, RF2, MS8, T45, Topas 19-2</td>
<td>6 / 6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S5</td>
<td>A2704-12, A5547-127, GTS40-3-2, MON89788</td>
<td>4 / 4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S6</td>
<td>TT51-1, Kefeng NO.6, T2A-1, KMD1, Bar68-1, t1c-19, RJ5</td>
<td>7 / 7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Monitoring results of the simulated samples.**
The coverage of the MACRO platform for known GMOs.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cover 2011</th>
<th>Total 2011</th>
<th>Coverage</th>
<th>Cover 2012</th>
<th>Total 2012</th>
<th>Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>55</td>
<td>57</td>
<td>96.5%</td>
<td>80</td>
<td>82</td>
<td>97.6%</td>
</tr>
<tr>
<td>Rice</td>
<td>12</td>
<td>12</td>
<td>100%</td>
<td>12</td>
<td>12</td>
<td>100%</td>
</tr>
<tr>
<td>Canola</td>
<td>24</td>
<td>24</td>
<td>100%</td>
<td>26</td>
<td>26</td>
<td>100%</td>
</tr>
<tr>
<td>Soybean</td>
<td>17</td>
<td>21</td>
<td>81.0%</td>
<td>20</td>
<td>24</td>
<td>83.3%</td>
</tr>
<tr>
<td>Cotton</td>
<td>26</td>
<td>26</td>
<td>100%</td>
<td>34</td>
<td>34</td>
<td>100%</td>
</tr>
<tr>
<td>Tomato</td>
<td>11</td>
<td>11</td>
<td>100%</td>
<td>14</td>
<td>14</td>
<td>100%</td>
</tr>
<tr>
<td>Sugar Beet</td>
<td>7</td>
<td>7</td>
<td>100%</td>
<td>7</td>
<td>7</td>
<td>100%</td>
</tr>
<tr>
<td>Papaya</td>
<td>3</td>
<td>3</td>
<td>100%</td>
<td>3</td>
<td>3</td>
<td>100%</td>
</tr>
<tr>
<td>Others</td>
<td>34</td>
<td>34</td>
<td>100%</td>
<td>40</td>
<td>41</td>
<td>97.6%</td>
</tr>
<tr>
<td>Total</td>
<td>189</td>
<td>195</td>
<td>96.9%</td>
<td>236</td>
<td>243</td>
<td>97.1%</td>
</tr>
</tbody>
</table>
One-step Visual Detection of Multiple Nucleic Acids in a Microcapillary Array
Basic principle and workflow

1. **Primer set in chitosan**
   - Drying

2. **Detection array**
   - Invert
   - Reaction mix
   - Insert
   - Sample loading
   - Remove

3. **Result**
   - Loading
   - Reaction mix

4. **Camera**
   - 60 - 65 °C
   - 30 min - 1 h

5. **LED**
   - UV

6. **Modification**
   - No modification
Sensitivity evaluation should this array has high sensitivity in different GM samples testing.
The accurate results of the practical sample tests indicate a good practicability of this array.
Outline:

- The development of GMOs
- GMO labeling regulations
- GMO detection methods
- GMO certified reference materials
1. Four types CRMs will be developed for GMO analysis

- Genomic DNA
- Matrix-based
- Plasmid DNA
- Protein
2. Establishment of the technical systems for matrix-based CRMs

- Identification of candidate material
- Pre-grind candidate material at low temperature
- Mixed with fixed concentration based on mass fraction
- Grinded with high homogeneity at low temperature
- freeze-dried treatment
- Packing
- Evaluation of Uncertainty
  - Value from inter-laboratory test
    - Homogeneity test
    - Stability test
Identification of the candidate materials

Planted three generations, and only homozygous individual plant was kept for self-copulation in each generation, we get 5 KG GM TT51-1 homozygous seeds.
Homogeneity study indicated the good homogeneity between different bottles and in one bottle.

Stability study indicated the period of validity is 36 months at least.
Values of the GM rice RM s expressed with copies ratio

The inter-laboratory ring trial was used for confirming the values;

The values of RM s were quantified by qRT-PCR method and pure homozygous rice leave DNAs;

The total U for RM s were calculated according to the U of homogeneity, stability, and Values confirmation.

Update, the matrix CRMs for 16 GM events were developed, including GM soya, GM Rice, GM maize, and GM cotton.

<table>
<thead>
<tr>
<th></th>
<th>TT51-1c</th>
<th>TT51-1d</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM</td>
<td>1.042</td>
<td>0.556</td>
</tr>
<tr>
<td>u</td>
<td>0.038</td>
<td>0.019</td>
</tr>
<tr>
<td>char</td>
<td>0.022</td>
<td>0.011</td>
</tr>
<tr>
<td>u</td>
<td>0.051</td>
<td>0.029</td>
</tr>
<tr>
<td>lts</td>
<td>0.135</td>
<td>0.073</td>
</tr>
</tbody>
</table>
3. Establishment of the technical systems for plasmid DNA CRMs

- Plasmid construction
- Plasmid DNA extraction
- Specificity and Commutability study
- Evaluation of Uncertainty
  - Value from inter-laboratory test
    - Homogeneity test
    - Stability test
- Bottling
In 2008, we constructed the double targets plasmid as calibrator for GM soybean analysis.
Constructed several plasmid RMs for GM maize, rice and cotton events

Event specific sequence
Schematic diagram of plasmid pSOY and pCanola
Specificity evaluation

Sensitivity evaluation
- Easily obtaining through microbial culture
- High purity and stability
- Low costs in production
- Stable copy number ratio of GM/Taxon fragment

Update, the plasmid RMs for 48 GM events were developed, including GM soya, GM Rice, GM maize, GM canola, and GM cotton.
Summary:

- A lot of GM crops and animals were developed, and under the pipeline of commercialization;
- The detection methods were well developed, and successfully used in GM contents monitoring and inspection;
- Several new methods were developed for high throughput, low cost, and quick analysis;
- The systems for CRMs were established, and which will help us to produce the CRMs of the GM events developed by Chinese researchers.
Family Album

http://zhanglab.sjtu.edu.cn/english/
Thanks for your attention!