Application of bioinformatics in GMO detection

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Summary

Intensified production and release of GMOs into the environment and market are correlated with the development of evaluation requirement and tools for GMO screening. In the last years, application of integrated approaches based on molecular detection techniques and bioinformatics tools has been noted. The examples of bioinformatics systems, methods, and IT achievements are described in this paper.

Key words:
GMO, detection, bioinformatics.

1. Introduction

The general worldwide practice of releasing of a genetically modified organism (GMO) is that, in advance, it is necessary to conduct a detailed risk evaluation concerning its potential effects on health and environment. When such assessment is applied to an unknown GMO, it is necessary to announce the risk level of this GMO as compared to the already known ones. With the rapid development of molecular techniques, the likelihood of obtaining such new GMO more frequently is getting considerably higher. The other side of the intensified GMO production

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biotechnologia
3 (74) 24–35 2006

is the development of clear evaluation requirements and tools for unknown GMO detection. The deliberately obtained GMO and its release are strictly following the commercial side of the product or its marketing. On the other hand, obtaining an unknown GMO could be a consequence of a research study, beyond public knowledge and experience.

The release of GMOs in the environment and marketing of GMO-derived food products are strictly regulated in the European Union and other regions (e.g. European Commission, 1997, 2001, 2003; FMBJ, 2000; MAFK, 2000). Moreover, the 'Cartagena Biosafety Protocol' agreement governs the trade and transfer of living GMOs across national borders, and allows governments to prohibit the import of genetically modified food when there is a concern over its safety (1). The pressure from the green groups and consumer organizations has also raised the general public's awareness of the GMO issue. As a result, it is important for food exporters, importers and retailers, as well as for competent authorities responsible for food safety, to know the extent and the nature of GMO ingredients in the products they handle.

The alteration, which renders an organism a GMO, usually consists of the insertion of a recombinant piece of DNA into the genome of the organism. Here, the inserted DNA is called the insert. Detection of the insert can be done using the methods that target modified genes, i.e. DNA, or gene products, i.e. RNA or protein. Presently, the majority of the methods applied in diagnostics target the modified DNA (2,3) as DNA is a rather stable molecule and the most common detection method, polymerase chain reaction (PCR), is very sensitive (4). Any PCR-based detection strategy depends on a detailed knowledge of the DNA sequence of the insert in order to select the appropriate oligonucleotide primers (for a comprehensive review of PCR-based detection methods, see Holst-Jensen et al., 2003). Hence, PCR is inappropriate for the use in the direct detection of unknown GMOs. A challenge in GMO screening in the near future is the rapid development of GM plants that feature new or multiple genes and genetic control elements. New technologies and instruments will be needed that offer high throughput detection of multiple or unknown inserts (3,5). To ensure that, competent authorities and others responsible for food and environmental safety and compliance with legislations can do their job. It is urgent to provide suitable analytical tools for discovering unknown GMOs. As an attempt to attain these targets, the DNA chip design described herein is meant to create theoretical basis for detection of unknown GMOs, i.e. GMOs that are not described in current literature (6). Given that the approach is deemed likely to work from a theoretical point of view, the experimental part of the design can start up. However, the extensive costs in terms of reagents and manpower may not justify the setting up of the experiments unless the theoretical basis is sound.

A great impact in recent time has had the development and application of integrated approaches based on molecular detection techniques and bioinformatics tools. The major benefit from the development of such bioinformatics resources is signifi-

cant time reduction to perform bio-sequences retrieval and analyses through the user-friendly web query and processing system that helps and guides end-users to find the data and, finally, the analyses that best fit their need with the implementation of a work flow logic. This fact is very important in the time-consuming operations, in which the researcher has to perform data retrievals and analysis manually, in many steps. Another important advantage of using such bioinformatics methods and resources is the economical efficiency by combining of *in silico* methods and laboratory tools.

2. GMO analyses within the EU: a short update

The given list of methods for the detection, identification and quantification of GMOs is authorized in the EU. These methods have been submitted to ring trial evaluation and are listed below, grouped per type of method and type of organism (soybean, maize). The categories are:

– Qualitative PCR methods for screening of foodstuffs containing soybean,

– Qualitative PCR methods for screening of foodstuffs containing maize,

– Qualitative PCR methods for screening of foodstuffs containing maize and soybean,

– Real-time PCR screening method for quantification of GMOs in foodstuffs containing soybean,

– Real-time PCR method for the quantification of GM-soybean,

– Real-time PCR method for the quantification of GM-maize,

– Immunoassay for detection and semi-quantification of GM-soybean,

– Immunoassay for detection and semi-quantification of GM-maize.

The listed methods allow detection and/or quantification of Roundup Ready soybean GTS 40-3-2 and of the five authorised GM maize varieties Bt-11, MON-810, Bt-176 and T25 in the following food matrices:

Foodstuffs derived from soy: soybean, soy flower (all types), soy meal, acidified soybeans, texturised vegetable protein, and biscuits.

Foodstuffs derived from maize: maize, maize flour (all types), and polenta.

Foodstuffs derived from soy and maize: baby food, raw material for food supplements biscuits.

The methods used for legislative implementation should produce acceptably accurate, precise, and reproducible results for the given analyte. Their performance should therefore be tested in collaborative studies according to the harmonized international protocols (e.g. IUPAC, AOAC, ISO), and should cover a wide range of laboratories, usually no fewer than eight. Information on the validation status of the listed methods is included in the tables. More detailed information on the methods' performance could be obtained by consultation of the compendium of the validated methods. This compendium, compiled by the European Commission Joint Research Centre can be downloaded from **http://biotech.jrc.it/methodsdatabase.htm**

3. Quantification of transgenic events and use of bioinformatics

3.1. Detection and quantification of GMOs

Polymerase chain reaction (PCR) methods are very useful techniques for detection and quantification of genetically modified organisms (GMOs) in food samples. These methods rely on the amplification of transgenic sequences and quantification of the transgenic DNA by comparison to an amplified reference gene. Reported here is the development of specific primers for the rapeseed (*Brassica napus*) *BnACCg8* gene and PCR cycling conditions suitable for the use of this sequence as an endogenous reference gene in both qualitative and quantitative PCR assays. Both methods were assayed with 20 different rapeseed varieties, and identical amplification products were obtained with all of them. No amplification products were observed when DNA samples from other *Brassica* species, *Arabidopsis thaliana*, maize, and soybean were used as templates, which demonstrates that this system is specific for rapeseed. In real-time quantitative PCR analysis, the detection limit was as low as 1.25 pg of DNA, which indicates that this method is suitable for use in processed food samples which contain very low copies of target DNA.

During the past decade, techniques have been developed that have allowed the introduction of candidate genes into plants and their regulated expression in different plant tissues, leading to the production of genetically modified organisms (GMOs) with characteristics of agronomical interest. These organisms have been extensively cultivated, and their derived products were first introduced into the food market in 1994 (7). However, acceptance of these GMO-derived products by the public has been controversial, and concerns about their safety persist among consumers. Such negative public response mandates the importance of providing complete information on food composition based on objective scientific studies (8). In Europe, marketing and distribution of GMO containing foodstuffs is controlled by European Union (EU) regulations 258/97, 1139/98, 49/2000, and 50/2000 (for food additives), as well as by National Food Ordinances. These regulations establish that food or food ingredients containing GMOs in concentrations >1% must be labeled to accurately reflect GMO content. Within the European Community (EC), strict control regarding commercialization of genetically modified organisms (GMOs) is exercised. Only five GMOs, including four transgenic maize (MON810, Bt176, Bt11, and GA21) and one transgenic soybean (Roundup Ready soybeans, Monsanto), are authorized within the EC. Furthermore, the EC requires labeling of foodstuffs containing GMOs and allows up to 0.9% adventitious presence of authorized GMOs in imported food (EC regulations 258/97 and 49/2000) (9).

Therefore, it is expedient to develop GMO detection and quantification methods that are specific, reliable, sensitive, and suitable to implement on a large number of samples. Polymerase Chain Reaction (PCR) amplification has proven to be one of the

most powerful techniques for the detection of specific DNA species. These techniques are especially useful for the identification and quantification of transgenic DNA in GMO food products because of their simplicity, specificity, and sensitivity. In addition, the high stability of DNA under the adverse conditions to which some foods are subjected during processing makes PCR-based methods particularly practical. PCR-based methods to qualitatively detect the presence of the transgene in a range of GMOs are presently available, and some of these methods have been validated by ring trials among European laboratories. PCR detection is often based on the amplification of transgenic target sequences such as the *CaMV 35S* promoter or the *Agrobacterium nos* terminator, present in most of the GMOs presently approved (9). Detection may also be based on the amplification of either the specific transgene introduced into the GMO or the specific insertion event. Examples of these methods are those based on the amplification of the *CP4-EPSP* gene in Roundup Ready soybeans (Monsanto Corp., St. Louis, MO) and the *Bt* genes in Maximizer maize Bt176, Bt-11 maize (Novartis Seeds AG, Basel, Switzerland), Liberty-Link T25 maize (Hoechst Schering AgrEvo GmbH, Berlin, Germany), or Yield Gard maize (Monsanto) (10).

The use of real-time quantitative PCR detection methods is a very accurate and fast system for the quantitative detection of GMOs in processed food samples. These methods normally rely on the amplification of transgenic specific sequences and their quantification, relative to an endogenous reference gene, gives an estimation of the total amount of target DNA in the sample. With this technology, the amount of GMO is calculated as a function of total plant-specific DNA in the food product. PCR primers and fluorogenic probes suitable for use with this technology have been published for genetically modified Roundup Ready soybeans and Maximizer maize Bt176. These systems require both the primers specific for the transgene and the species-specific primers complementary to an endogenous reference gene. Amplification of such reference sequences will allow the detection of DNA from the plant species of interest in food samples and, at the same time, assay for the quality of the extracted DNA, providing a means to quantify the amount of GMO in the processed food sample. Much effort has been expended to obtain reference genes for the analysis of genetically modified soybean and maize due to the economic importance of these food crops. Therefore, it is of great importance that detection methods are able to identify several transgenes in a single reaction. Routinely used GMO detection methods generally involve polymerase chain reaction (PCR) techniques to detect specific DNA sequences, often in a multiplex format in which two or more targets are simultaneously amplified in the same reaction (11). For example, recently there was described robust and specific multiplex PCR method to simultaneously detect five transgenic and two wild-type genes in raw materials and foodstuffs (12). This seven-target method detects the four transgenic maizes and one transgenic soybean authorized by the EC as well as two endogenous controls (zein gene for maize and lectin gene for soybean). The simultaneous ampli-

fication of multiple sequences in a single reaction saves time, reagents, and efforts in GMO detection analysis and decreases the number of reactions required to assess the presence of all possible GMOs (13).

Nevertheless, as recently highlighted by the case of controversial PCR evidence for transgenic contamination of maize in Mexico (4), post-PCR monitoring is required to confirm sequence identities (14). In this context, microarray technology can greatly improve the ease and speed of post-PCR amplicon analysis and identification.

One alternative is to combine the ligation detection reaction (LDR) with a universal array in a molecular approach to detect and quantify the amplified cryIA(b) gene from Bt176 maize (15). Universal arrays consist of a set of synthetic oligonucleotides named Zipcodes (16) or tags of similar thermodynamic characteristics (melting temperature) but different sequences: The sequences are totally unrelated to those under scrutiny. Gerry et al. first proposed LDR combined with a demultiplexing universal array as a powerful tool for sequence discrimination. This procedure requires the design of two adjacent probes specific for each target sequence.

3.2. IT and bioinformatics resources for GM detection

Few domains experienced an explosion of "data" production as Biology. Over the past two decades thousands of databases of biological knowledge have been produced ranging from very large initiatives, such as the Genome Project to specialized databases produced by small research groups around the world coming from *in vivo*, *in vitro* and *in silico* analysis.

The need for tools able to manage a large biological knowledge base is growing (17). Integrated bioinformatics platforms that make more effective use of repositories of structured molecular data integrated with bioinformatics analysis capabilities will be widely employed in the near future. The need to construct such a kind of bioinformatics instrument came from the European Community in the 2000, when the European Network of Genetic Modified Organism (GMO) Laboratories (ENGL) was created. They needed a Molecular Register containing data on molecular characterization of GMOs approved for placing on the market in the EU and the necessary on-line tools to analyse the related sequences. This project was considered of highest priority since such bioinformatics instrument is extremely necessary to support the ENGL in the detection and characterisation of engineered genetic constructs.

3.3. Web-based integrated platform

To satisfy these needs, the construction of an innovative web-based comprehensive prototype platform was planned. This platform was developed to integrate a

database, a database submission tool, a query and retrieval system tailored to the extraction of data and a set of analysis software able to meet and satisfy all the analysis needs of the ENGL. Collaboration agreement contracts for the development of the GMOs MOLREG package have been signed between the Institute for Health and Consumer Protection of the Joint Research Centre and:- the Section of Bioinformatics and Genomics of the ITB – Consiglio Nazionale delle Ricerche (CNR), Italy- the Robert Koch-Institut (RKI), Zentrum Gentechnologie, Germany. The CNR led the design and development of the GMOs MOLREG package as an easy to use web-based system consisting of a database storing GMOs data, a GMOs data submission component, a query component and a bioinformatics tool for running GMO's bio-sequences analysis. The design of the GMOs MOLREG database itself and the development of the web-submission component has been done by the CNR in collaboration with RKI and the JRC, while to design and develop the GMO Query component and the GMO bioinformatics analysis component, the CNR established a collaborative agreement with the Life Science Team of the IBM Semea Sud, a specialized services unit of IBM in Italy.

The database has been designed and developed to include information on the following aspects: general administrative/legislative information on the registered GMO, specific information on the origin of the GMO including a detailed molecular characterisation, detailed information on available GMO detection methods and certified reference materials, screening, identification, quantification methods and information on reference literature. The Database Management System (DBMS) used for this application is Oracle 8i, the design of the database has been made using Oracle Designer, while the submission tool has been developed using Oracle Forms.

The architecture of the query and analysis components of the GMOs MOLREG package has required the setup of a server environment hosting the elements needed to provide a web access to the user, a secure and friendly tool for the GMOs MOLREG data retrieval and a complete system suitable to perform sequence analyses among the GMO sequences and the data stored in public Databases. Access to the MOLREG package is reserved to the registered users, connectivity security is ensured by the HTTPS protocol. Data query, retrieval and sequence analysis sections of the package have been implemented with Java technology [J2EE for servlet, JSP 1.0 for GUIs, JDBC for DB interface]. An Application Server [Oracle 9iAS Enterprise Edition], a HTTP Server [Apache adapted to Oracle] and a Java Runtime Environment [Java Development Kit version 1.2.2] have been installed in order to run the previous components on the GMOs MOLREG server.

The need for running bioinformatics analysis on GMOs bio-sequences required that the Registry was deeply integrated with Bioinformatics analysis tools, using a novel approach based on XML, giving end-users the possibility to analyse molecular data stored into the Repository and/or correlate such data with external public biological databases (EMBL, SWISS-PROT, PROSITE, REBASE, TRANSFAC, UNIVEC). For this purpose, specific bioinformatics algorithms were required to be integrated in

the GMO Repository information infrastructure; they are some of the analysis program of the EMBOSS package (18), besides BLAST and FASTA.

An important aspect concerning the integration between the GMOs MOLREG database and the external bioinformatics analysis tools is that a specific XML-based description language has been developed in order to generalize the run time generated web user interface, both to manage algorithms' input parameters and to show the tool results. This XML language provides a interface layer between the GMOs MOLREG database and the underlying bioinformatics analysis package used. Additionally, a Web administration console that manipulates the XML descriptors was also created with a double end: to manage analysis programs updates; to attach, as a sort of plug-in, new bioinformatics programs.

Strong security features (encryption, profiling authorization and control, server certificates, etc.) were also implemented in order to protect the confidential business information flow during all the steps of the extraction/analysis processes. The query and retrieval system has been developed in Java by using the IBM Websphere Studio Application Developer 4.0.3 environment and deployed and successfully tested on Websphere Application Server, Oracle Application server as well as on Tomcat.

3.4. Bioinformatics tools and applications

There are several types of systems, used in bioinformatics for detection of GM motifs. Desktop applications are used for single probe experiments, by laboratory workers. These systems include desktop and client-server tools, provided by the bioinformatics and cheminformatics industry. There are more sophisticated systems, called algorithmic pipelines that are used on large-scale experimental systems or on industrial basis. These pipelines are using complex algorithmic tree approach to ease the integration of data flow and increase the speed of production.

Since unknown genetically modified organisms are not risk evaluated, they might pose a danger to health and environment. Presently, the majority of the methods applied in diagnostics target the modified DNA. Any PCR-based detection strategy depends on a detailed knowledge of the DNA sequence of the insert in order to select the appropriate oligonucleotide primers (for a comprehensive review of PCR-based detection methods, see (3). Hence, PCR is inappropriate for use in the direct detection of unknown GMOs.

3.5. Systems and methods

State-of-the-art integrated bioinformatics and computational biology systems are briefly characterized by several criteria: complex data integration input, multiple algorithmic engine, multiple analysis data integrity checkpoints, and last but not

least – annotation pipeline. Most of the current integrated bioinformatics systems are not publicly available, since they are custom designed for specific pharmaceutical tasks.

In this article, we will draw an example /do an *in silico* experiment/ using several of the major GM identification patterns. Many of these include c-nos terminator, 35S promoter, herbicide tolerance gene ***epsps***, overlap between CDPK promoter and insect resistance gene cryIAb, junction region of adh 1S-Intron 2 (IVS2) and pat gene, CaMV 35S terminator and pat gene, Edge fragment covering genomic maize DNA and CaMV promoter, CP4 EPSPS transgene, Junction between *Petunia hybrida* CTP and CP4 EPSPS sequence from *Agrobacterium* sp. Strain, N.1 intron from maize hsp70 gene and synthetic cryIAb gene, IVS6 int. from maize alcohol dehydrogenase 1 gene (adh1-1S) and synthetic cryIA (b) gene, etc.

The 35S promoter example

Our experiment will is to try to detect whether an unknown plant is actually a GM or transgenic plant. After the activities in the laboratory, the bioinformatics algorithms will analyze data and will validate if there should be reasonable doubt for the natural origin of the plant sample.

For the experiment we will execute a local alignment search, using BLAST. As query sequence we will use an unknown sequence that is provided by the sequencing:

tcgtagctacgatgctacgagtcacgacgatctacgactagctacgtgtcctatgctagc
CAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAG
AGGACACGCTCGAGGAATTCGGTACCCCGGGTTCGAAATCGATAAGCTTGGATCCTCTAG
Atcgatcgactgacgtatgcatcgtatgcatcgta

Using BLAST service at National Institute of Health (NCBI), we will input the artificial sequence into MegaBlast – a tool for identification of slightly different sequences.

```
# BLASTN 2.2.13 [Nov-27-2005]
# Fields: query id, subject ids, % identity, alignment length, mismatches,
# gap opens, q. start
# 134 hits found
1_4786 gi|312235|emb|X69707.1|SYNPART7   100.00 121   0   0   61
1_4786 gi|15982216|emb|AJ311873.1        100.00 88    0   0   61
1_4786 gi|15982216|emb|AJ311873.1        100.00 33    0   0   149
```

The first entry has an EMBL accession number X69707, which is exactly the synthetic construct CaMV 35S promoter and multiple cloning site (gi|312235|emb|X69707.1|SYNPART7[312235]). Using this result, we proved that the used artificial sequence plant has significantly high probability to be transgenic, because it contains a region (bold region in the query sequence) with 100% similarity to a CaMV 35S Promoter site.

4. Implementation

4.1. *In silico* evaluation of allergenicity of GM foods

Recent research has revealed that exposure the GM-plants to a viral infections can trigger new allergies and the combination of genetic material can lead to cross allergenicity. This research shows a possibility of introducing of amplifying the allergenicity of food by gene transfer in GM-plants.

4.2. Antigenic determinants prediction

The major antigenic determinant algorithms are focused in calculations for predicting secondary structure, hydropathy, hydrophobicity, flexibility and surface probability, which allow to predict significant epitopic regions in a primary protein sequence.

Most of these algorithms were developed in the late 70s, but now they are applied in a different field – bioinformatics. The main algorithms for *in silico* antigenic determinant prediction include:

a) Protein hydrophobicity – hydrophilicity algorithms:

– Fauchere, Janin and Manavalan – Protein hydrophobicity algorithm for prediction of surface and inside volumes in globular proteins.

– Kyte and Doolittle – a method for predicting the hydropathic character of a protein.

– Sweet and Eisenberg – an algorithm for predicting correlation of sequence hydrophobicities measures similarity in three-dimensional protein structure.

– Goldman, Engelman and Steitz (GES) – a method for identifying nonpolar transmembrane helices in amino acid sequences of membrane proteins.

b) Protein flexibility prediction algorithm:

– Karplus and Schulz – a method for prediction of chain flexibility in protein sequences.

c) Protein secondary structure prediction algorithms:

– GOR II method (Garnier and Robson) is a method for predicting secondary structures in protein sequences.

– Chou and Fasman – calculating conformational parameters for amino acids in helical, β-sheet, and random coil regions.

d) Protein "antigenicity" prediction (prediction of the distribution of polar and apolar residues along a protein sequence. Most commonly, such analysis has the goal of predicting membrane-spanning segments (highly hydrophobic) or regions that are likely exposed on the surface of proteins (hydrophilic domains) and therefore potentially antigenic).

– Parker – an algorithm based on hydrophilicity scale derived from high-performance liquid chromatography retention data and correlation of predicted surface residues with antigenicity.

– Protrusion Index (Thornton) – a method for detection of continuous antigenic determinants in the protruding regions of protein sequences.

– Antigenic index – prediction of the antigenicity of a sequence based on its hydrophilicity, predicted side chain flexibility, surface probability, and the predicted turns. Using these *in silico* methods and the primary protein sequence, an analysis of the epitopic profile could be easily made. Based on these data a comparison between the standard and the probes would give exact answer if the probe is a genetically modified plant or not. The future aspects of this problem might be resolved using complex *in silico* models of all gene-gene, gene-protein, and protein-protein interactions of the new transgene plant. Using these techniques, firstly, it will be easy to ensure food safety of the agricultural production and, secondly, it will be a cost effective method of identification of GM-plants.

5. Conclusions

a) Molecular identification techniques together with the latest bioinformatics tools and IT achievements provide integrative system for easy, fast and significant information retrieval and analysis of GM plants.

b) Examining the use of methods of bioinformatics together with standard laboratory tools in GM detection, we may obtain the major benefits they provide.

c) Using bioinformatics methods is proven to be economically more effective than using laboratory kits. Comparison of *in silico* methods and laboratory tools shows significantly higher cost effectiveness of software solutions. In recent days, the algorithms for data integration and analysis have been developed in a way of decreasing the error level, due to the system complexity.

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