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Application of Next Generation Sequencing (NGS) in characterisation of unauthorised GMO in food and feed

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Outline

EU legislation framework on GMO detection and UGM

Next Generation Sequencing (NGS)

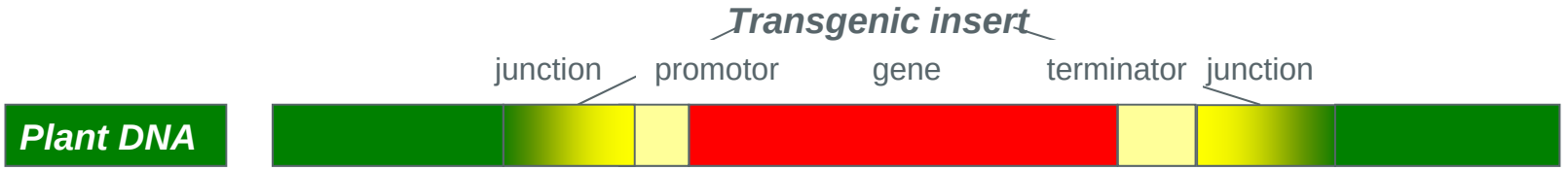
Whole genome sequencing for characterisation of UGMM B. subtilis in feed addtitve

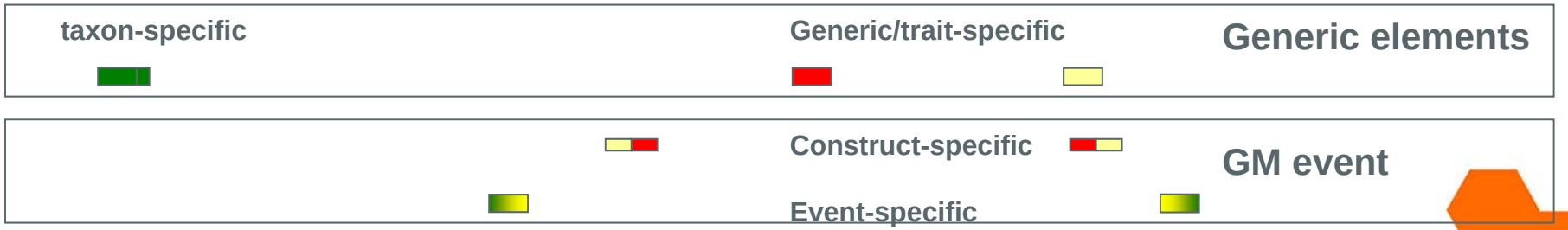
Targeted Genome seqiuencing for detection of plant UGM in food and feed

GMO detection: EU legislation framework

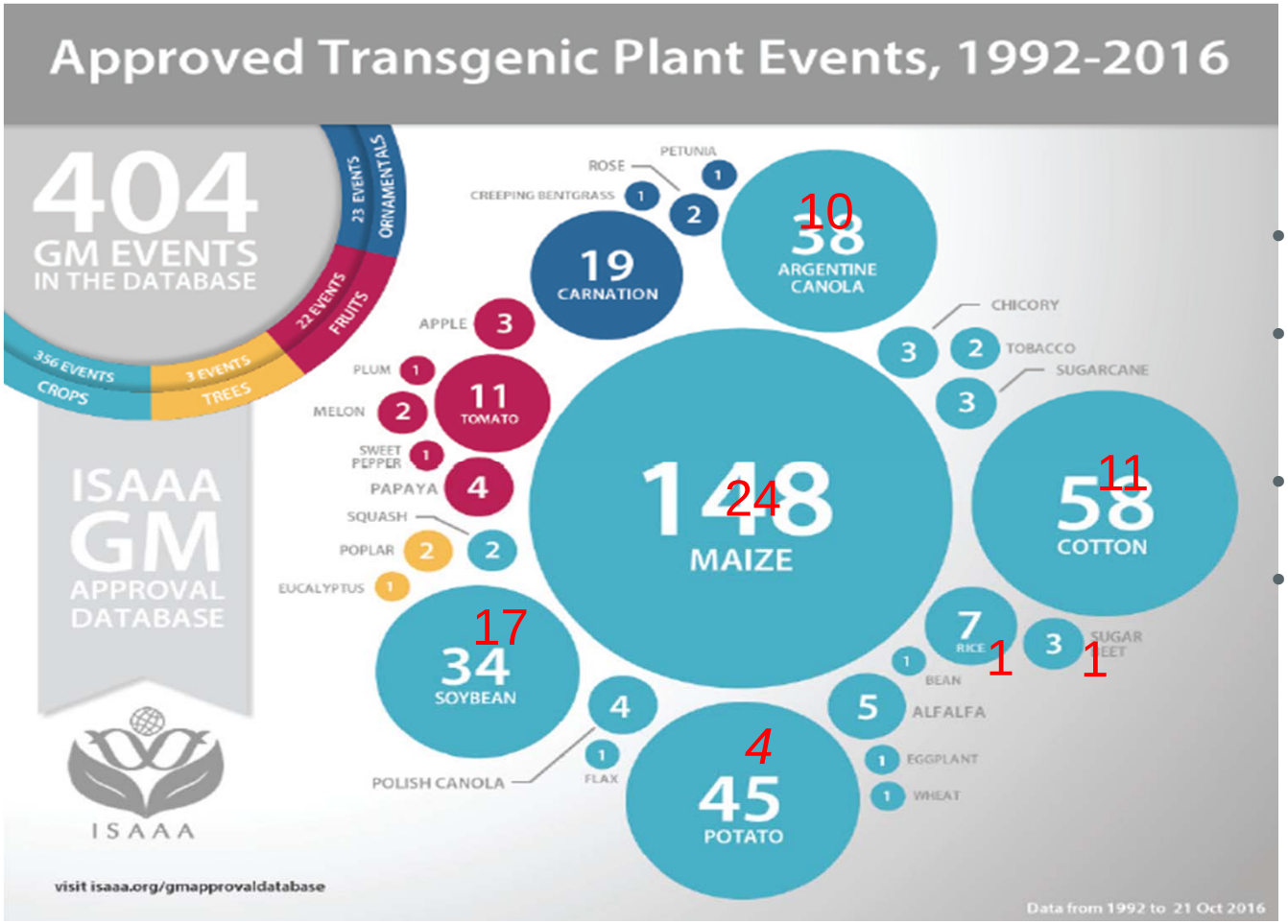
Legislation framework (EC/1829/2003, EC/1830/2003, EC/641/2004)

GM event developer		*Market*	*Control Authorities*	*Official control labs*
Application for authorisation Safety assessment Event-specific method	**EFSA: safety assessment** **EU-RL GMFF: Official reference methods**	Seed production Primary products Food Feed Labelling and traceability Consumer's choice	Controls in function of labelling – sampling food and feed Threshold of 0.9%	Analysis of food and feed **Real time PCR** Screening Identification Quantification



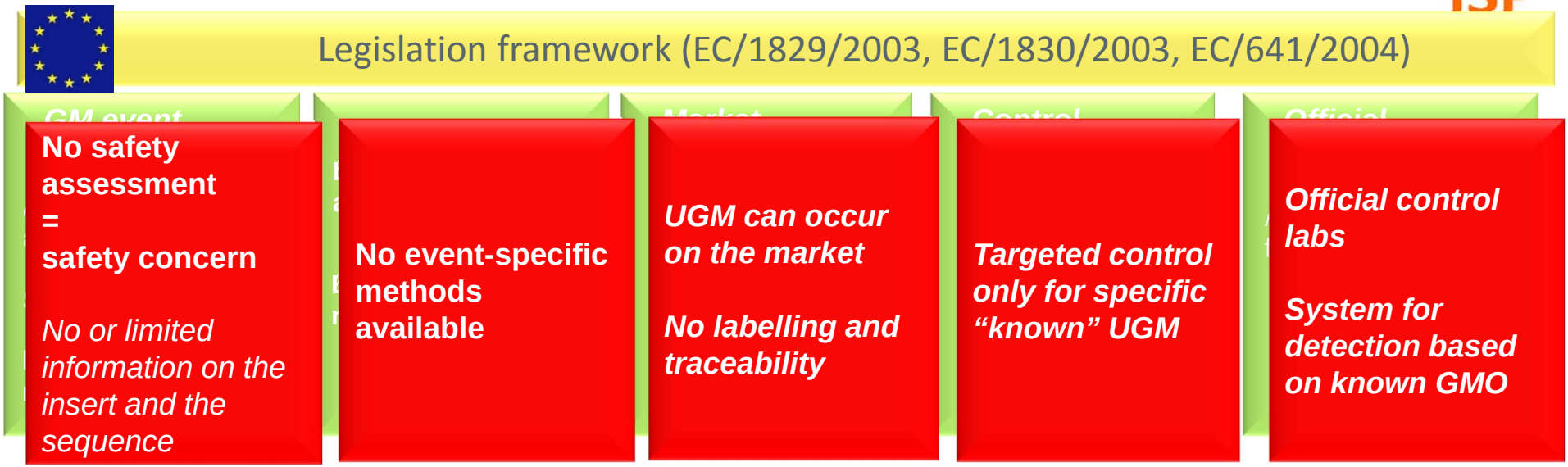


GM plants worldwide



- *Increasing number*
- *New transgenic elements / cassettes*
- *New species*
- *Produced by commercial companies or governmental research institutions*

GMO detection: unauthorised GMO



GM analysis: search for known GMO

- **Based on insert /sequence information => no such information available for UGM**
- **Screening with common transgenic elements occuring in EU authorised events, but also in UGM => presence of UGM can be « masked » by the presence of authorised GMO**
- **Identification with event-specific method => no event-specific methods for UGM**

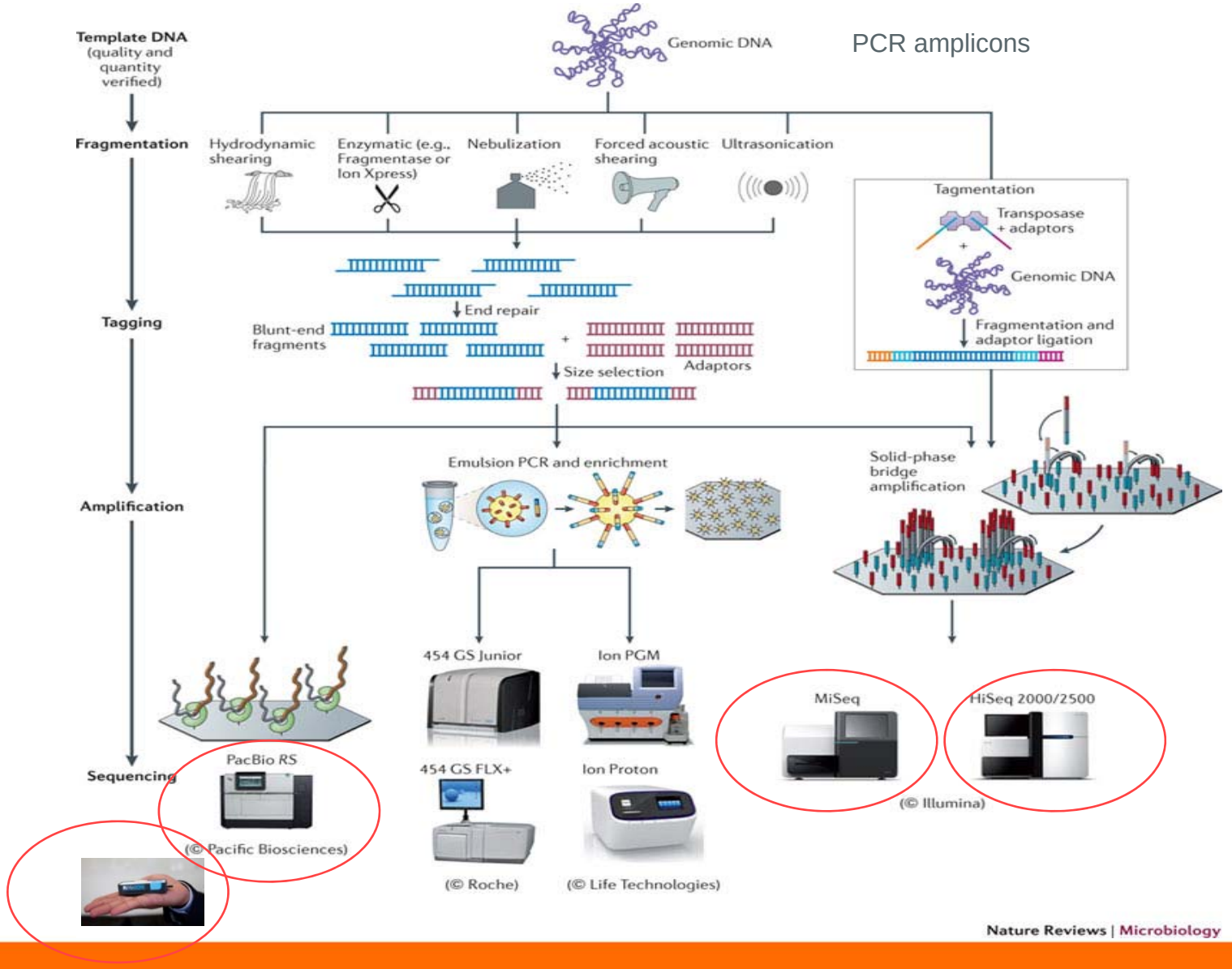
The system for authorised GMO does not work well for UGM!

New tools and approaches needed!

Next Generation Sequencing:

- High-throughput sequencing
- Rapid
- Different sequencing technologies / platforms
- Different approaches for data analysis depending on the sequencing strategy
- Two different strategies
    - Whole genome sequencing (WGS): *allows characterisation of sample without prior knowledge on the sequence - characterisation of GM B. subtilis in vitamin B2 samples*
    - Targeted genome sequencing (TGS): *minimal prior knowledge on the sequence is required – characterisation of plant GMO*

Next Generation Sequencing: workflow

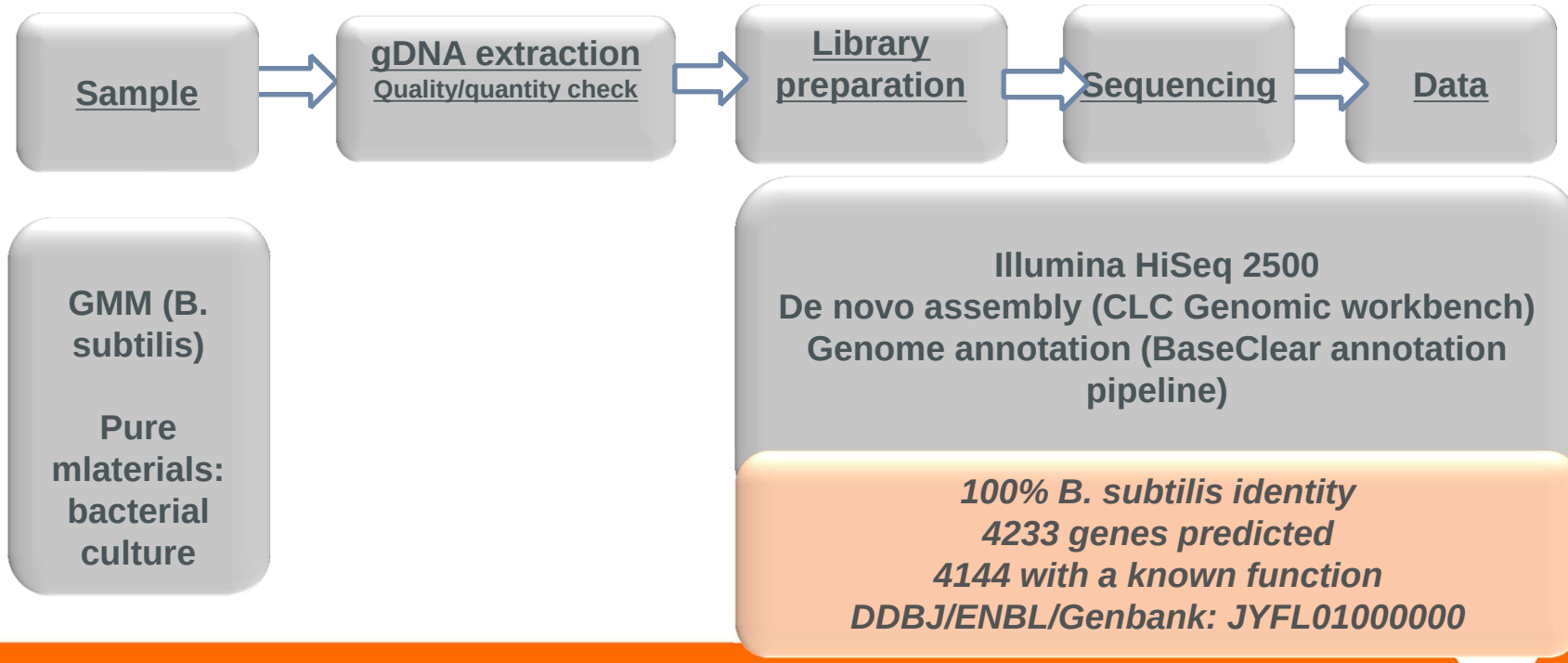


WGS: characterisation of GM *B. subtilis* in feed additives

Rapid Alert System for Food and Feed (RASFF) notification:
RASFF 2014.1249 (Germany)

No appropriate method available

Characterisation of the gDNA sequence of *B. subtilis* extracted from samples related to the notification



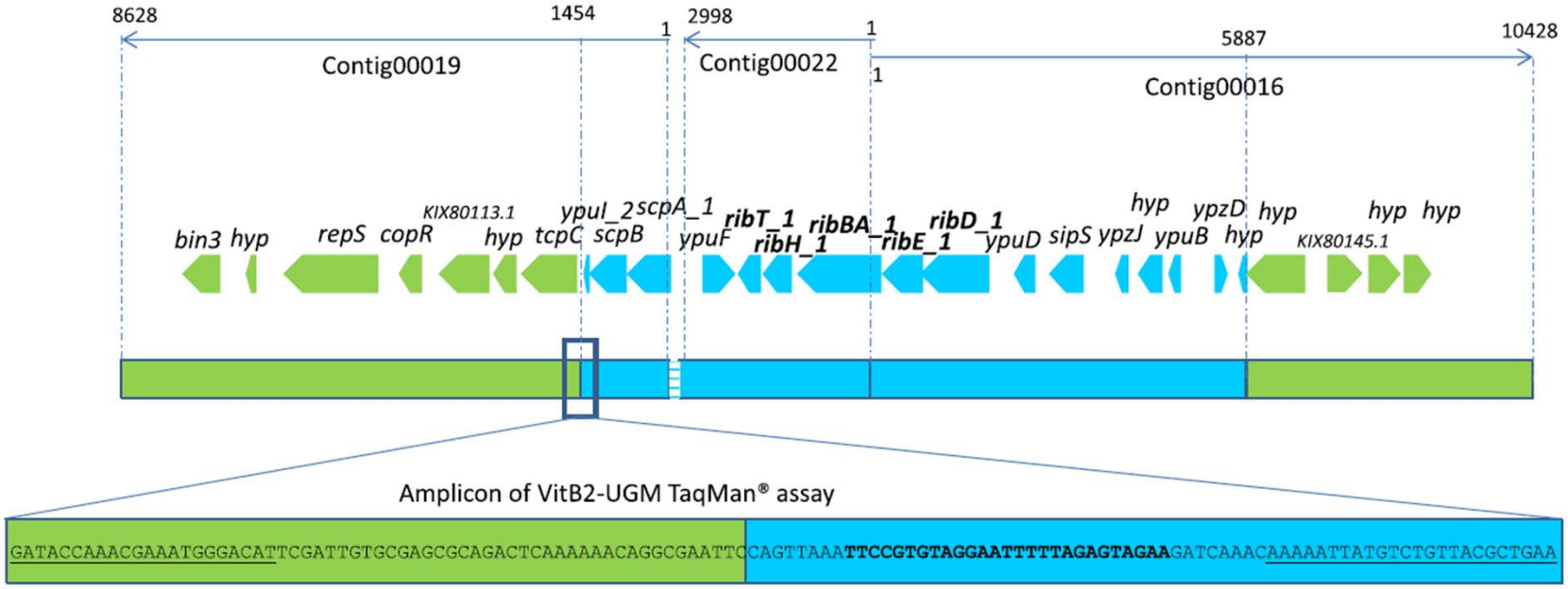
WGS: characterisation of GM *B. subtilis* in feed additives

Identification of 3 overlapping contigs containing the riboflavin biosynthesis operon - ribA gene

Identification of region with similarity to plasmid vectors

Design of PCR assay and verification of the amplicon sequence

Design of TaqMan qPCR assay



WGS: characterisation of GM *B. subtilis* in feed additives

- Sequence data were obtained from applying WGS
- The obtained sequencing data were used to develop qPCR method
- The qPCR method was in house validated according to the ENGL / EU-RL GMFF criteria (Method Performance Requirements, 2015)
- Applied in routine analyses for feed additive samples (Vit. B2)

genomeA
Journals.ASM.org

Genome Sequence of EU-Unauthorized Genetically Modified *Bacillus subtilis* Strain 2014-3557 Overproducing Riboflavin, Isolated from a Vitamin B2 80% Feed Additive

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E.B.-P. and S.C.J.D.K. contributed equally.

P.P. and N.H.R. contributed equally.

This paper announces the genome sequence and annotation of the genetically modified (GM) *Bacillus subtilis* strain 2014-3557 overproducing riboflavin (vitamin B2). This GM-strain is unauthorized in the European Union. Nevertheless, it has been isolated from a lot of vitamin B2 (riboflavin) 80% feed grade imported to Europe from China.

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Citation Barbau-Piednoir E, De Keersmaecker SCJ, Wuyts V, Gau C, Pirovano W, Costessi A, Philipp P, Roosens NH. 2015. Genome sequence of EU-unauthorized genetically modified *Bacillus subtilis* strain 2014-3557 overproducing riboflavin, isolated from a vitamin B2 80% feed additive. Genome Announc 3(2):e00214-15. doi:10.1128/genomeA.00214-15.

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Riboflavin (vitamin B2) cannot be biosynthesized by vertebrates, whereas plant and most microorganisms are able to do so (1). Therefore, vitamin B2 is used as a food and feed additive. As an alternative to costly chemical synthesis, microbial fermentation processes of riboflavin were developed for industrial production. Naturally producing or riboflavin-overproducing microorganisms transformed by a genetic engineering, chemical, or physical process can be used. *Bacillus subtilis*, a Gram-positive, rod-shaped bacterium, is exploited in industry for this purpose (1–3).

In July 2014, Germany detected a viable *Bacillus subtilis* strain which harbored a non-naturally occurring combination of DNA sequences in a lot of vitamin B2 feed additive imported from China. This strain was considered as genetically modified and unknown; therefore it is unauthorized in the European Union. In September 2014, the European Rapid Alert System for Food and Feed (RASFF) created a notification to alert the other European countries about the presence of the unauthorized GM-*Bacillus subtilis* in this particular vitamin B2 feed additive (https://webgate.ec.europa.eu/rasff-window/portal/ [enter reference 2014.1249]).

Consequently, the French competent authorities investigated this kind of product imported in France. A French National Reference Laboratory (NRL) for GMO isolated a yellow substance (presumably overproduction of riboflavin)-secreting bacterial strain from three samples of vitamin B2 feed additives imported from China.

To further characterize this finding, whole-genome sequencing was performed on one of the three isolates with an Illumina HiSeq2500 run using a paired-end library. Sequencing yielded 10,914,314 paired-end reads (350-fold coverage), which were assembled *de novo* using CLC Genomics Workbench version 7.5.1 (CLC Bio). The resulting draft genome was further linked into scaffolds with SSPACE (4) based on paired-end read linkage. Finally, a total of 39 gap-closed scaffolds were generated consisting of 143 contigs with a maximum gap-closed scaffold size of 1,018,461 bp and a minimum size of 370 bp. After filtering of these 39 scaffolds, three were discarded as two matched with *Homo sapiens* and one with *Haemonchus placei*. The total sequence length post-filtering is 4,175,764 bp, and has a G+C content of 44.32%.

Genome annotation was performed on the assembled scaffold sequences using the BaseClear annotation pipeline which is based on the Prokka Prokaryotic Genome Annotation System. This annotation confirmed the organism as *Bacillus subtilis* with 100% identity. 4,233 genes were predicted, of which 4,144 have a known function, including several encoding for proteins involved in riboflavin synthesis and transport. The genome sequence of this *Bacillus subtilis* strain will be useful for further characterization of its GM nature and in developing a specific method for its detection in food and feed additives by enforcement laboratories.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. JYFL00000000. The version described in this paper is version JYFL01000000.

Barbau-piednoir *et al. BMC Biotechnology* (2015) 15:103
DOI 10.1186/s12896-015-0216-y

BMC Biotechnology

RESEARCH ARTICLE Open Access

Use of next generation sequencing data to develop a qPCR method for specific detection of EU-unauthorized genetically modified *Bacillus subtilis* overproducing riboflavin

Elodie Barbau-piednoir[1], Sigrid C. J. De Keersmaecker[1], Maud Delvoye[1], Céline Gau[2], Patrick Philipp[2] and Nancy H. Roosens[1*]

Abstract

Background: Recently, the presence of an unauthorized genetically modified (GM) *Bacillus subtilis* bacterium overproducing vitamin B2 in a feed additive was notified by the Rapid Alert System for Food and Feed (RASFF). This has demonstrated that a contamination by a GM micro-organism (GMM) may occur in feed additives and has confronted for the first time the enforcement laboratories with this type of RASFF. As no sequence information of this GMM nor any specific detection or identification method was available, Next GenerationSequencing (NGS) was used to generate sequence information. However, NGS data analysis often requires appropriate tools, involving bioinformatics expertise which is not always present in the average enforcement laboratory. This hampers the use of this technology to rapidly obtain critical sequence information in order to be able to develop a specific qPCR detection method.

Methods: Data generated by NGS were exploited using a simple BLAST approach. A TaqMan® qPCR method was developed and tested on isolated bacterial strains and on the feed additive directly.

Results: In this study, a very simple strategy based on the common BLAST tools that can be used by any enforcement lab without profound bioinformatics expertise, was successfully used to analyse the *B. subtilis* data generated by NGS. The results were used to design and assess a new TaqMan® qPCR method, specifically detecting this GM vitamin B2 overproducing bacterium. The method complies with EU critical performance parameters for specificity, sensitivity, PCR efficiency and repeatability. The VitB2-UGM method also could detect the *B. subtilis* strain in genomic DNA extracted from the feed additive, without prior culturing step.

Conclusions: The proposed method, provides a crucial tool for specifically and rapidly identifying this unauthorized GM bacterium in food and feed additives by enforcement laboratories. Moreover, this work can be seen as a case study to substantiate how the use of NGS data can offer an added value to easily gain access to sequence information needed to develop qPCR methods to detect unknown and unauthorized GMO in food and feed.

Keywords: Identification, Event-specific, GMO, Unauthorized GM-*Bacillus subtilis*, Riboflavin, Vitamin B2, qPCR

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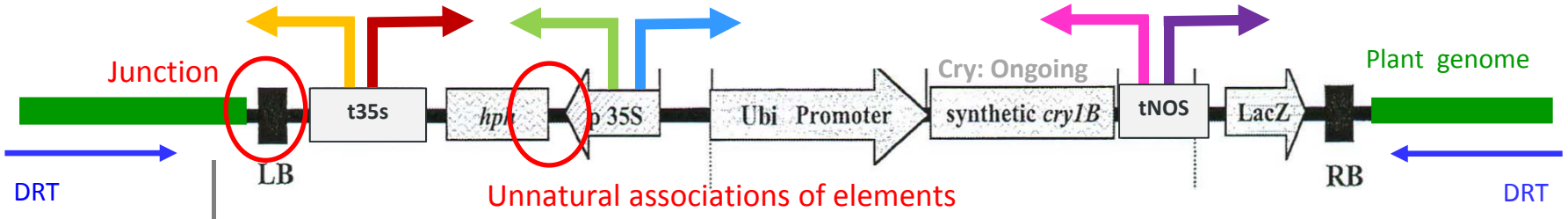
Targeted genome sequencing: characteristaion of plant GMO

DNA walking → obtaining DNA fragments of interest

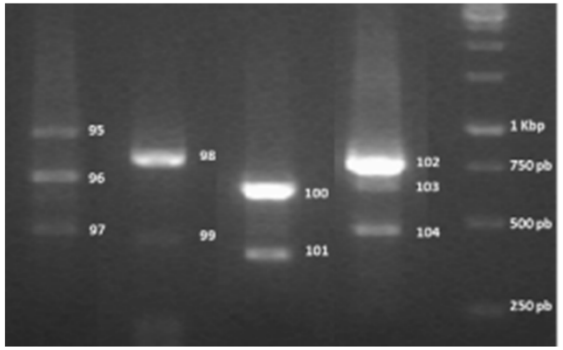
Detected in qPCR screening

→ Combination of target-specific primers and degenerate random tagged primers (DRT)



Exponential amplification by **PCR** of the sequences of interest

Visualisation on gel



Fraiture et al. 2014, Food Chemistry

Fraiture et al. 2015, Food Chemistry

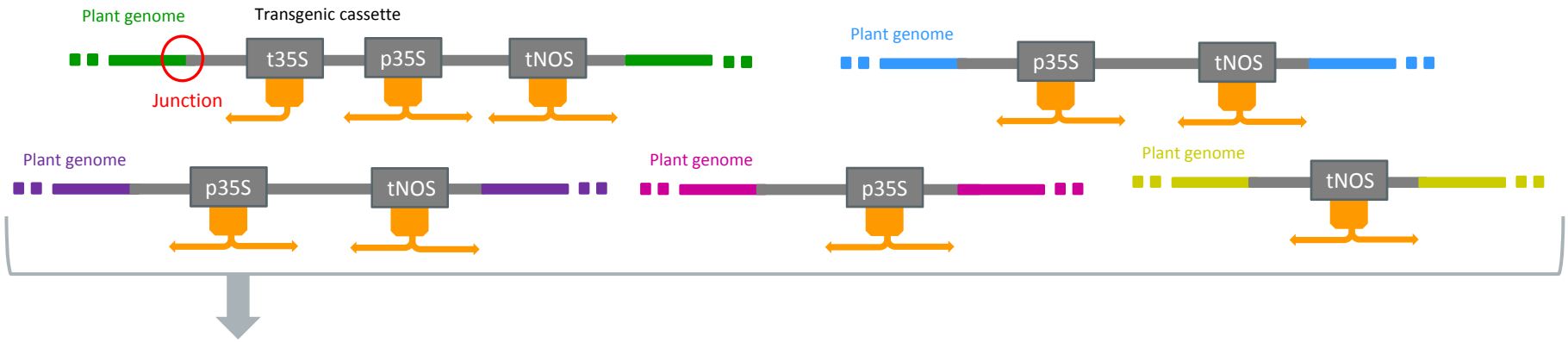
Fraiture et al. 2015, BMC Biotechnology

DNA walking coupled to NGS

→ Obtaining sequences from DNA fragments of interest

❖ Main limitation of DNA walking coupled to Sanger Sequencing:

→ Matrix with multiple GMOs containing the same targeted elements

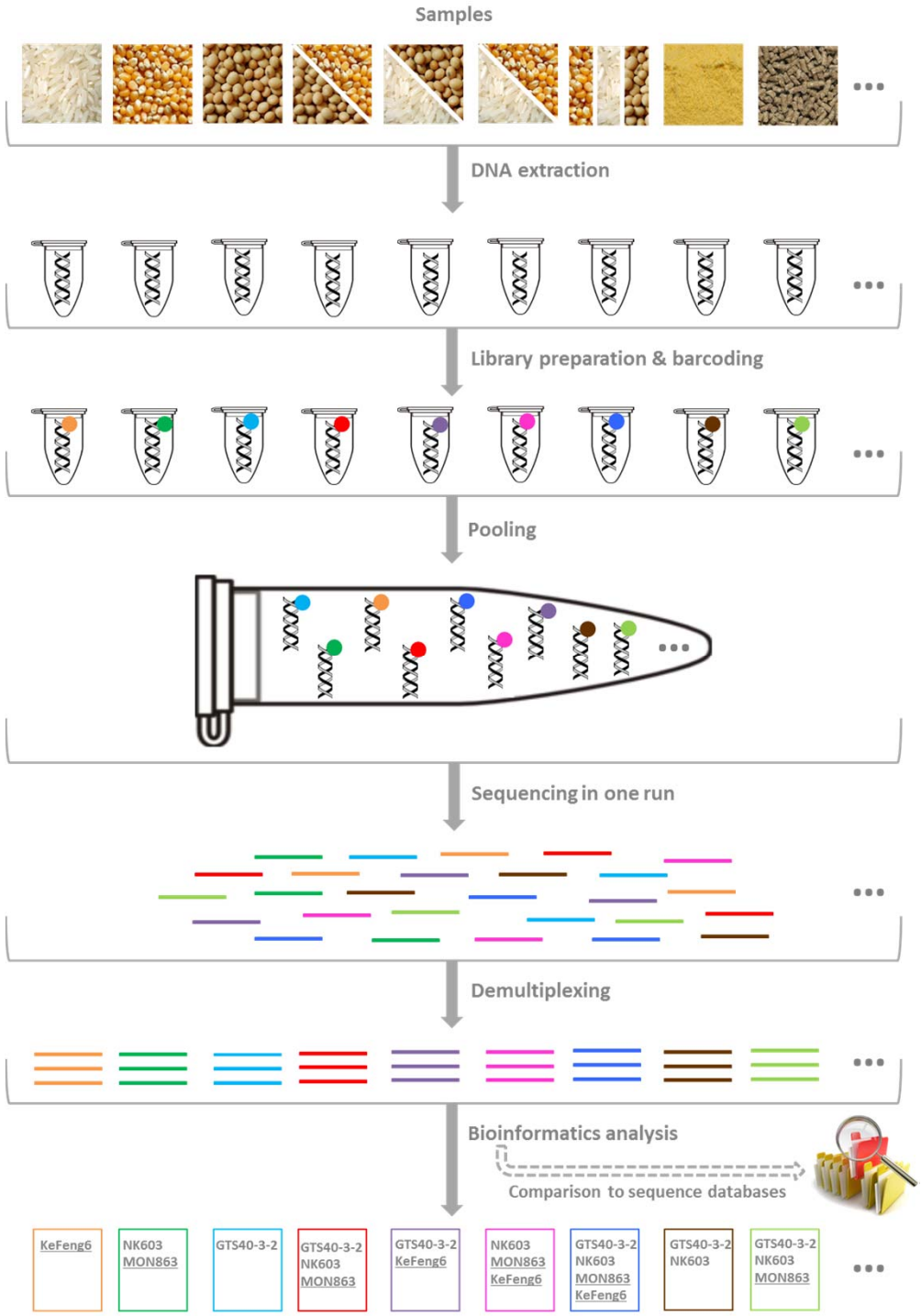


NGS

→ **Massive parallel DNA sequencing (High-throughput)**

DNA walking coupled to NGS

workflow



DNA walking coupled with NGS: selection of NGS platform

- Able to deal with heterogenic library size
  - Amplicon size range going from ~ 200 bp to 6 Kbp
- Long read length (up to 60 Kbp)

Able to sequence the whole amplicons

No library shearing and de novo assembly

User-friendly bioinformatics analysis

- Sequencing Cost per Gb : ~ 2000 €

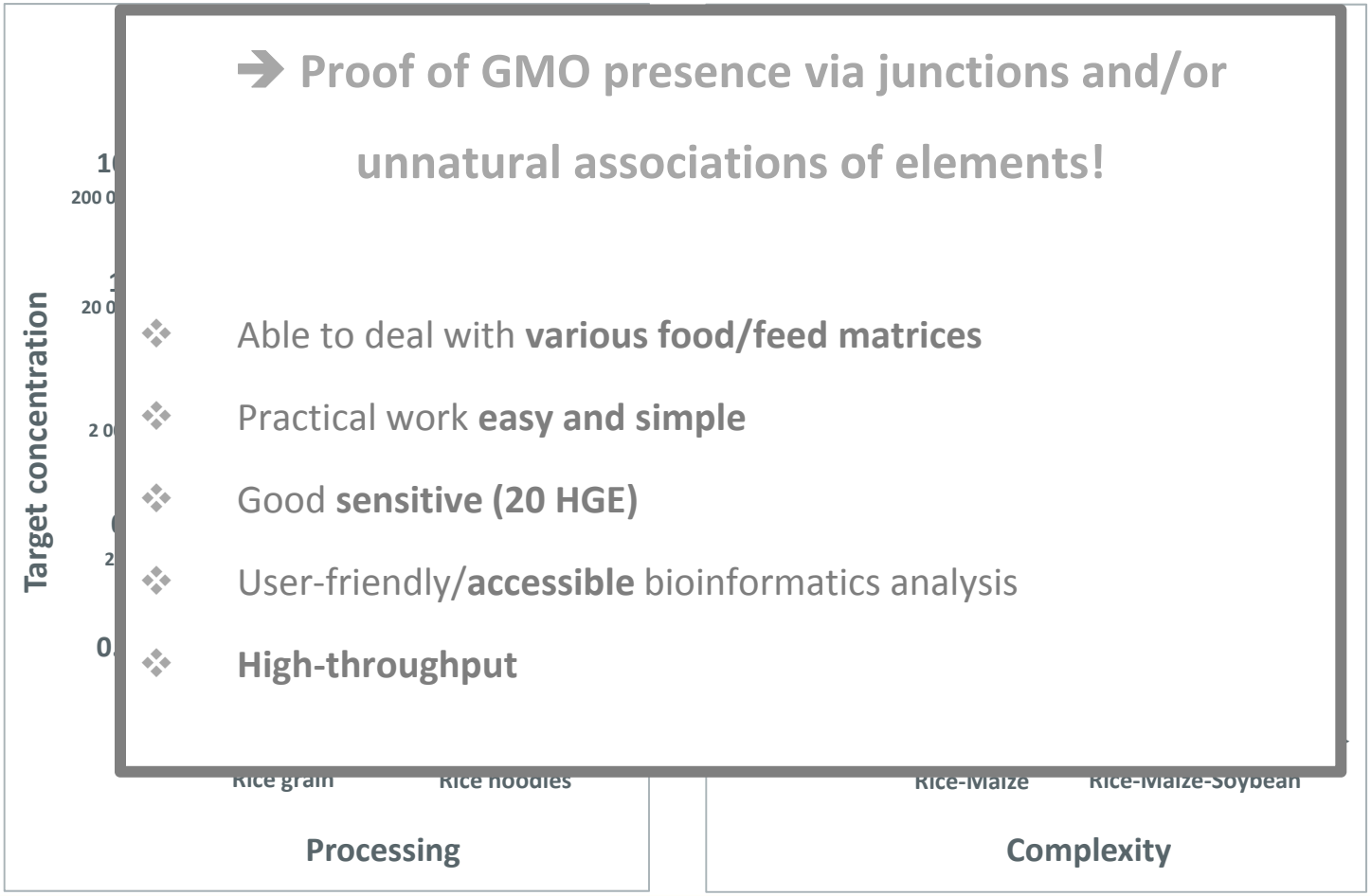
PACIFIC BIOSCIENCES

DNA walking coupled to PacBio

- Tests on typical food/feed matrices encountered in GMO routine analysis
  - 10 samples, individually barcoded, sequenced together in one run

➔ **Proof of GMO presence via junctions and/or unnatural associations of elements!**

- Able to deal with **various food/feed matrices**
- Practical work **easy and simple**
- Good **sensitive (20 HGE)**
- User-friendly/**accessible** bioinformatics analysis
- **High-throughput**



PACIFIC BIOSCIENCES

DNA walking coupled to PacBio

ISP WIV

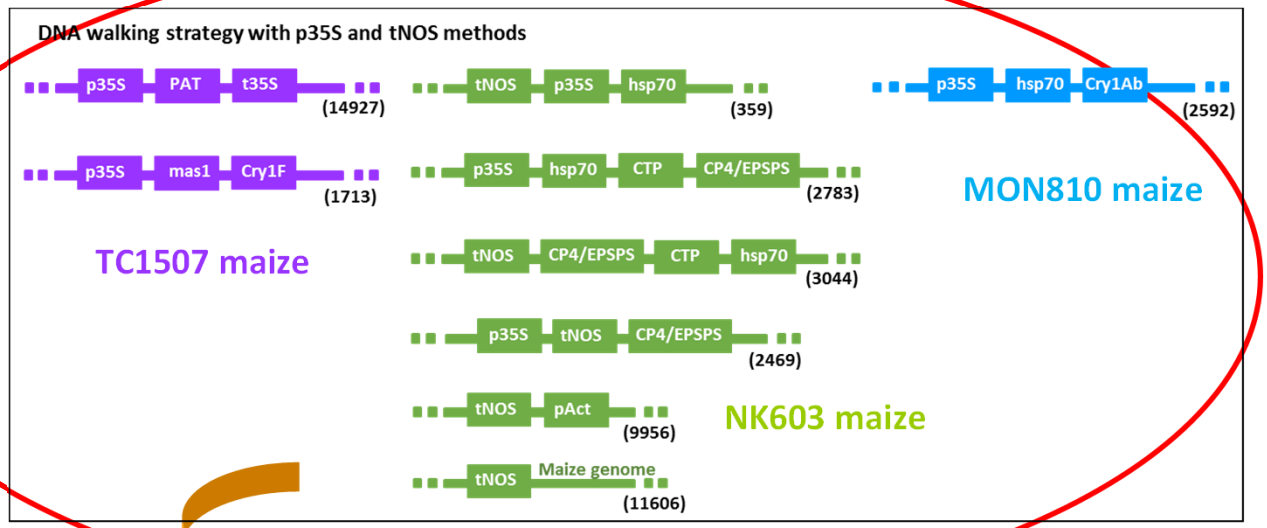
- Real-life sample (Kuwait)

Screening markers	Observed signals
PLD	- (C_t: 40; T_m: 70.8°C)
ADH	+ (C_t: 24; T_m: 75.4°C)
LEC	- (C_t: 31; T_m: 79.3°C)*
CRU	- (C_t: 40; T_m: 73.4°C)
p35S	+ (C_t: 26.3; T_m: 76°C)
tNOS	+ (C_t: 29.9; T_m: 71.8°C)
pFMV	- (C_t: 39.8; T_m: 74.4°C)
PAT	+ (C_t: 24.4; T_m: 76.3°C)
BAR	- (C_t: 40; T_m: 72.7°C)
CP4-EPSPS	+ (C_t: 28.3; T_m: 84.3°C)
Cry3Bb	- (C_t: 36; T_m: 80.6°C)*
t35S pCAMBIA	- (C_t: 40; T_m: 71.5°C)

*Trace

Possible to identify unauthorized GMO

Comparison to in-house database



Proof

All unnatural associations of elements and junctions correspond to authorized GMO

Verification via the standard GMO detection system

DSS

CoSYPS

Van den Bulcke et al., 2010

List of potentially detected GMO	
3272 maize	MIR162 maize
98140 maize	MON 810 maize
Bt11 maize	MON 87427 maize
DAS 40278-9 maize	MON 87460 maize
DAS59122 maize	NK603 maize
GA21 maize	T25 maize
MIR 604 maize	TC1507 maize

Identified GMO

TC1507 maize (C_t: 26)

NK603 maize (C_t: 30.6)

MON 810 maize (C_t: 39) <LOD

Bt11 maize (C_t: 39.7) <LOD

Not possible to identify unauthorized GMO

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An integrated strategy combining DNA walking and NGS to detect GMOs

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ABSTRACT

Recently, we developed a DNA walking system for the detection and characterization of a broad spectrum of GMOs in routine analysis of food/feed matrices. Here, we present a new version with improved throughput and sensitivity by coupling the DNA walking system to Pacific Biosciences® Next-generation sequencing technology. The performance of the new strategy was thoroughly assessed through several assays. First, we tested its detection and identification capability on grains with high or low GMO content. Second, the potential impacts of food processing were investigated using rice noodle samples. Finally, GMO mixtures and a real-life sample were analyzed to illustrate the applicability of the proposed strategy in routine GMO analysis. In all tested samples, the presence of multiple GMOs was unambiguously proven by the characterization of transgene flanking regions and the combinations of elements that are typical for transgene constructs.

1. Introduction

Concerns regarding the traceability of food and feed products in the market and the right of consumers to know the exact ingredients in the food and feed have led to the establishment of legislations concerning the introduction and control of genetically modified organisms (GMOs) in the food and feed chain. In enforcement laboratories worldwide, the presence of GMOs in food and feed matrices is routinely monitored using qPCR analyses. More precisely, the presence of GMOs is initially assessed by qPCR screening using a panel of methods that target a broad range of common GMO elements. This step, can also discriminate the presence of certain genetically modified (GM) events. From the positive and negative signals observed in these screening methods, a list of potential GM events present in the tested matrix is created, and the corresponding event-specific methods are then used to confirm their presence (Broeders, Papazova, Van den Bulcke, & Roosens, 2012; Fraiture, Herman, Taverniers, De Loose, Deforce, Roosens, 2015c). However, this system is not specifically designed to identify unknown GMOs. Indeed, in the situation where no correspondence is established between a set of positively confirmed known GMOs and the signals observed during screening, the presence of an unknown GMO can be inferred but remains to be proven using alternative methods. This is in part because several targeted screening elements are derived from natural organisms (e.g., p35S from cauliflower mosaic virus (CaMV) and tNOS from *Agrobacterium*) that may naturally be present in the tested sample. In addition, most of these screening elements are commonly found in both European Union (EU)-authorized and unauthorized GMOs, obscuring their independent detection. In fact, the explanation of observed qPCR screening signals with positive observations of known EU-authorized GMOs does not prove the absence of EU-unauthorized GMOs *per se* (Broeders, De Keersmaecker, & Roosens, 2012; Broeders, Papazova et al., 2012; Holst-Jensen et al., 2012; Ruttink et al., 2010).

To resolve this issue, an integrated DNA walking strategy was recently developed to strengthen the current qPCR system for the detection of EU-unauthorized GMOs (Fraiture et al., 2014; Fraiture, Herman, Taverniers, De Loose, Nieuwerburgh et al., 2015; Fraiture, Herman, Lefèvre et al., 2015). Following a positive

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Trends in Biotechnology

CellPress

Special Issue: Computation and Modeling

Opinion

How Can We Better Detect Unauthorized GMOs in Food and Feed Chains?

Marie-Alice Fraiture,[1] Philippe Herman,[1] Marc De Loose,[2] Frédéric Debode,[3] and Nancy H. Roosens[1,*]

Current GMO detection systems have limited abilities to detect unauthorized genetically modified organisms (GMOs). Here, we propose a new workflow, based on next-generation sequencing (NGS) technology, to overcome this problem. In providing information about DNA sequences, this high-throughput workflow can distinguish authorized and unauthorized GMOs by strengthening the tools commonly used by enforcement laboratories with the help of NGS technology. In addition, thanks to its massive sequencing capacity, this workflow could be used to monitor GMOs present in the food and feed chain. In view of its potential implementation by enforcement laboratories, we discuss this innovative approach, its current limitations, and its sustainability of use over time.

The Current GMO Detection System Makes it Difficult to Detect Unauthorized GMOs

To guarantee their safety and traceability in the food and feed chain as well as the freedom of choice for consumers, legislations regarding **GMOs** (see Glossary) have been established in several countries around the world. Most of these GMO legal frameworks aim notably at regulating the introduction of GMOs into the food and feed chain. To apply for an authorized introduction of a GMO, an applicant should carry out a case-by-case environmental risk assessment and provide information about accurate instructions and conditions for use and labeling. However, some legislation requirements, such as the labeling threshold (between 0.9% and 5%), vary among different jurisdictions. The term 'unauthorized GMOs' concerns GMOs released in the market of a certain country without prior authorization.

Two main categories of unauthorized GMOs may be found in the market. First, GMOs can be considered unauthorized because they have been approved in some countries (such as the USA, Canada, or Japan) but not yet in others [such as those in the European Union (EU)], a situation referred to as 'asynchronous approvals', or because their time-limited regulatory approval has expired and was not renewed [1]. However, this category of unauthorized GMOs could be considered to be safe because they were fully characterized and are traceable on the market using their official **event-specific methods**. By contrast, a more problematic scenario can also lead to the presence of unauthorized GMOs on the market: an accidental or deliberate release of 'experimental' GMOs from laboratories or field trials (e.g., Bt10 maize, Liberty Link 601 rice, FP967 flax, Bt63 rice, PRSV papaya, and MON71800 wheat) [2]. Unauthorized GMOs in this category usually have not received any regulatory approval in any country, so they could be considered to be 'unsafe' and 'unknown'. In addition, no or few event-specific or **construct-specific methods** are available to ensure their traceability in food and feed

Trends

Most European Union (EU)-unauthorized GMOs can be detected by enforcement laboratories via the current GMO detection system.

However, based on the current approach, distinguishing EU-unauthorized GMOs from EU-authorized GMOs is almost impossible.

One possible way to overcome this issue is a new workflow using NGS technology.

This new workflow may improve the ability of the current system to detect EU-unauthorized GMOs.

The high-throughput property of NGS technology, enabling the generation of massive amounts of sequence data from several samples in parallel, may also enable the monitoring of GMOs that are present in the food and feed chain.

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Concluding remarks

NGS provides possibilities to prove straightforward the presence of (U)GM in the sample

TGS in combination with DNA walking => promissing alternative of the current detection strategies allowing identification of UGM

The sequences obtained from this analysis allow development of new PCR methods to be applied for targeted screening for UGM =>implementable in routine GMO analysis

Implementation in GMO testing laboratories

- *Analysis still relatively expensive*
- *Choice of sequencing platform*
- *Adequate computer infrastructure*
- *Strong bioinformatics support*
- *Availability of sequences databases*

Scientific Institute of Public Health

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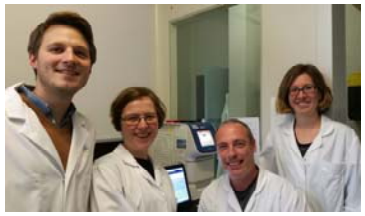
Dr. Nina Papazova
GMOlab: GMO analysis, NRL-GMO

Dr. Kevin Van Este
BioInformatics platform

4 bioinformaticians and 1 sofware engineer

Lab: Routine analyses GMO, Sanger sequencing, NGS, R&D



Loïc Lèfevre
Els Vandermassen
Dirk Van Geel
Maud Delvoye

Stefan Hoffman
NGS

R&D: 1 postdoc, 6 PhD students

Dr. Marie-Alice Fraiture,
DNA walking, detection of UGM

Thank you for your attention!