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Review

Biotech rice: Current developments and future detection challenges in food and feed chain

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ABSTRACT

Background: To improve agricultural practices and the food/feed security, plant breeding techniques were developed, including transgenesis commonly using *Agrobacterium tumefaciens* or biolistic technologies. To guarantee the traceability of GMO in food/feed chain and the consumer's freedom of choice, regulatory frameworks were established in many countries around the world, such as in Europe. Their implementations, including detection systems usually based on qPCR, are becoming complex and expensive regarding the number of analysis to perform. Moreover, the dispersion of publicly available information about developed GMO prevents to accurately estimate the efficiency of the standard detection system applied to unauthorized GMO.

Scope and approach: To illustrate this problem, the case of rice, one of the leading staple crops, was investigated. An overview of worldwide developed biotech rice generated by transgenesis was thus conducted, based on 1067 peer-reviewed publications, and analysed regarding *inter alia* their expressed genes of interest and the corresponding traits, their transformation processes and the elements composing their transgenic cassettes. From this work, the power and weakness of the standard detection system, notably used by the European enforcement laboratories, are evaluated. To strengthen this system, especially with unauthorized GMO, additional strategies are suggested. Moreover, given the growing interest for biotech rice produced by new plant breeding techniques, related challenges for their detection are discussed.

Key findings and conclusions: According to all collected information, suitable detection strategies, combining qPCR to additional technologies (e.g., DNA walking and NGS), are proposed to cover most of inventoried biotech rice. The present approach, including the data centralization to subsequently suggest appropriated detection strategies, can be extended to biotech events from different species.

1. Introduction

To cope with the challenge of increasing the crop production, the evolution of current agricultural practices was envisaged via the support of conventional breeding methods by contemporary approaches. Therefore, various strategies have been used to develop biotech crops, such as transgenic crops, also named genetically modified (GM) crops or genetically engineered crops, opening new possibilities to reach the expected crop nutritional necessity and to ensure food security (Ahmad et al., 2012; He, Xia, Peng, & Lumpkin, 2014). As observed for several crops, genetic engineering had *inter alia* been applied to rice (*Oryza sativa*), currently one of the most important cereal crops that is cultivated in many countries through the world. For more than 3.5 billion people, rice is a staple food providing more than 20% of their daily calories intake, especially in developing countries. In addition, this crop is also intended to feed animals. In 2014, the annual production of paddy rice was estimated at 741.3 million tons (corresponding to 494.4 million tons of

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milled rice) where the majority was grown in Asia (674.4 million tons; 91%), mainly in China (208.1 million tons), India (155.5 million tons), Indonesia (70.6 million tons), Bangladesh (52.4 million tons) and Vietnam (44.9 million tons). The rest of paddy rice is harvested in Africa (27.6 million tons), South America (24.8 million tons), North and Central America (12.9 million tons), Europe (4.1 million tons) and Oceania (0.9 million tons) (FAO, 2015; Fraiture et al., 2014; Khush, 2013). Therefore, even if no biotech rice is nowadays cultivated worldwide at a commercial scale, its potential significance is clearly obvious in the near future (De Steur et al., 2014).

With the aim to guarantee the traceability on the markets as well as the freedom of choice to the consumers, several genetically modified organism (GMO) labelling systems have been established in several countries with a threshold varying from 0 to 5%. The labelling is either mandatory (e.g., in Australia, Brazil, Chile, China, EU, India, Indonesia, Israel, Japan, Philippines, Russia, Saudi Arabia, South Korea, Taiwan and Thailand) or voluntary (e.g., in Argentina, Canada and USA). On the European Union (EU) market, commercialised food and feed products containing at least 0.9% of EU authorized GMO have to be labelled to guarantee the freedom of choice of the consumers while the zero tolerance is applied on unauthorized GMO (Davison, 2010; Kamle & Ali, 2013). The implementation of these legislations is mainly carried out using real-time PCR (qPCR) technology, allowing to detect, identify and quantify GMO (Fraiture, Herman, Taverniers, De Loose, Deforce et al., 2015). Three main steps are traditionally followed in GMO routine analysis. First, the presence of GMO is detected by screening. It includes simplex or multiplex methods targeting the most common transgenic elements, such as p35S (35S promoter from Cauliflower mosaic virus (CaMV)) and tNOS (nopaline synthase terminator from *Agrobacterium tumefaciens*). In addition, some more discriminative markers are used to reduce the number of subsequent identification. In case of positive signals, the identity and the quantity of GMO are afterwards determined via event-specific methods. If the signals observed during the screening step do not correspond to any of the authorized GM events, the presence of unauthorized GMO is then suspected (Broeders, De Keersmaecker, & Roosens, 2012, Broeders, Papazova, Van den Bulcke, & Roosens, 2012).

Given the ongoing and further expected expansion of GMO in terms of number, diversity and cultivated areas, the implementation of labelling legislations is becoming even more complex. Furthermore, the presence of some GM events could be prohibited or not according to the jurisdiction in reason of the asynchronous authorisations between many countries. Moreover, unlike the present commercialised GM crops which have been mainly developed by American and European companies, more and more GMO produced by national technology centres in developing countries are intended for local consumption. Consequently, these GM crops will probably not be submitted for EU approval. Therefore, the frequency of unauthorized GMO on the EU market is likely to significantly increase due to accidental contamination of non-transgenic raw material and processed food/feed matrices. In addition, unauthorized GMO concerns also GM crops that are currently unknown to the competent authorities (Broeders, De Keersmaecker, et al., 2012; Holst-Jensen et al., 2012; Parisi, Tillie, & Rodríguez-Cerezo, 2016; Stein & Rodriguez-Cerezo, 2009). The complexity of this problematic is particularly well illustrated by GM rice for which no events are nowadays authorized on the EU market. First, the problem of asynchronous approvals has been encountered with products originating from the USA. More precisely, the herbicide tolerant LLRICE601, in 2006, and LLRICE62, in 2007, both produced by Bayer CropScience and exclusively authorized in the USA, were identified in commercial rice matrices. Second, the insect resistant Bt Shanyou 63 and KeFeng-6 were found in food products originating from China in 2006 and 2010, respectively. These GM rice, produced by Asian research centres, were probably accidentally spread. Besides, the illegal propagation of seeds from field trials as well as their planting by Chinese farmers have been reported (Fraiture et al., 2014; Ruttink et al., 2010; Wang & Johnston, 2007; Wang, Zhu, Lai, & Fu, 2011). Finally, the presence of unknown GM rice was also reported by the RAPID Alert System Database (http://ec.europa.eu/food/safety/rasff/index_en.htm), allowing notably to notify the detection of unauthorized GMO on the EU market, such as in 2010 and 2011 in products imported from China, contaminated in all likelihood by accident (Fig. 1). Furthermore, still according to the RAPID Alert System Database, practically 50% of analysed food/feed samples between January 2012 and May 2015 contained unauthorized GM rice, including Bt63 and GM Basmati rice, imported from Asia, mainly China. Due to the high level of EU unauthorized GM rice, the EU commission has notably decided to implement "Emergency measures regarding unauthorized genetically modified rice in rice products originating from China and repealing Decision 2008/289/EC" (Commission Implementing Decision no. 2011/884/EU).

The success of the qPCR strategy is directly linked to the availability of information on the targeted sequences, such as from transgenic elements (element-specific markers), association of elements (construct-specific markers) or from junctions between the transgenic cassettes and the plant genomes (event-specific markers). In addition, the quantification of identified GM events requires the availability of Certified Reference Materials (CRM) and taxon-specific methods (Broeders, Papazova et al., 2012; Holst-Jensen et al., 2012). Conversely to EU authorized events for which all these data are accessible in the Compendium of reference methods for GMO analysis, the identification of EU unauthorized events by qPCR strategy could be difficult (Fraiture, Broeders et al., 2015). Indeed, the lack of centralized information about unauthorized transgenic crops is problematic. For instance, for GM rice, only three herbicide tolerant (LLRICE601, LLRICE06 and LLRICE62), four insect resistant (Bt63, Huahui-1, Tarom molaii and GM rice 101096), one fungi resistant (GM rice 101097), six multiple biotic stress resistance (NIA-OS002-9, NIA-OS012-8, NIA-OS004-8, NIA-OS003-1, NIA-OS005-3 and NIA-OS006-4), two abiotic stress resistance (S–C and As-d) and two rice seed edible vaccines against Cedar pollen allergy (7Crp#10 and OsCr11) are currently reported in publicly available GMO databases. Those GM rice lines are mainly described according to the expressed genes of interest and the corresponding traits, the transformation methods used, the transgenic elements contained in the vectors and the related biosafety information (Biosafety Clearing-House (https://bch.cbd.int/), Biosafety Scanner (http://en.biosafetyscanner.org/), CERA (http://www.cera-gmc.org/?action=gm_crop_database), GMO Compass (http://www.gmo-compass.org/eng/gmo/db/), GMO register (http://ec.europa.eu/food/dyna/gm_register/index_en.cfm)).

Therefore, in this review, after a brief description of the biotech rice history including the transformation technologies used, an overview of transgenic rice events developed through the world was built on the basis of more than 1000 available peer reviewed publications. All data were collected and analysed regarding notably the genes of interest expressed and their origins, the vectors and transgenic elements that composed them, the transformation technologies used and the status of the transgenic rice (laboratory development stage or field trial). In this way, the information on biotech rice centralized in this review can be used to complete publicly available databases as well as to develop and strengthen GMO detection strategies.

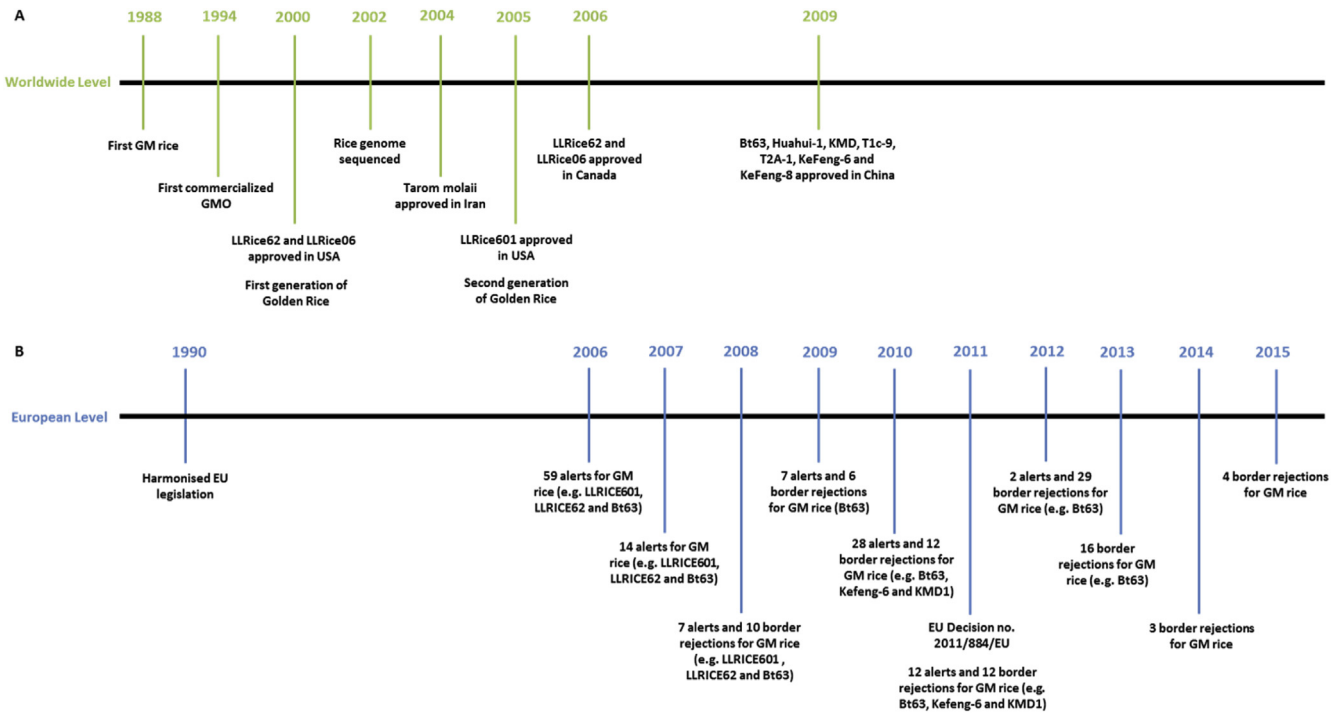

Fig. 1. Timeline of transgenic rice history at the worldwide level (A) and of transgenic rice impact on the EU market (B).

2. Biotech rice history

As efficient genetic engineering techniques and its genome sequence estimated at 430 Mb are available, rice represents currently a key crop model to develop biotech plants (Kathuria, Giri, Tyagi, & Tyagi, 2007). Since most of the biotech rice are generated using transgenesis techniques to date, this section concerns essentially transgenic rice, also called GM rice. However, due to the rise of biotech rice originated from new plant breeding techniques (NPBT), the sub-section 2.4 is entirely devoted to it. The rice crops generated by these promising alternatives are also named NPBT rice.

2.1. Transformation technologies

In 1988, the first transgenic rice plants were successfully developed by electroporation-mediated or polyethylene glycol-mediated protoplast transformation methods (Fig. 1). Based on electroporation and polyethylene glycol-mediated technologies, the fertility recovery of transgenic rice was then respectively reported in 1989 and 1990 (Hiei, Komari, & Kubo, 1997; Kathuria et al., 2007).

In 1991, the biolistic transformation method, also named particle or microprojectile bombardment, was successfully used to generate transgenic rice plants. This direct DNA delivery system is often characterized by integration in multiple copies of transgenes into the recipient genome. Moreover, the transgenic rice plants present the risk of an unstable and aberrant expression of the gene of interest because of its likely fragmentation and rearrangement induced by this transformation technology (Hiei et al., 1997; Hoque, Mansfield, & Bennett, 2005; Kathuria et al., 2007).

Although *Agrobacterium tumefaciens*-mediated transformation is used since the 1980s, its application, initially limited to dicotyledonous plants, was slightly modified to be adapted on monocotyledonous plants such as rice (Hiei et al., 1997; Hoque et al., 2005). In 1990, the first transgenic rice calli were produced after application of the *Agrobacterium*-mediated transformation method. Afterwards, transgenic rice plants were regenerated from Agrobacterium-transformed calli (Hiei et al., 1997; Kathuria et al., 2007). To improve this strategy, several factors affecting the transformation efficiency have been studied. For instance, the activation of the T-DNA transfer process is promoted via the addition of phenolic compounds, such as acetosyringone, a potent inducer of virulence genes participating to the recognition of the host by *Agrobacterium*. Moreover, the bacterial strains and vectors used are decisive, particularly with recalcitrant rice genotypes such as some Indica cultivars. The choice of the competent rice tissue (age, cell type, cell cycle stage) has been also highlighted as a key parameter. In addition, the culture conditions are determining in the success of the transformation. Indeed, instead of kanamycin and G418 antibiotics, hygromycin B is preferably used as an antibiotic during the selection step of transformed rice as no natural resistance to hygromycin is present in rice. In addition, this antibiotic does not impact the transgenic rice regeneration and fertility (Hiei et al., 1997). Even if food/feed and environmental safety concerns were raised in the past, to date only theoretical assumptions without evidence-based arguments have demonstrated any significant impact of antibiotic resistance marker genes related to the potential risk of horizontal gene transfers from transgenic plants to neighbouring bacteria and plants as well as a potential increase of antibiotic resistance among consumers. Anyway, partly due to regulatory requirements in the EU for phasing-out antibiotic resistance marker genes such as npt II in GMO, several strategies have been developed to remove microbial selection markers from transgenic plants (Breyer, Kopertekh, & Reheul, 2014; Hiei et al., 1997). One alternative is to replace the microbial selection markers by vegetal selection markers that naturally confer an antibiotic resistance or by herbicide tolerance genes. In addition, other marker genes can be used instead of the classical microbial markers via three main strategies. First, the positive selection marker approach confers a metabolic or developmental advantage to the transformed cells without implying the death of

untransformed cells. Second, just the opposite, the negative selection marker approach leads to a metabolic or developmental disadvantage in the transformed cells. Third, the reporter genes allow to visually select transgenic plants (Breyer et al., 2014; Hiei et al., 1997). In order to remove definitely the selection markers in the selected transgenic plants, several techniques, based on the site-specific recombination (e.g., Cre/loxP and MAT), the intra-chromosomal homologous recombination, the intra-genomic transpositional mechanism (e.g., Ac/Ds transposable element) or the segregation via co-transformation, have been performed. This last approach is the simplest and widely used method that involved the introduction of two T-DNA regions, one with the gene of interest and one with the selection marker gene, allowing to subsequently generate transgenic plants presenting only one T-DNA by sexual crossing in successive generations (Breyer et al., 2014).

Unlike to direct DNA delivery transformation system, Agrobacterium strategy generates transgenic plants presenting a more predictable pattern of integration and a non-rearranged segment of DNA being inserted into the genome at a low copy number (Hoque et al., 2005). However, although the *Agrobacterium tumefaciens* strategy has generated progress in crop biotechnology, the less-known rhizosphere bacterium *Ensifer adhaerens* represents a potential alternative as it has been used to transform plants including Arabidopsis and potato. Besides, this approach has recently been applied to rice (Wendt, Doohan, & Mullins, 2012; Zuniga-Soto, Mullins, & Dedicova, 2015).

2.2. Currently developed transgenic rice

2.2.1. Data collection

As observed for other transgenic crops, the number and the diversity of transgenic rice is significantly increasing with time. Therefore, a large inventory of the currently developed transgenic rice has been carried out allowing to provide crucial information to enforcement laboratories in order to detect and identify them. This kind of strategy was previously suggested notably regarding the detection of unauthorized GMO (Ruttink et al., 2010). Based on the analysis of available online databases (Biosafety Clearing-House, Biosafety Scanner, CERA, GMO Compass, GMO register) as well as 1067 peer-reviewed articles published in 242 different journals, a list of transgenic rice was drawn up. Initially, the scientific literature was reviewed using the Scopus database (www.scopus.com/home.url) to cover the period from January 1991 to October 2015 with the coupled keywords “transgenic rice” (the search with the keywords “Genetically Modified Rice” or “GM Rice” provided far fewer data). In a second step, the relevant scientific literature was selected based on several keywords, including “herbicide”, “insect”, “resistance”, “tolerance”, “stress”, “abiotic”, “biotic”, “bacteria”, “virus”, “fungi”, “pathogen”, “drought”, “salt”, “salinity”, “cold”, “disease”, “deficiency”, “heat”, “metal”, “chill”, “oxidative”, “iron”, “water”, “submergence”, “yield”, “grain”, “biomass”, “size”, “fortification”, “nutritional”, “texture”, “carotene”, “vaccine”, “allergen”, “pharmaceutics”, “health”, “therapy”, “inflammation”, “immunogenicity”, “microbe”, “improvement” and “development”. Furthermore, all pertinent peer-reviewed publications adjacent to the selected scientific literature were analysed (Additional file 1: Inventory of transgenic rice).

All the identified transgenic rice were recorded in an excel file according to their trait (one kind of trait per excel sheet), including herbicide tolerance, insect resistance, bacteria resistance, fungi resistance, virus resistance, multiple biotic stress resistance, abiotic stress resistance, abiotic and biotic stress resistance, grain yield improvement, nutritional quality improvement, pharmaceutical production and other innovations. Each transgenic rice was described in so far as possible in terms of rice variety, gene of interest expressed, donor organism of the gene of interest, vector used, transgenic elements used, transformation method used, knowledge level (KL) classification (ENGL ad hoc working group on “unauthorised GMOs”, 2011), status of the transgenic rice (laboratory development stage or field trial), year of publication, developer country and references of the related peer-reviewed publications. Moreover, if necessary, more details on the expressed traits were added (Additional file 1).

2.2.2. Geographical distribution

In order to have a worldwide picture of the developed transgenic rice, the geographical distribution of all inventoried developments described in peer-reviewed publications was investigated. Although some transgenic rice are developed in American (11.5%), European (8.9%), Oceanian (1.1%) and African (1%) laboratories, the majority of these research and development (R&D) studies is performed in Asia (77.5%), especially in China (47.8%) and Japan (20.2%) (Fig. 2).

2.2.3. Developed traits

Among all inventoried transgenic rice from all analysed articles and databases, most of the them have only been tested at the laboratory level (75.4%), but a significant amount (20.6%), approved or not for commercial cultivation, have already been subject to field trials. It should be noted that these data were not available for 4% of the listed transgenic rice. Concerning the traits identified in all inventoried transgenic rice, herbicide tolerance (6.1%) and insect resistance (20.3%), as found in the first transgenic rice lines approved for commercialisation, are still observed. Nevertheless, these traits are less developed in the course of time (Fig. 3 and Additional file 2A-B). In fact, progressively, more and more transgenic rice present at the R&D step display a high diversity of new traits such as the resistance to biotic stress (17.2%), in targeting one specific category of phytopathogens (bacteria (5.8%), fungi (7.1%) or virus (4.4%)) as well as more than one category of phytopathogens (2.1%) (Fig. 3). Moreover, the development of transgenic rice resisting to several abiotic stress (30.2%, e.g., salinity, drought, cold and heavy metal) is increasing over time (Fig. 3 and Additional file 2C). Some transgenic rice present also a resistance to both biotic and abiotic stresses (1.5%). In addition, some R&D centres focus their energy to improve directly the yield of grains (4.5%) (Fig. 3). The introduction of new traits in rice could also improve the consumer's quality of life in terms of nutrition and health. To this end, the modification of the rice grain nutritional composition (8.5%), including micronutrients (e.g., iron, zinc, manganese), vitamin (e.g., A and B9/folate) and essential amino acids for biofortification, is an auspicious strategy (Fig. 3). This is actually the case with the folate biofortification which could prevent some birth defects. To reach the minimum daily level in folate, between 137 g and 281 g of biofortified rice have to be consumed (De Steur et al., 2014). A second well-known example is the Golden Rice Project, rewarded with the “Patents for Humanity Award” in 2015, with the aim to develop transgenic rice with β-carotene biofortified grain in order to struggle against the vitamin A deficiency leading notably to blindness in children (Golden Rice Project (http://www.goldenrice.org/)). Following to several improvements from the first generation of golden rice, the second generation of golden rice, transformed with a maize (*Zea mays*) phytoene synthase (psy) gene and a bacterial (*Erwinia uredorova*) phytoene desaturase (crt1) gene, yields a higher β-carotene concentration. The consumption of 77 g and 122 g of golden rice, by respectively young children and pregnant women, provides the minimum daily intakes in vitamin A (De Steur et al., 2014; Paine et al., 2005). After three seasons of field trials in the Philippines, the grains from the Golden Rice event R (GR2-R) presented the expected beta-carotene level. However, as the

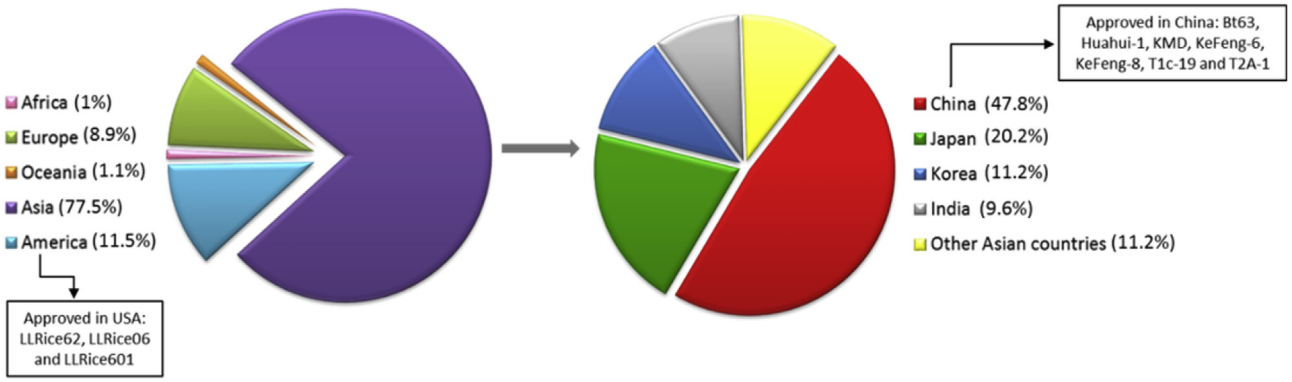

Fig. 2. Contribution (%) of the five continents to the transgenic rice development in term of peer-reviewed publications, including a zoom on the Asian countries.

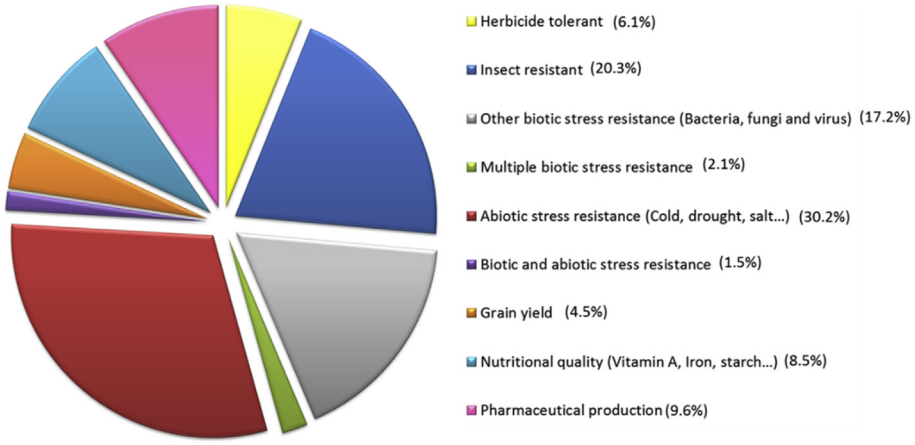

Fig. 3. Observed traits (%) in the inventoried transgenic rice.

average yield was inferior to the local varieties, new assays will be carried out with other Golden Rice versions such as GR2-E (James, 2014). In addition, transgenic rice are developed in the pharmaceutical field (9.6%) to produce cytokines, vaccines, antibodies, albumin or other therapeutic proteins (Fig. 3). Molecular farming of rice is envisaged to treat several critical health conditions such as allergy, autoimmune disorders, infectious diseases or even Alzheimer disease (Azegami, Itoh, Kiyono, & Yuki, 2014). Among the 1673 transgenic rice listed in the current study, 413 of them are directly involved in fundamental research (e.g., study of development, flowering, signalling) or in the implementation of new strategies such as optimisation of the transformation protocol and the production of marker-free transgenic rice.

In order to give rise to all these new properties in transgenic rice, a broad-spectrum of different gene families have been identified for each trait, including 16 gene families for herbicide tolerance, 23 gene families for insect resistance, 25 gene families for bacterial resistance, 41 gene families for fungi resistance, 13 gene families for virus resistance, 155 gene families for abiotic stress resistance, 29 gene families for grain yield improvement, 35 gene families for nutritional grain quality improvement and 62 gene families for drugs production (Table 1).

2.2.4. Transformation vectors

Among all inventoried transgenic rice (1673 transgenic rice), approximately 200 different vectors were used for their transformation. The *Agrobacterium tumefaciens*-based method was predominantly used (75.7%; 1267 transgenic rice) compared to the biolistic one (13.5%) or other methodologies (2.6%). For 8.2% of the inventoried transgenic rice, the transformation method was not mentioned. Regarding more precisely the vectors used, most of inventoried transgenic rice (36.9%, 617 transgenic rice) were transformed using the family vector pCAMBIA. Based on all analysed articles published from 2001, the use of the pCAMBIA vector has increased over time to reach a plateau of around 30% from 2004 to 2013. Since 2014, the use of this vector has even increased as more than half of the described transgenic rice was transformed with a pCAMBIA cassette (Fig. 4). It was already shown in 2007 that 30% of transgenic plants have been developed using vectors from the pCAMBIA family (Fraiture et al., 2014). Although the pCAMBIA family vector has been mainly used, transgenic rice collected in the present bibliographical study were also transformed with other vectors such as pBI (3.7%), pIG (2.5%), pSB (2.1%), pANDA (2%), pCIB (1.8%), pBIG (1.7%), pGA (1.6%), pGPTV (1.1%) and pPZP (1%). For the rest of the inventoried transgenic rice, 30.5% of them presented a large variety of vectors only anecdotally used. It should also be mentioned that 15.1% of inventoried transgenic rice were transformed with vectors for which their name were not properly identifiable. However, for these transgenic rice with unnamed vectors, information about the elements composing their transgenic cassettes was available for 6% of them.

2.2.5. Elements found in transgenic rice

In order to collect more information about all inventoried transgenic rice (1673 transgenic rice), the elements composing their transgenic cassettes were studied. Similarly to EU-authorized

Table 1
Examples of gene families used to acquire new proprieties in transgenic rice.

Traits	Gene families	References
Herbicide tolerance	Acetolactate synthase (Als)	Endo, Shimizu, & Toki, 2012
	Bialophos resistance (Bar)	Christou, Ford, & Kofron, 1991
	Protoporphyrinogen oxidase (PPO)	Chun et al., 2013
	5-enolphyruvylshikimate-3-phosphate synthase (EPSPS)	Zhao, Lin, & Shen, 2011
	Glutathione *S*-transferase	Hu, Qu, Xiao, & Huang, 2009
	Cytochrome P450	Ohkawa & Ohkawa, 2002
	atrazine chlorohydrolase	Zhang et al., 2014
Insect resistance	Cry	Breitler et al., 2004
	GNA lectin	Sudhakar et al., 1998
	ASAL lectin	Yarasi, Sadumpati, Immanni, Vudem, & Khareedu, 2008
	Trypsin inhibitor	Su et al., 2011, Yang et al., 2013
	Potato proteinase inhibitor II (PINII)	Bhutani et al., 2006
Bacteria resistance	Receptor kinase-like protein	Afroz, Qureshi, Zahur, Rashid, & Rashid, 2012
	WRKY transcription factor	Liu, Bai, Wang, & Chu, 2007
	Thaumatin-like protein	Shah, Singh, & Veluthambi, 2013
	Ferredoxin-like amphipathic protein	Tang et al., 2001
Fungus resistance	Chitinase	Sridevi, Parameswari, Sabapathi, Raghupathy, & Veluthambi, 2008
	Pi-d2	Chen et al., 2010
	Defensin	Kanzaki et al., 2002
	AFP antifungal protein	Coca et al., 2004
	ACC synthase	Seo et al., 2011
	Cecropin	Coca et al., 2006
Virus resistance	Coat protein	Sivamani et al., 1999
	Spike protein	Chaogang et al., 2003
	Replicase	Verma, Sharma, Devi, Rajasubramaniam, & Dasgupta, 2012
Abiotic stress resistance	Stress associated protein	Ben Saad et al., 2012
	D1-pyrroline-5-carboxylate synthetase	Hien et al., 2003
	Na^+/H^+ antiporter	Ohta et al., 2002
	Basic region/leucine zipper transcription factor	Tang N et al., 2012
	Aquaporin	Ayadi et al., 2014
	Disulfide isomerase-like protein	Chen, Pan, et al., 2012, Chen, Wang, et al., 2012
Grain yield	C4 pyruvate orthophosphate dikinase	Fukayama et al., 2001
	Cytosolic dehydroascorbate reductase	Kim et al., 2013
	Phytochome	Garg et al., 2006
Nutritional grain quality	Phytoene synthase (PSY)	Ye et al., 2000
	Carotene desaturase (crt)	Ye et al., 2000
	Ferritin	Masuda et al., 2012
	Waxy	Terada et al., 2000
	ScAcr3p	Duan, Kamiya, Ishikawa, Arao, & Fujiwara, 2012
Pharmaceutical products	Major T-cell epitope	Suzuki, Yang, & Takaiwa, 2012
	Lactoferrin	Humphrey, Huang, & Klasing, 2002
	Lysozyme	Humphrey et al., 2002
	Stilbene synthase	Baek et al., 2014
	Human serum albumin	He et al., 2011
	Major outer membrane protein	Zhang et al., 2008

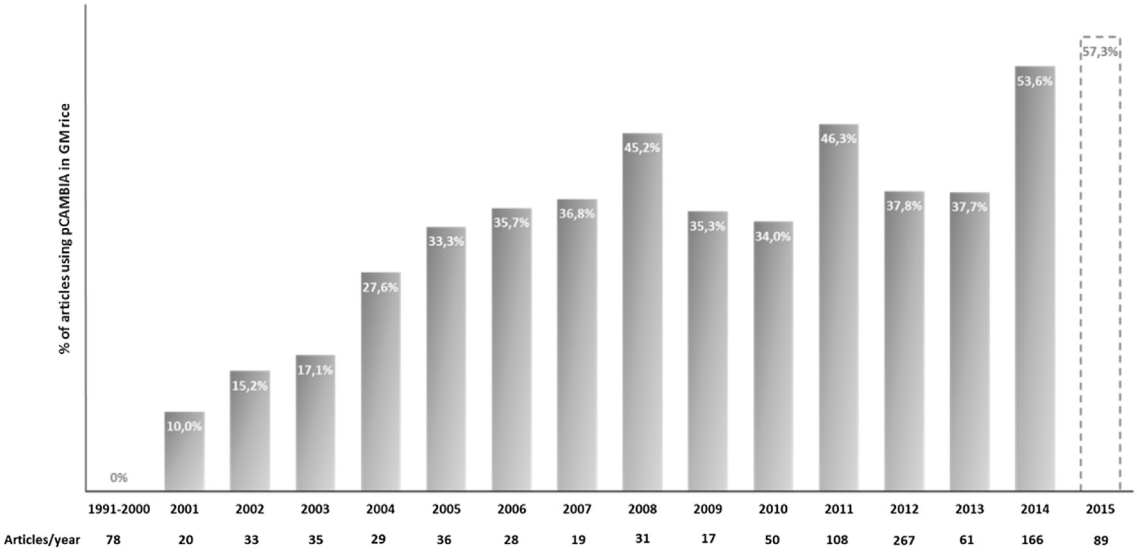

Fig. 4. Percentage (%) of analysed peer-reviewed publications, by year, presenting the use of pCAMBIA vector to generate transgenic rice. The number of articles recorded per year is indicated below the corresponding period. Since the current year of 2015 is not entirely covered, the corresponding bar chart is built in dotted line.

GMO (GMO Register), the collected transgenic rice contain frequently the p35S promoter (69.8%, 1167 transgenic rice) and the tNOS terminator (62.6%, 1048 transgenic rice). Furthermore, some transgenic elements were often found in the collected transgenic rice, such as the UBI promoter (27.6%), the NOS promoter (8.4%), the ACT promoter (5.4%), the terminator t35S (38.5%; from pCAMBIA family vector or not), the PINII terminator (3.3%) and the GUS gene (18.4%). For the elements intended for the plant selection, the hygromycin B (54%) and kanamycin (9.5%) resistance genes (respectively *hph* and *npt II*) are mainly used. Among the 185 different transgenic elements identified, 175 different transgenic elements are only occasionally observed. It should also be mentioned that no transgenic element was identified for 11.1% of inventoried transgenic rice, including 9.1% of them transformed with an unnamed vector.

2.3. Commercialisation of transgenic rice

Since the first approved GMO for commercialisation (Flavr-Savr™ tomato) in 1994 in the United States, in 1995 in Canada and in Mexico and in 1997 in Japan, as well as the first significant transgenic crop cultivation surface reported in 1996, 181.5 million hectares of transgenic plants in 28 countries have been planted in 2014. This represents an increase of more than 100 fold from the 1.7 million planted hectares in 1996 (Fig. 1) (CERA, James, 2014). As rice is one of the most important crops, many transgenic lines have been developed to improve agricultural productivity. In 1999, Liberty Link® (Bayer CropScience) rice varieties LLRICE06 and LLRICE62 were approved for release into the environment in the United States. These transgenic rice contains the bar gene from *Streptomyces hygroscopicus* in one complete copy (LLRICE62) or in several complete and partial copies (LLRICE06). This gene, encoding for phosphinotricin-*N*-acetyltransferase (PAT), confers herbicide tolerance by catalysing the conversion of the active form of glufosinate ammonium (L-phosphinotricin) in its inactive form. In 2000 and 2006, these two transgenic rice were notably then respectively approved for food/feed use in the USA and Canada (CERA). Between 1998 and 2001, another herbicide tolerant Liberty Link® rice (LLRICE601), expressing also the bar gene, has been subjected to field trials in order to be approved for release into the environment in 2006 in the USA (CERA, Quirasco, Schoel, Chhalliyil, Fagan, & Gálvez, 2008). However, none of these Liberty Links® rice lines have been planted in any country for commercialisation (Cao et al., 2011; De Steur et al., 2014).

In 2004, an insect resistant rice (Tarom molaii), containing the Bt gene Cry1Ab was approved for commercialisation in Iran (Fig. 1). However, this authorisation was suspended by the National Biosafety Council of Iran in 2005 due to an inter-ministerial lack of consultation in order to assess the dossier (Biosafety Scanner, De Steur et al., 2014).

In 2009, after several field trials in 2008 in collaboration with China National Rice Research Institute and Food and Environmental Safety Assessment, China's Ministry of Agriculture has delivered biosafety certificates for commercial production of two rice cultivar Minghui 63 lines (Bt Shanyou 63, also called Bt63 or TT51-1) and Huahui-1 in Hubei province. These insect resistant transgenic rice were previously tested in controlled field trials between 1999 and 2000, approved for environmental release between 2001 and 2002 and two preproduction field trials were performed between 2003 and 2004. These Bt rice contain the fused Cry1Ab/Cry1Ac genes, which confer insect resistance, under the control of the rice actin 1 gene promoter (pAct1) and NOS terminator (tNOS) (Cao et al., 2011; Chen, Shelton, & Ye, 2011, Lu C, 2010). Compared to the WT variety, these Bt rice present a superior yield of 6–9% with a decreased use of pesticides of 80% (He Z. et al., 2014). Concerning the potential commercialisation of transgenic rice, two insect resistant (Kemingdao 1 (KMD1; cv. Xiushui 11) and B827) and one bacterial resistant (Xa21) lines have already been submitted for regulatory assessment in China (Babekova, Funk, Pecoraro, Engel, & Busch, 2009; De Steur et al., 2014). Besides, in 2009, five insect resistant rice lines (KMD, T1c-19, T2A-1 and KeFeng variety (6 and 8 which both contain Cry1Ac and SCD genes)) have been approved by the National Biosafety committee of China, following preproduction tests, without any biosafety delivered certificate (Fig. 1) (Chen et al., 2011).

2.4. New plant breeding techniques applied to rice

In 1990, a harmonised EU legal framework regulating the deliberate release of GMO has been established (Directive 90/220/EEC). Even if this legislation has been revised and updated overtime, GMO are still defined as organisms “in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination” (Directive 2001/18/EC, Regulation (EC) n° 1829/2003). Given that this definition depends on process-based approaches used to produce GMO, the status of some NPBT, whether or not they are generating plants which fall into the scope of the EU GMO legislation, is still under discussion. These techniques, not technically achievable in the 90's, are *inter alia* the gene editing technology using zinc finger nucleases (ZFN) or oligonucleotide directed mutagenesis (ODM). More recently, other gene editing techniques were developed relying on meganucleases, transcription-activator like effector nucleases (TALEN) and the CRISPR/Cas system. In addition, cisgenesis, intragenesis, RNA-dependent DNA methylation (RdDM), grafting (non GM scion on GM rootstock or the opposite), reverse breeding and agro-infiltration (true agro-infiltration, agro-inoculation or infection and floral dip) are considered as belonging to the NPBT. Even if the status of new crop varieties developed with NPBT is still undefined in the EU, decisions regarding some products obtained through NPBT have already been taken in Argentina, Australia, New Zealand and the USA (Araki, Nojima, & Ishii, 2014; Lusser, Parisi, Plan, & Rodríguez-Cerezo, 2011; Pauwels, Podevin, Breyer, Carroll, & Herman, 2014). In the coming years, the use of these NPBT could become widespread. Commercial breeders have already adopted some of these approaches towards the production of the first potential commercialised next-generation biotech crop. Research in this field is being conducted for crops such as herbicide tolerant oilseed rape and maize, herbicide tolerant and insect resistant cotton, fungal disease resistant potatoes, drought stress tolerant maize, scab disease resistant apple and reduced amylose content potatoes. In addition, more traits and/or crops developed by companies have still not been disclosed (Lusser et al., 2011; Wolt, Wang, & Yang, 2015).

Via some of these NPBT, new rice varieties presenting an agronomic interest have also been developed. At our knowledge, these studies, carried out essentially in the USA (27.3%), China (36.4%) and Japan (36.4%), concern up to now only seven NPBT rice that present either an herbicide tolerance, a bacterial resistance, an abiotic stress resistance or a nutritional benefit. First, regarding the herbicide tolerance, the mutation of two amino acids (W548L, tryptophan to leucine, and S627I, serine to isoleucine) in the rice acetolactate synthase (ALS) gene generated by gene targeting via homologous recombination has allowed to provide a bispyribac-sodium (BS) herbicide hyper-tolerant rice line. Compared to BS tolerant plant from conventional breeding, the level of BS herbicide tolerance was superior. This rice line used as feed has also an additional nutritional value since these mutations have increased the branched-chain amino acid content that are not synthesized by animals (Endo et al., 2007, 2013). The tolerance to BS herbicide has also been

acquired in rice via the biolistic introduction of chimeric RNA/DNA oligonucleotides to induce specific mutations (Pro171, Trp548 and Ser627) in the ALS gene (Okuzaki & Toriyama, 2004). By combining the TALEN technology to the chimeric RNA/DNA oligonucleotides strategy, the development of transgenic rice with a tolerance to glyphosate herbicide has been attempted by the replacement of the base C^{317} by a base T inside the OsEPSPS gene (Wang et al., 2015). Second, biotech rice with a resistance to bacterial blight stress was developed via the TALEN-mediated mutation of the natural binding site of the *Xanthomonas oryzae pv. Oryzea* TAL effector AvrXa7 or PthXo3 localised upstream of the Os11N3 (OsSWEET14) gene (Li, Liu, Spalding, Weeks, & Yang, 2012). Third, the abiotic stress tolerance of rice was also investigated. Based on the CRISPR/Cas9 system, several genes were successfully targeted in rice, including OsDERF1, coding for the AP2 domain containing protein, that is implied in the drought stress resistance (Zhang, Chen, et al., 2014, Zhang, Zhang, et al., 2014). Finally, NPBT have allowed to improve the nutritional quality of rice. Indeed, the TALEN technology has been used to create fragrant rice from a rice variety devoid of this propriety following to a targeted knockout of OsBADH2 gene (Shan et al., 2013, 2015). Moreover, precise mutations were introduced by gene targeting based on homologous recombination in the OASA2 gene, coding for an α-subunit of anthranilate synthase that is involved in rice tryptophan biosynthesis. Compared to non-transformants, mature seeds from the obtained rice line present a higher accumulation of tryptophan without any phenotypic modifications. At the nutritional value level, these tryptophan fortified rice plants represent an interesting benefit in both human and livestock diets (Saika et al., 2011).

3. Detection methods targeting biotech rice and correlated challenges

In order to assess existing GMO detection strategies regarding all collected biotech rice, several DNA-based methods were investigated. By this way, the related benefits and difficulties in the detection of biotech rice originating from transgenesis or NPBT are discussed.

3.1. Transgenic rice detection

3.1.1. Knowledge level classification

In 2011, the European Network of GMO Laboratories (ENGL) *ad hoc* working group "Unauthorized GMOs" suggested a GMO classification, based on the knowledge level (KL) about the sequence of the insert. Given that the availability of this information is crucial for DNA-based GMO detection analysis, the inventoried transgenic rice for which the elements composing their transgenic cassettes are known, representing 1487 transgenic rice, were classified according to the four categories of this KL classification system, ranging from KL-1 to KL-4. For GMO from the KL-1 category, the DNA sequences from the inserts and the transgene flanking regions are known. After detection by element-specific and construct-specific markers in qPCR screening analysis, their identification is thus carried out using event-specific methods. Among all listed transgenic rice, only 0.2% (LLRICE62, LLRICE601 and LLRICE06) belong to this category. Concerning GMO from the KL-2 category, their transgenic cassettes are identical to GMO from the KL-1 category. However, their transgene flanking regions are unknown. Therefore, even if their presence could still be detected using element-specific and construct-specific methods, no event-specific method allows to identify them. In this work, no collected transgenic rice was associated to the KL-2 category because their genetic constructs do not correspond to the ones found in fully characterized GMO from the KL-1 category. For the KL-3 category, GMO present a transgenic cassette where at least one known transgenic element is found in GMO from the KL-1 category. In addition, their transgene flanking regions are unknown. The majority of the listed transgenic rice (97.6%; 1451 transgenic rice) was classified in this KL-3 category. Within this KL category, the transgenic rice present frequently the p35S promoter and/or the tNOS terminator (93%, 1383 transgenic rice). More precisely, 22.5% (335 transgenic rice), 14.5% (216 transgenic rice) and 56% (832 transgenic rice) of the listed transgenic rice contain respectively only the p35S element, only the tNOS element or both of these elements. Therefore, the transgenic rice from the KL-3 category could be detected using element-specific methods targeting notably these common transgenic elements. However, similarly to GMO from the KL-2 category, no event-specific method makes their identification feasible. Among the KL-3 category, transgenic rice approved for commercialisation in some parts of the world, including Huahui-1, TT51-1, KeFeng-6, KeFeng-8, KMD1, T1c-19 and T2A-1, were notably reported. Finally, GMO assigned to the KL-4 category are only transformed with novel genetic elements and their transgene flanking regions are unknown, making their detection impossible with conventional qPCR approaches. However, only thirty-three transgenic rice (2.2%) corresponded to this criterion.

3.1.2. Detection strategies

As mentioned before, classically, three steps are successfully applied in GMO routine analysis through qPCR, the gold standard technology.

First, the presence of GMO is detected in the screening phase using element-specific and construct-specific markers. Several screening markers have besides been successfully tested on transgenic rice approved for commercialisation (e.g. LLRice601, LLRice62, TT51-1, KeFeng-6 and KMD1) as well as other transgenic rice lines (e.g. Bt rice and KMD2) (Table 2) (Akiyama et al., 2007; Fraiture et al., 2014; Gu, Wu, Li, Li, & Yang, 2009; Kluga et al., 2012; Made, Degner, & Grohmann, 2006; Quirasco et al., 2008; Reiting, Grohmann, Moris, & Mäde, 2013). As most of the inventoried transgenic rice for which information about the elements composing their transgenic cassettes is available belongs to the KL-1, KL-2 and KL-3 groups (97.8%, 1454 transgenic rice), existing screening markers allow to target them (see sub-section 3.1.1) (Fig. 5). Although the collected information regarding the transgenic elements is based on the name and not on the sequence (see sub-section 2.2), and consequently must be used with caution, this allows to estimate the efficiency of the current qPCR GMO detection system to target EU-unauthorized GMO. By this way, 93% of inventoried transgenic rice are covered in a first line through a minimum set of two screening markers targeting the highly frequent p35S and tNOS elements (see sub-section 3.1.1). In order to increase the coverage, six additional screening markers are proposed to be applied after the initial qPCR screening using the p35S and tNOS markers. More precisely, given that many inventoried transgenic rice contained the t35S element (from the pCAMBIA family or not), the corresponding screening markers were also selected to be applied in a second line. In using two screening markers, which one is specific to the t35S element from the pCAMBIA family vector while the other one targets all t35S elements not originating from the pCAMBIA family vector, a coverage of 94.8% is reached. Among the rest of inventoried transgenic rice belonging to the KL-3 category without possessing at least one of these elements (3%), 1.7% of them contained the Cry1Ab and/or the Cry1Ac elements. Therefore, the use of the Cry1Ab/Ac screening marker in third line allows to cover 96.5% of inventoried transgenic rice. Concerning the remaining 1.3% of collected transgenic rice for which the detection is still possible, the pUBI (0.8%), pNOS (0.1%) or Bar (0.4%) elements were observed in their transgenic cassettes.

Table 2
Representative examples of qPCR detection methods tested on transgenic rice approved for commercialisation. Those validated at the EU level are indicated by an asterisk.

Multiplexing	Method	Target	LLRICE601	LLRiCE62	TT51-1 (Bt63)	Huahui 1	KeFeng-6	KeFeng-8	KMD1	T1c-19	T2A-1	References
Simplex	**Element-specific**	p35S	X	X			X		X			Kluga et al., 2012
Simplex	**Element-specific**	tNOS			X		X		X			Kluga et al., 2012
Simplex	**Element-specific**	Cry1Ab/Cry1Ac			X		X		X			Kluga et al., 2012
Duplex	**Element-specific**	p35S	X	X			X		X			Kluga et al., 2012
	Element-specific	tNOS			X		X		X			Kluga et al., 2012
Simplex	**Element-specific**	p35S	X	X								Quirasco et al., 2008
Simplex	**Element-specific**	t35S		X								Quirasco et al., 2008
Simplex	**Construct-specific**	p35S-Bar	X	X								Quirasco et al., 2008
Simplex	**Construct-specific**	Cry1Ab/Cry1Ac-tNOS			X							Made et al., 2006
Simplex	**Construct-specific**	p35S-hpt					X		X			Reiting et al., 2013
Simplex	**Construct-specific**	hpt-t35S					X					Reiting et al., 2013
Simplex	**Construct-specific**	hpt-tNOS							X			Reiting et al., 2013
Duplex	**Construct-specific**	Cry1Ac-tNOS (two probes)					X					Akiyama et al., 2007
Simplex	**Event-specific**	LLRICE601*	X									Mazzara et al., 2006a
Simplex	**Event-specific**	LLRICE62*		X								Mazzara, Grazioli, Savini, & Van Den Eede, 2006b
Simplex	**Event-specific**	TT51-1			X							Wu et al., 2010, Cao et al., 2011, Wang et al., 2014
Simplex	**Event-specific**	Huahui 1				X						Li, Wang, Zang, Sui, & Zhao, 2013a
Simplex	**Event-specific**	KeFeng-6					X					Su et al. 2011, Guertler et al., 2012, Wang et al., 2011, Wang et al., 2014
Simplex	**Event-specific**	KeFeng-8						X				Wang, Zhu, Lai, & Fu, 2012
Simplex	**Event-specific**	KMD1							X			Babekova et al., 2009, Wang et al., 2014
Simplex	**Event-specific**	T1c-9								X		Qian & Wang, 2013
Simplex	**Event-specific**	T2A-1									X	Qian & Wang, 2013
Pentaplex	**Taxon-specific**	PLD	X	X	X							Köppel et al., 2010
	Construct-specific	p35S-Bar*	X	X								Köppel et al., 2010
	Event-specific	LLRICE601*	X									Köppel et al., 2010
	Event-specific	LLRICE62*		X								Köppel et al., 2010
	Event-specific	TT51-1			X							Köppel et al., 2010

With the help of the corresponding screening markers, the possible maximum coverage (97.8%) could thus be achieved. However, due to its natural presence in maize, the pUBI marker should be used with caution. Concerning the rest of the inventoried transgenic rice (2.2%), they remain uncovered by the set of screening markers since they belong to the KL-4 group.

Nevertheless, the maximum of coverage is only one aspect of the screening analysis. Indeed, the second one is the discriminative power, which allows to reduce the subsequent number of reaction to perform in the identification step. This step consists to identify GM events using the corresponding event-specific methods, targeting the transgene flanking regions, as well as differentiate EU-authorized and EU-unauthorized GMO. Therefore, even if the minimum set of two screening markers (p35S and tNOS) allows to cover a large spectrum of GMO, the six additional screening markers contribute to increase the discriminative power of the analysis.

However, this identification step is only applicable to GMO for which information about the insertion sites is known, such as for all transgenic rice approved for commercialisation (e.g. LLRice601, LLRice62, TT51-1, Huahui-1, KeFeng-6, KeFeng-8, KMD1, T1c-19 and T2A-1) or few other transgenic rice lines (e.g. Golden Rice 2) (Table 2) (Babekova et al., 2009; Cao et al., 2011; Guertler, Huber, Pecoraro, & Busch, 2012; Jacchia et al., 2015; Köppel, Zimmerli, & Breitenmoser, 2010; Li, Zhang, Wan, & Jin, 2013; Mazzara, Cordeil, & Van den Eede, 2006, Mazzara, Grazioli, Savini, & Van Den Eede, 2006; Qian & Wang, 2013, Su, Xie, Wang, & Peng, 2011; Wang et al., 2011, 2014, 2012; Wu et al., 2010). For the majority of

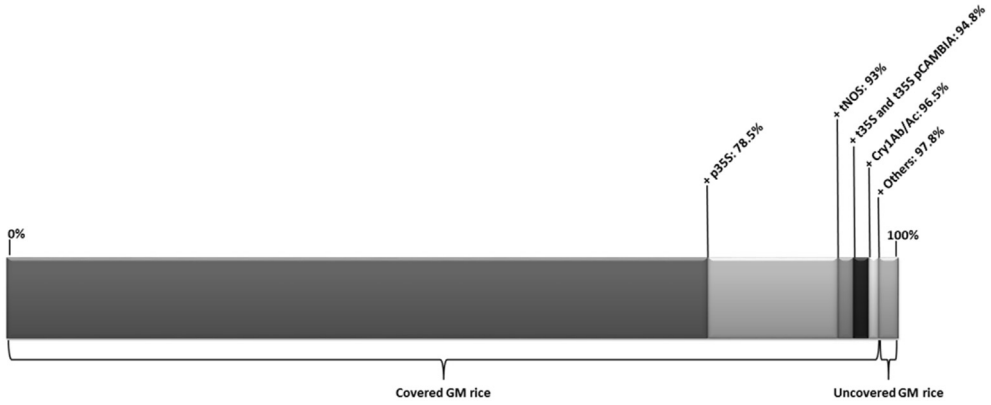

Fig. 5. Covering (%) of inventoried transgenic rice, for which data regarding the elements composing their transgenic cassettes are known, through qPCR screening markers. In successively combining the p35S, tNOS, t35S, t35S pCAMBIA, Cry1Ab/Ac and other markers (pUBI, Bar and pNOS), most of these inventoried transgenic rice is targeted (97.8%). Uncovered transgenic rice belong to the KL-4 group (2.2%).

inventoried transgenic rice, classified in the KL-3 and KL-4 groups, their identification requires the development of new event-specific methods, which depend on the availability of their sequences. Consequently, only very few unauthorized GMO, originating mainly from the KL-2, KL-3 and KL-4 groups, could be identified using the current qPCR GMO detection system, especially with food/feed matrices composed of several different GMO. Indeed, although the unauthorized GMO could be detected, their discrimination is difficult in using the signals obtained with the qPCR technology because same elements are found both in EU-authorized and EU-unauthorized GMO. In this context, the use of the t35S pCAMBIA screening marker will be helpful in order to target specifically some EU-authorized GMO (~30%) since no EU-authorized GMO has nowadays been transformed with pCAMBIA constructs (Fraiture et al., 2014).

To overcome this challenge, the DNA walking technology is proposed as an additional tool allowing to characterize, in any given genome, unknown nucleotide sequence flanking from a short known DNA region, earlier detected through the qPCR screening analysis (Table 3). By this way, this approach allows to unequivocally demonstrate the presence of a GMO in food/feed matrix by the characterization of the transgene flanking regions and the unnatural associations of elements. A semi-routine strategy was developed and successfully applied to GM rice and maize by using methods specific to the p35S, tNOS and t35S pCAMBIA elements. Given that the cassette and the insertion sites for most of the inventoried transgenic rice are only partially known (KL-2 and KL-3 categories), the uncharacterized regions could thus be revealed by applying this DNA walking strategy (Fraiture et al., 2014, Fraiture, Herman, Taverniers, De Loose, Van Nieuwerburgh et al., 2015, Fraiture, Herman, Lefèvre et al., 2015). However, as a minimum of knowledge is required, this strategy cannot deal with GMO containing exclusively unknown transgenic elements (KL-4 category). In that case, Next-Generation-Sequencing (NGS) offers a potential solution, especially in using the whole-genome-sequencing (WGS) approach (Table 3). To this end, a strategy of de novo assembly could be applied. Even if some difficulties could be encountered with large and complex plant genomes as well as

Table 3
Representative examples of alternative detection methods tested on transgenic rice.

Technology	Method	LLRiCE62	TT51-1 (Bt63)	KeFeng-6	KMD1	T1c-19	Other GM rice	References
dPCR	Construct-specific p35S-Bar	X						Köppel & Bucher, 2015
	Event-specific Bt63		X					Li et al., 2015
	Event-specific KeFeng-6			X				Li et al., 2013b
	Event-specific KMD1							Köppel & Bucher, 2015
LAMP	Element-specific Cry1Ab				X			Li et al., 2013
	Element-specific p35S			X		X		Zhang et al., 2012
	Element-specific tNOS		X	X		X		Zhang et al., 2012
	Element-specific bar					X		Zhang et al., 2012
	Event-specific Bt63		X					Chen, Pan, et al., 2012, Chen, Wang, et al., 2012
	Event-specific KeFeng-6			X				Chen, Pan, et al., 2012, Chen, Wang, et al., 2012
	Event-specific KMD1				X			Chen, Pan, et al., 2012, Chen, Wang, et al., 2012
RPA	Element-specific p35S			X	X			Xu, Li, Jin, & Wan, 2014
	Element-specific tNOS		X	X	X			Xu et al., 2014
MLSEB	Specific probe GM rice						X	Huang et al., 2015
DNA walking	Anchored to t35S pCAMBIA, p35S and tNOS						X	Fraiture et al., 2014, 2015a, 2015b
	Anchored to p35S and t35S	X						Spalinskas, Van den Bulcke, Van den Eede, & Milcamps, 2012, 2013
NGS	Whole genome sequencing using Illumina platform	X						Wahler, Schauser, Bendiek, & Grohmann, 2013
	Whole genome sequencing using Illumina platform		X					Yang et al., 2013
	Whole genome sequencing using Illumina platform						X	Willems et al., 2016

with food/feed matrices composed of several different GMO, the analysis could be facilitated in combining the strength of different NGS platforms. For instance, reads from the Illumina technology could be aligned on substitutes of reference genomes generated by the PacBio technology (Fraiture, Broeders et al., 2015). However, even if it seems to offer promising solutions in the GMO detection field, the NGS technology is presently not easily implementable routinely by the enforcement laboratories and still requires a long-time frame to get results and qualified bioinformaticians dealing with NGS data. Among the available NGS data analysis tools, none are really dedicated specifically to GMO, except one developed by Yang et al., 2013. This last approach allows to map the reads, corresponding partially to the reference host genome, to the transgenic cassette sequence. In this way, the number of inserts and their transgene flanking regions could be determined (Yang et al., 2013). Regarding more universal bioinformatics tools, the CLC Genomics Workbench commercial software can be used. It presents the advantage to require only few bioinformatics background and provide easily interpretable output formats compared to other softwares like the Command-Line-Tools. However, with this user-friendly tool, the range of possibilities is limited by the available pre-designed workflows (Willems et al., 2016). This kind of pre-designed workflows could also be created by qualified bioinformaticians in order to simplify the analysis for bioinformatics novices, with the advantage of being over time improved in-house if needed. In addition, even if the WGS approach could realistically be applied on a food/feed matrix exclusively composed of one kind of GM crop, such analysis still remains unreasonable, in term of cost, to detect GMO present at trace level. In the frame of the DECATHLON project, bioinformatics pipelines will be besides developed and assessed for appropriate implementations notably in GMO routine analysis. (DECATHLON; Willems et al., 2016).

Besides the considerations upstream, alternatives approaches have also been considered to overcome some limits inherent to the qPCR technology, such as an insufficient throughput to deal with the increasing number of GMO, a relative quantification requiring certified materials that are essentially available for EU-authorized GMO and an incompatibility to carry out analysis directly on-field. These approaches allow notably to improve the speed, the transportability (e.g., loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA)), the quantification accuracy (e.g., digital PCR (dPCR)) or the throughput (e.g., multi-labelling system based on electrochemical biosensor (MLSEB)) of the analysis (Table 3).

3.2. NPBT rice detection

Although only few applications have been nowadays reported for rice, the use of NPBT, including the most recent one like Crisp/Cas9, is expected to increase. Therefore, in order to assess the theoretical potential to detect these new plant varieties in a near future, the applicability of existing technologies has been carried out. Concerning the detection and the identification by qPCR of the plants generated from NPBT, several difficulties have already been highlighted by Lusser et al., 2011 in function of the technique used. First, as only small modifications are induced, with or without a repair template, the detection, but not the identification, of plants coming from the ZFN technology of respectively the ZFN-1 and ZFN-2 categories is feasible with the help of a minimum of available information. However, in case plants are modified by the ZFN-3 category, which induces large modification (e.g. gene), their detection and identification, are possible with a priori knowledge whereas, without any available information, only the detection step is conceivable. To attempt to prove these modifications from ZFN approaches, a strategy of full genome sequencing could be envisaged if no information is available. Second, for the mutations produced by the ODM method, only their detection could be performed based on prior information. But, similarly to ZFN approaches, the NGS technology could be applied when no information is available. Third, given that cisgenic plants and plants obtained through intragenesis present a large modification, PCR assays, using event-specific markers designed on the known sequence of the unnatural association of elements, can allow their detection and identification with a known case whereas sequencing strategies have to be investigated if no information is available. Fourth, the identification of RdDM plants is not applicable since the modifications generated could also occur naturally. Fifth, regarding reverse breeding, agro-infiltration and grafting, no discrimination is possible with plants coming from conventional breeding methods, preventing any detection and identification (Lusser et al., 2011).

It is thus clear that the precise mutations, as integrated in the reported NBPT rice (see sub-section 2.4), will be highly difficult to detect. Since each transgenic plant submitted for an approval on the EU market should be associated to a corresponding detection or identification method, it represents thus a real challenge for most of the plants coming from NPBT listed up to now, especially in absence of any prior information (Lusser et al., 2011). However, in case of the competent authorities agree finally that NPBT organisms do not fall under the GMO legislations, no related detection system will be mandatory.

4. Conclusion

Based on all the information collected from the inventoried biotech rice in the present study, the possibility to detect them have been evaluated, allowing to suggest suitable detection strategies.

On the one hand, the detection of transgenic rice could be considered in function of the KL classification. Indeed, the current qPCR strategy is still valid to detect GMO belonging to the KL-1, KL-2 or KL-3 categories since 97.8% of inventoried transgenic rice, for which information about the elements composing their transgenic cassettes are known, are covered by this way. To this end, a set of eight screening markers, including p35S and tNOS elements which are highly observed both in EU-authorized and EU-unauthorized GMO, was suggested. In addition, to increase the discrimination power of the analysis, the use of the t35S pCAMBIA marker allows to specifically target around 30% of EU-unauthorized GMO. Concerning their identification, the current qPCR strategy is however only applicable for GMO belonging to the KL-1 category. Therefore, for the majority of collected transgenic rice, classified in the KL-3 category, the observed screening qPCR positive signals, suggesting that GMO are present in the tested sample, need to be confirmed by alternatives approaches. Moreover, unlike the qPCR system, among the potentially detected GMO, some of the alternatives approaches could discriminated EU-authorized and EU-unauthorized GMO present within the same food/feed matrix. Indeed, in using DNA walking techniques, sequences from the transgene flanking regions and from unnatural associations of elements could be characterized in order to irrefutably prove the presence of GMO (Fraiture, Herman, Lefèvre et al., 2015). However, as a minimum of prior knowledge is required, this strategy is not convenient for the few transgenic rice belonging to the KL-4 category. In that case, only whole-genome-sequencing approaches seems relevant, even if some progresses are still required *inter alia* in terms of sensitivity, availability of reference genomes and ease of bioinformatics analysis.

On the other hand, for the rice produced by NPBT, regardless of the fact that no decision has yet been taken at the EU level regarding their potential GMO status, most of the introduced

genetic modifications are too similar to those obtained with conventional breeding or natural processes, making their detection without prior knowledge challenging, or even technically impossible. The detection of some of these biotech plants could be envisaged in a near future using NGS approaches. Taking into account that the NGS technology and related analysis will be improved, the integrated modifications could be located via whole genome sequencing approaches. Moreover, for some of these biotech rice, the knowledge of their sequences, via developers or NGS analysis, could allow designing appropriated qPCR TaqMan® markers with probes specific to the integrated mutations.

Using biotech rice as a study case, the present approach could definitely be extended to biotech events belonging to other species. Indeed, similarly, publicly available information for non-rice biotech events developed worldwide, also usually scattered, could be centralized. By this way, clue information, regarding notably the integrated genetic elements, could be highlighted in order to subsequently establish an appropriated detection strategy. According to the collected data, the efficiency of the current qPCR GMO detection system used by the enforcement laboratories could be assessed. Moreover, if necessary, this detection system, especially for the unauthorized GMO, could be strengthened with additional methods, such as DNA walking and NGS.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tifs.2016.03.011.

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