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Application of New Technologies in the Safety Assessment of Genetically Modified Feed

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Application of New Technologies in the Safety Assessment of Genetically Modified Feed

Thesis by:

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Bachelor of Animal Sciences (First-Class Hons), Master of Agricultural Sciences

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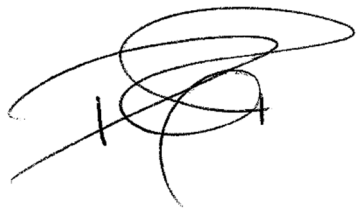
Abstract

Agricultural biotechnology, such as genetic modification, has been proposed as a way to face food security and climate change. All new crop varieties with altered genetics must be subjected to safety assessments to fulfil regulatory requirements, prior to marketing and/or commercial release. The aim of this thesis is to undertake part of the safety assessment of transgenic plants, using emerging molecular biology technologies. Initially, the molecular characterisation of two genetically modified plants were performed using long-read DNA sequencing. Results demonstrate the capability of this technique to characterise transgenes located in complex and/or repetitive regions of the plant genomes, in a fast and cost-effective way with simple and robust bioinformatic pipelines that would be broadly applicable and accessible. Then, a reliable methodology was developed using droplet digital PCR tools for the detection of transgenes in a variety of complex pasture-based products relevant to livestock industries. Later, a toxicological assessment of the high-energy perennial ryegrass was performed using a metabolomics approach. The current study indicates that the new technologies can provide a highly reliable and efficient solution for the safety assessment. This thesis represents a contribution in the use of new technologies to improve the risk assessment procedure of genetically modified plants.

Declaration

This is to certify that:

1. This thesis comprises my original work only except where indicated in publication prefaces.
2. Due acknowledgement has been made to all materials used in the text.
3. This thesis is fewer than 100,000 words in length, exclusive of words in tables, bibliographies and appendices.



Paula Andrea Giraldo Parra

Date 31/03/2020

Preface

This thesis contains the following original manuscripts by P.A. Giraldo Parra as the primary researcher and author:

1.	Full title:	**Safety assessment of genetically modified feed: is there any difference from food?**
	Authors:	P.A. Giraldo, H. Shinozuka, G.C. Spangenberg, N.O.I. Cogan, and K.F. Smith
	Candidate contribution:	80%
	Status:	Published
	Journal name:	Frontiers in Plant Sciences
	DOI	10.3389/fpls.2019.01592
2.	Full title:	**Rapid and detailed characterisation of transgene insertion sites in genetically modified plants via nanopore sequencing.**
	Authors:	P.A. Giraldo, H. Shinozuka, G.C. Spangenberg, K.F. Smith and N.O.I. Cogan
	Candidate contribution:	80%

Status:	In progress
Journal name:	Pending

3. Full title: **Development and application of droplet digital PCR tools for the detection of transgenes in pastures and pasture-based products.**

Authors:	P.A. Giraldo, N.O.I. Cogan, G.C. Spangenberg, K.F. Smith and H. Shinozuka
Candidate contribution:	80%
Status:	Published
Journal name:	Frontiers in Plant Sciences
DOI	10.3389/fpls.2018.01923

4. Full title: **Evaluation of endophyte toxin production and its interaction with transgenic perennial ryegrass (*Lolium perenne* L.) with altered expression of fructosyltransferases.**

Authors:	P.A. Giraldo, C. Elliott, P. Badenhorst, G. Kearney, G.C. Spangenberg, N.O.I. Cogan, and K.F. Smith.
Candidate contribution:	55%
Status:	Published
Journal name:	Transgenic Research

DOI 10.1007/s11248-018-0087-9

For all publications included in this thesis, co-authors of published work have completed The University of Melbourne's co-author authorisation form. All co-authors have certified that the contribution of the candidate is greater than 50% and is the primary author of these publications.

The candidate's advisory panel has approved inclusion of the published work listed in this thesis. The principal supervisor of this work, Professor K.F. Smith, has signed The University of Melbourne's declaration for a thesis with publication.

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This segment is for all of you. Yes you, the few who are going to read this small piece of myself. You should consider yourself guilty for all your encouragement and support, always assuring me that I was going to do a good work in this PhD. You know who you are, and this is your entire fault.

I dedicate this thesis to Dr. Noel Cogan for all scarified Sundays checking my terrible writing and making sense of what I wanted to say. To Dr. Hiroshi Shinozuka for his valuable comments and exhaustive checking and Dr. Kevin Smith for teaching me what is required to do a PhD, patience. Who would thing that among a polite British, a meticulous Japanese, a calmed Australian and a crazy Colombian, we would do such wonderful team.

Figure 1: Logo of the Department of Agriculture Victoria

List of Abbreviations

%	Percent
~	Around
°C	Degrees Celsius
µg	Micrograms
µL	Microliters
µM	Micromoles
1SST	Sucrose-sucrose 1-fructosyl transferase
6G-FFT	6-glucose fructosyl transferase
amu	Atomic mass unit
AMV	Alfalfa mosaic virus
Biotech	Biotechnology
BLAST	Basic local alignment search tool
BLASTn	Basic local alignment search tool nucleotide
Bt	*Bacillus thuringiensis*
cv.	Cultivar
ca.	Circa
CCS	Circular consensus sequence
cm	Centimeter
Ct	Cycle threshold
Cul4	Cullin 4
CV	Coefficient of variation

ddPCR	Droplet digital polymerase chain reaction
DJPR	Department of Jobs, Precincts and Regions
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded deoxyribonucleic acid
E. coli	*Escherichia coli*
EC	Embryogenic callus
EFSA	European Food Safety Authority
ERA	Environmental risk assessment
ETIP	Engineered Transgene Integration Platform
EU	European Union
g	Grams
Gbp	Giga base pairs
GC	Guanine-cytosine
gDNA	Genomic deoxyribonucleic acid
GE	Genome edited/Genome editing
GF	Gene flow
GM	Genetically modified/Genetic modification
GMO	Genetically modified organism
GOI	Gene of interest
GOI-	Gene of interest negative
GOI+	Gene of interest positive
h	Hours
HGT	High molecular weight
hmg	High mobility group
hph	Hygromycin B phosphotransferase
IgE	Immunoglobulin E
kbp	Kilo base pairs
kg	Kilograms
LAMP	Loop-mediated isothermal amplification

LC-MS	Liquid-chromatography mass-spectrometry
LMW	Low molecular weight
Lp	*Lolium perenne*
LSD	Least significant difference
ME	Metabolizable energy
mg	Milligrams
min	Minutes
MJ	Megajoules
mL	Milliliters
mM	Micromoles
mm	Millimeters
ng	Nanograms
NGS	Next generation sequencing
nM	Nanomoles
OGTR	Office of the Gene Technology Regulator
ONT	Oxford Nanopore Technologies
ORF	Open reading frame
PacBio	Pacific Biosciences
PCR	Polymerase chain reaction
ppm	Parts per million
qPCR	Quantitative polymerase chain reaction
RbcS	Ribulose-1,5-bisphosphate carboxylase/oxygenase
s	Seconds
SDN-1	Site-direct nuclease 1
SE	Standard endophyte
SGS	Second generation sequencing
SSR	Single sequence repeat
ST	Standard toxic
T1/F1	Filial 1 hybrid
T-DNA	Transgenic deoxyribonucleic acid

TGS	Third generation sequencing
t-nos	NOS terminator
UK	United Kingdom
US	United States
UTP	Uridine triphosphate
WSC	Water soluble carbohydrates
WTO	World Trade Organization
ZFN	Zinc finger nuclease

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Chapter 1

General Introduction

Food security is one of the major concerns in our growing global population, creating significant challenges for agricultural production. In addition to the increasing food demand, climate change and environmental degradation are reducing the available agricultural land, generating important questions and significant challenges regarding the future directions for the agriculture industry (Dibden, Gibbs, and Cocklin, 2013). Agricultural biotechnology, including GM techniques, has a potential to reduce environmental footprint, by improving food quality and increasing productivity (Barros, et al. 2019).

GM crops have been globally produced for over 20 years and have delivered significant economic benefits to the agricultural sector. Biotech-delivered crops are the technology with the fastest adoption in the history of agriculture, there are c. 18 million farmers planting GM crops in 28 countries, wherein c. 97.1 million hectares were planted by small and resource-poor farmers from developing countries in 2015 (James, 2015). A meta-analysis of 147 studies on the impacts of GM crops estimated that adoption of GM crops, has reduced chemical pesticide use by 37%, but increased crop yields and farmer profits by 22% and 68%, respectively (Klümper and Qaim, 2014).

GM crops can be traded as food and feed products (Panel on Genetically Modified Organisms, 2010). The products are classified as GM food, when the direct consumers are mainly humans, and the products only intended for animal consumption are regarded as GM feed. However, a range of GM crops, such as maize, soybean and canola, are used as both food and feed. Most GM crops available in the market, except for alfalfa (*Medicago sativa* L. ssp. sativa) and creeping bentgrass (*Agrostis stolonifera*) have been assessed as GM food, because they can be consumed by humans. Commercialisation of GM cultivars of the major grain species, such as canola [rapeseed (*Brassica napus* L.)], maize (*Zea mays*

L.), and soybean (*Glycine max* (L.) Merr.) is relatively common in developed countries, which dominate the global trade in these commodities (Marmiroli et al. 2008). Such GM crops are mainly used as animal feed, and it has been estimated that between 70 to 90% of all GM crops are used in farms to feed animals (Flachowsky et al., 2012). In the US and EU, representing the world's largest feed markets, 98% of GMO imports are used for animal feed (Popescu and Nicolescu, 2016).

Although grains as feed supplementation for animals have played an important role in agricultural productivity, forage crops are essential to the livestock industry and sustainable agriculture worldwide (Wang and Brummer, 2012). Eighty per cent of the world's cow milk and 70% of the world's beef and veal are produced from temperate grasslands. In the US, the proportion of forage in the total nutrients consumed is 61% for dairy cattle, 83% for beef cattle and 91% for sheep. In Western Europe, 17% of the total land area consists of permanent grassland. In Australia, 70% of the area is covered by grasslands (Wilkins and Humphreys, 2003).

Implementation of molecular breeding technologies in plant species intended for animal consumption, can lead to the development of genetic solutions for current limitations in forage quality, pest and disease resistance, nutrient efficiency, tolerance to abiotic stresses and the targeted modification of growth and development (Smith, et al., 2007, Ramessar, et al., 2010). However, to release new transgenic cultivars, it is necessary to satisfy a variety of regulatory requirements, of which the main aim is to assess a potential impact on human, animal and environmental health.

The scientific evidence that must be provided in the safety assessment of GM crops can vary among different legal jurisdictions (Alexandrova, et al. 2005). However, a detailed molecular characterisation of the transgene insertion, development of tracking and tracing methodologies to ensure legality and traceability, and environmental studies to enable coexistence frameworks, are common studies in the safety assessment of GM crops (Van Haver, et al. 2003). Other studies, such as toxicological, allergenicity, nutritional, and horizontal transfer, have been performed following a case-by-case approach considering newly emerging scientific knowledge and technologies (König, et al. 2004). Therefore, the project goal of the current study is to review in detail the GM

deregulation process, with particular emphasis on GM feed, and development of contemporary tools and methods to characterise transgenic events.

Research questions

GM crops are marketed as GM food or feed products (Panel on Genetically Modified Organisms, 2010). A GM product is classified as food when there is a possibility that the product is consumed by humans, irrespective of whether the genetic improvement has been made to benefit animal consumption. Only products that cannot be consumed by humans are assessed as GM feed. For example, maize, soybean and canola, can be used for both human and animal consumption. Therefore, the safety assessment for GM maize, soybean and canola is conducted as a GM food. Forage products are not digested in the human gastric system, and GM forage products may be considered as GM feed (Canadian Food Inspection Agency – Novel Feeds, 2019). To date, few crops have been assessed as GM feed. Some examples are alfalfa (*Medicago sativa* L. ssp. *sativa*), creeping bentgrass (*Agrostis stolonifera* L.), and the Kentucky bluegrass (Scotts miracle Gro). However, relatively little information is publicly available about the assessment procedure of such GM feed crops. Therefore, the first research question addressed in this thesis is: What is the difference in the safety assessment of GM feedstuffs as opposed to human food? Hence, Chapter 2 of this thesis reviews the scientific literatures on biosafety assessments for food, applying it where appropriate, to GM feedstuffs.

The safety assessment of new transgenic plants aims to provide an evaluation of their potential impact on humans, animals and the environment. The first step in the safety assessment is the molecular characterisation of the transgene (Schouten, et al. 2017). A full characterisation of a new GM crop is a comprehensive description of the structural information of the transgene and stability of the trait (Li, et al. 2017). It is the foundation of subsequent GM product safety assessments before commercialisation, and also serves as a baseline for the development of detection and identification tools to satisfy traceability and labelling requirements (European Parliament, 2003). Since such characterisation is performed at the molecular level, the same methods and techniques may be used for both GM feed and food. More than 20 years have passed since development of some methods which have been applied for this purpose. New molecular

technologies may provide less expensive and laborious solutions with a higher accuracy, especially in plant genomes with complex structures and genetic redundancy (Ren, et al. 2018). Therefore, the second research question raised in this thesis is: how could a full molecular characterisation of a new transgenic plant be conducted in a more accurate and effective way across a range of crop species? Accordingly, Chapter 3 of this thesis focuses on the development of an accurate and efficient approach for full molecular characterisation of GM plants across a broad range of crop species.

GM traceability is the second step that needs to be addressed in the safety assessment of GM plants. Development of tracking and tracing strategies is not only important to ensure legality and traceability, but also to comply with GM labelling regulations (European Parliament, 2003). The method chosen to comply to traceability and labelling requirements, should be sensitive enough to detect the transgene(s) at levels below the corresponding jurisdiction tolerance threshold (e.g. 5% in US, 1% AU and 0.9% in EU) (Ramessar, et al., 2010). Additionally, it should be able to detect the transgene(s) from raw agricultural commodities ensuring traceability of the product at all stages of the supply chain. Due to the fact that until today a majority of the GM products available in the market were assessed as GM food, detection methods have been typically developed for matrices or substrates for human consumption, such as grain or flour. However, in case of GM feedstuffs, both unprocessed and highly processed materials, including fresh leaves, dry leaves (hay), pollen, seeds, tillers or stems, silage and/or faecal matter, are commonly traded (EFSA, 2018). Establishment of methods to guarantee to detect the transgene(s) from such materials are required. Therefore, the third research question is: how can we use emerging technologies, such as ddPCR, to detect transgenes in different agricultural products? In Chapter 4, a method to detect transgenes in a range of agricultural products is developed, using a high-energy transgenic ryegrass as a study case.

In a process of transgenic deregulation, the issue to be subsequently addressed is about the environmental safety and animal health. Environmental safety issues and coexistence strategies are already well-studied in outcrossing forage species and cross-pollinated grain crops, such as canola and maize. Hence, existing principles such as development of detection techniques, physical barriers and agronomic management can be applied to

other forage crops (Smith and Spangenberg, 2016). Thus, environmental safety studies are reviewed in Chapter 2, but field trials were not required. Toxicological assessments are conducted in Chapter 5, to characterise unintended changes and detect active substances or compounds that could have unexpected toxic effects for non-targeted organisms (Van Haver, et al. 2003). All toxicity assessments for GM material are required to be performed based on a case-by-case approach, considering the toxicological profile of new introduced substances (Domingo, 2007). For this purpose, high-energy ryegrass was used as a case study, which is not pathogenic or capable of causing any disease in other plants, humans or animals, but it can contain fungal endophytes, known as *Epichloë festucae*. Due to production of a range of alkaloids, a presence of the fungal endophytes in the transgenic plants can unintentionally affect the crop quality as animal feed (OGTR 2008). In terms of techniques, high-throughput '-omics' profiling techniques have been suggested as an accurate approach to detect unintended effects in GM crops (Ricroch, 2013). Therefore, the fourth research question of this thesis is: Does the modification of the fructan biosynthesis pathway in high-energy perennial ryegrass, as host of different endophytes, alter the concentration of alkaloids produced from the fungi? Chapter 5 involves the use of metabolomic techniques to quantify alkaloid concentration in high-energy perennial ryegrass crossed into a range of cultivar backgrounds with varying endophytes.

When integrating all safety studies performed in this research project along with future feed and nutritional safety studies in animals, a range of questions and challenges arise, as a result the final research question of this thesis is: What are the challenges that safety assessments of GM plants are facing, and which strategies can be implemented to solve them? A general discussion of what has been done in the evaluation of GM plants is stated in Chapter 6, highlighting the areas that need to be improved and proposing possible strategies to assist them.

Research aims

- To review the scientific literatures on biosafety assessments of GM food and compare them with those of GM animal feedstuffs.

- To develop an accurate and effective method to characterise GM plants at the molecular level.
- To establish a sensitive and reliable method for traceability of GM forages.
- To assess the concentrations of different alkaloids when high-energy perennial ryegrass is colonised by different fungal endophyte strains.
- To perform a retrospective study about the safety assessment of GM crops to raise questions for the upcoming challenges and propose strategies to face them.

Thesis outline

The thesis is divided into 6 chapters, where Chapter 2 describes this study in relation to the current scientific literature and research. In the chapter, a general background on GM crops is discussed, followed by the regulatory framework and labeling policies for GM crops in different jurisdictions. Approaches for molecular characterisation, GM traceability, and analytical methods to comply with labelling requirements, environmental safety studies, feed safety studies, toxicological studies and allergenicity studies are also discussed. Finally, a critical conclusion on safety assessments of GM crop intended for animal feeding is presented.

In Chapter 3, a fast, simple and reliable approach for molecular characterisation of GM products using novel technologies, such as the nanopore sequencing technologies, is developed. For this purpose, two GM plants were used to exemplify the effectiveness of the method; canola and white clover (*Trifolium repens* L.). This is the first time this approach is applied as a GM analysis strategy to identify and characterize a GM transgene insertion at the molecular level.

Chapter 4 describes the development and application of ddPCR tools for the detection of transgenes in pastures and pasture-based products. The information presented in the chapter provides guidance and resources for pasture-based biotechnology applications that are required to comply with traceability requirements. This study has been published as a research article in *Frontiers in Plant Science* (Frontiers Media, Lausanne, Switzerland).

In Chapter 5, a toxicological assessment of GM high-energy perennial ryegrass, through its evaluation of endophyte toxin production in different genotypic backgrounds and endophyte strains. This chapter provides guidance on future approaches of transgenic breeding requirements, in relation to endophyte toxin evaluation and assist in the comprehensive evaluation of a potentially commercialisable transgenic event. The manuscript was published in *Transgenic Research* (Springer Nature Switzerland AG, Basel, Switzerland).

Chapter 6 contains the general discussion, future directions and conclusions of this PhD thesis. The main findings of the aforementioned experiments are collectively discussed and appropriate recommendations and implications for the safety assessment of GM plants are exposed.

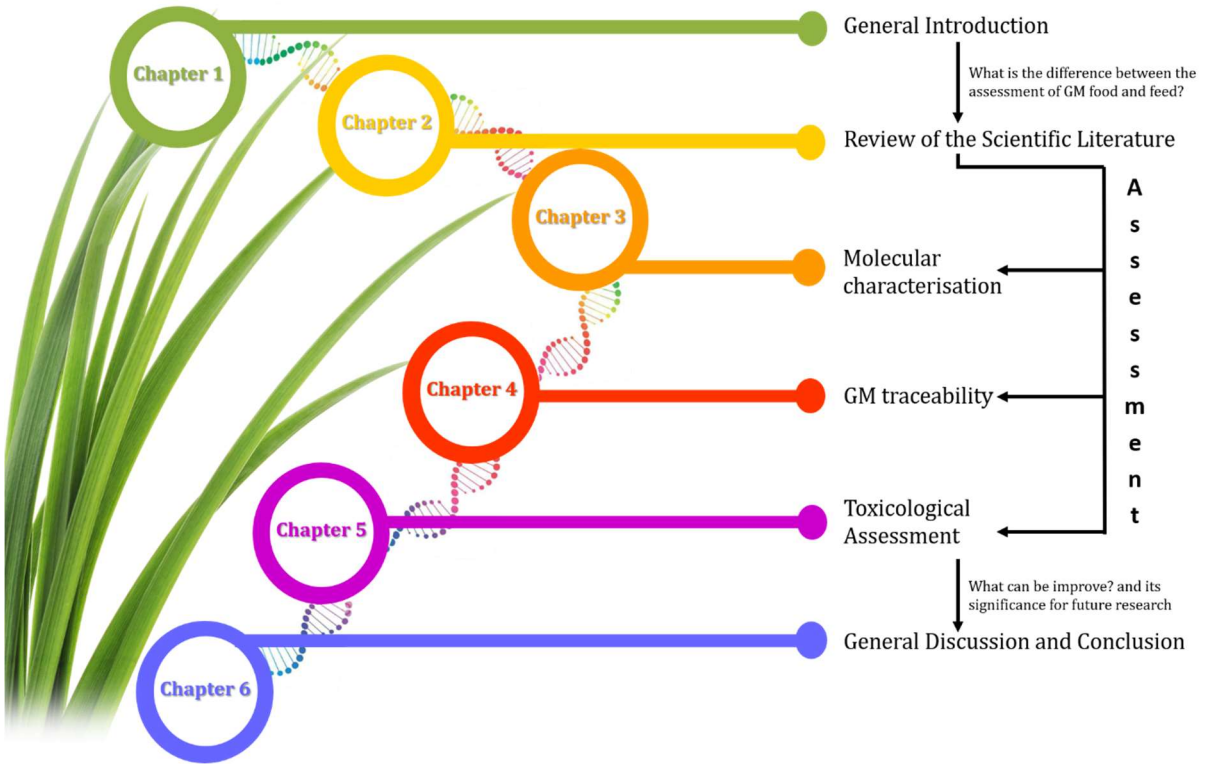

Figure 2: Thesis logic and structure

Hypotheses

- There are significant differences in the safety assessment of GM feedstuffs as opposed to GM human food.
- The Nanopore sequencing technologies simplifies, increases accuracy and reduces labor time in the molecular characterisation of GM plants.
- Droplet digital PCR increases the sensitivity and reliability of GM forages traceability, as compared with qPCR.
- The presence of a modified fructan biosynthesis transgene in perennial ryegrass does not affect endophyte persistence and quantitative alkaloid.

Chapter 2

frontiers in Plant Science

REVIEW
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Safety Assessment of Genetically Modified Feed: Is There Any Difference From Food?

*Paula A. Giraldo[1,2], Hiroshi Shinozuka[2], German C. Spangenberg[2,3], Noel O.I. Cogan[2,3] and Kevin F. Smith[1,4]**

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Food security is one of major concerns for the growing global population. Modern agricultural biotechnologies, such as genetic modification, are a possible solution through enabling an increase of production, more efficient use of natural resources, and reduced environmental impacts. However, new crop varieties with altered genetic materials may be subjected to safety assessments to fulfil the regulatory requirements, prior to marketing. The aim of the assessment is to evaluate the impact of products from the new crop variety on human, animal, and the environmental health. Although, many studies on the risk assessment of genetically modified (GM) food have been published, little consideration to GM feedstuff has been given, despite that between 70 to 90% of all GM crops and their biomass are used as animal feed. In addition, in some GM plants such as forages that are only used for animal feeds, the assessment of the genetic modification may be of relevance only to livestock feeding. In this article, the regulatory framework of GM crops intended for animal feed is reviewed using the available information on GM food as the baseline. Although, the majority of techniques used for the safety assessment of GM food can be used in GM feed, many plant parts used for livestock feeding are inedible to humans. Therefore, the concentration of novel proteins in different plant tissues and level of exposure to GM feedstuff in the diet of target animals should be considered. A further development of specific methodologies for the assessment of GM crops intended for animal consumption is required, in order to provide a more accurate and standardized assessment to the GM feed safety.

Keywords: traceability, environment, toxicological, allergenicity, forage, transgenic crops, regulatory framework, genome-editing

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INTRODUCTION

The growth of the human population will create significant challenges for agricultural production, making food security a growing issue. Nearly 870 million people suffer from malnutrition, most of them in the developing countries of Africa, Asia, and South America (World Hunger, 2013). Additionally, climate change and environmental degradation are currently reducing the available agricultural land, creating additional challenges to fill the increasing food demand (Hanjra and Qureshi, 2010). The use of modern biotechnology, including genetic modification techniques, has been proposed as a way

to reduce the environmental footprint, by improving food quality and increasing productivity (Barros et al., 2019).

Animal derived food is a major contributor to human nutrition and health, largely through supplying protein. It also plays a crucial role in rural economies of most developing countries, particularly in dry areas (Godfray et al., 2010). In order to produce enough protein for the growing global population, farming systems are increasing the pressure on land, water resource use and biodiversity conservation. Solutions to face climate change and high demand for natural resources are urgently needed, especially in the area of forage production (Sakadevan and Nguyen, 2017), since it compromises nearly 80% of the world agricultural land and provides the grazing feed-base for the dairy and red meat industries (Bruinsma, 2017).

Implementation of molecular breeding technologies in forage crops can enhance the agricultural sector through increases in productivity, more efficient use of natural resources and decreases in environmental impacts (Ramessar et al., 2010). Breeding programs target the development of genetic solutions for forage quality limitations, pest and disease resistance, nutrient acquisition efficiency, tolerance to abiotic stresses, and the targeted modification of growth and development (Smith et al., 2007).

Transgenesis is a classical DNA modification methodology, which enables production of crops with desired traits based on the introduced transgene(s). Genome editing technologies were more recently established, which enable alteration of the DNA to produce defined multination(s) and/or insertion of foreign gene(s) at the targeted site(s), in contrast to GM where the insertion is random (Sprink et al., 2016). Genetic modification is typically defined as alteration of the genetic material of an organism in a way, which does not naturally occur (Wolt et al., 2015). Crop cultivars developed through use of transgenesis, therefore, have been regard as GM organisms (Hundleby and Harwood, 2019).

Transgenic crops can be further divided into four classifications/classes, according to the structure and strategy for transgenesis (Lin and Pan, 2016). Most GM commercially available today are categorized as the first class of transgenics, also called single trait transgenics. These crops typically contain common transgenic elements, such as the 35s promoter sequence of cauliflower mosaic virus, and nopaline synthase terminator (nos-T) from *Agrobacterium* (Shaw et al., 1984; Wehrmann et al., 1996). The second class transgenics have stacked modified traits, and these varieties usually result from hybrid crosses of first-generation GM crop varieties. The hybrid cross procedure may increase economic values of the GM variety with a lower development cost. The third class of transgenics are so-called near-intragenics, these are GM crops where the transgene construction originates from the host with some minimal modifications. The last class are more related to true intragenic or cisgenic technologies, where the transgene is comprised of only products and elements from the host, without modifications and the only difference with its conventional counterpart is the specific order and insertion loci of the transgenes (Rommens et al., 2007; Jacobsen and Schouten, 2009; Lin and Pan, 2016).

On the other hand, genome editing technologies use biological tools such as sequence-specific nucleases to generate desired modifications within plant genomes, allowing the introduction of one or more transgenes at a specific locus, the removal of unwanted DNA from the host, or the control in the expression of endogenous or synthetic genes (Songstad et al., 2017).

Since approvals for commercialization of early-generation transgenic crops, safety issues for human consumption have been mainly considered. GM crops as animal feed and GM forage have not been considered as the primary target of the regulatory framework. Although there are some animal feedstuffs that crossover with human consumption (e.g., grain), there are many plants and plant parts that are not directly consumed by humans but are exclusively used by livestock as feed. This review article aims to summarize and discuss the elements needed for the safety assessment of GM crops for animal feed purposes, using the available information on the current practice of safety assessment that the product would be subjected to, as the baseline.

GM FEED

The market share of GM products has rapidly increased from commercialization of the early generation of GM crops in the 1990s, (I.S.A.A.A., 2017). The major GM crops available in the market are soybean (*Glycine max* L. Merr.) with 77% of the global area for individual crops (94.1 million hectares), maize (*Zea mays* L.) at 32% (59.7 million hectares), cotton (*Gossypium arboretum* L.) at 80% (24.21 million hectares), and canola (*Brassica napus* L.) 30% (10.2 million hectares). The commercial use of transgenesis-delivered crops cultivars has also recently expanded to more species including sugar beet (*Beta vulgaris* L.), papaya (*Carica papaya* L.), squash (*Cucurbita* L.), eggplant *Solanum melongena* L.), potatoes (*Solanum tuberosum* L.), and apples (*Malus pumila* Miller, 1768), and these products are already commercially available in US (I.S.A.A.A., 2017). A meta-analysis including 147 food and feed crops, also has revealed that the adoption of GM technology has decreased the use of chemical pesticides by 37%, increased crop yields by 22%, and increased farmer profits by 68% (Klümper and Qaim, 2014).

GM crops can be traded as food and feed products (Panel on Genetically Modified Organisms, 2010). The products are classified as GM food, when the direct consumers are mainly humans, and the products only intended for animal consumption are regarded as GM feed. However, a range of GM crops, such as maize, soybean, and canola, are used as both food and feed (**Figure S1**). Most GM crops available in the market, except for alfalfa (*Medicago sativa L.* ssp. sativa) and creeping bentgrass (*Agrostis stolonifera*) have been assessed as GM food, because they can be consumed by humans. On the basis of biomass, between 70 to 90% of all GM crops, however, are estimated to be used in farm as animal feed (Flachowsky et al., 2012).

In contrast to GM food crops, only a few types of GM forage products have been commercially released. Alfalfa is an economically important legume forage mainly in temperate regions. The first GM forage crop commercialized in US was the Roundup Ready® Alfalfa from Forage Genetics International (Nampa, ID, US), which can be categorized as a first generation transgenic (van Deynze et al., 2004).The herbicide-resistance trait was produced through inserting two copies of an *Agrobacterium*-derived gene (*cp4 epsps*) of which translational product (EPSPS;

5-enolpyruvylshikimate-3-phosphate synthase) contribute to glyphosate-tolerance. Such insertion of the foreign gene allows post-emergence applications of glyphosate-based herbicides for weed control (Putnam et al., 2016).

Following to Roundup Ready® Alfalfa, another first-generation transgenic, HarvXtra™ Alfalfa (Forage Genetics International) was developed and commercially released. Using RNA interference (RNAi)-based gene suppression mechanics, the lignin content and composition are modified in this cultivar. A transgene cassette including an inverted repeat of the interference-targeting sequence was introduced using *A. tumefaciens* for suppression of caffeoyl-CoA 3-O-methyltransferase (CCoAOMT), which is involved in lignin biosynthesis. The inverted repeat was designed to be transcribed under control of the phenylalanine ammonia-lyase (PAL)2 promoter from common bean (*Phaseolus vulgaris* L.) for vascular tissue-specific expression, allowing the desired suppression of lignin biosynthesis without negative effects on forage yield (Barros et al., 2019).

Perennial ryegrass (*Lolium perenne* L.) is also one of most important forage species in the temperate regions. Although several transgenic perennial ryegrass plants with potentially economically useful traits have been developed, none of them has been commercially released. Using a similar approach to HarvXtra™ Alfalfa, low-lignin perennial ryegrass individuals have been generated (Hu et al., 2013). In the transgenic perennial ryegrass, lignin biosynthesis-related genes were down-regulated based on the RNAi mechanisms. As the transgenic low-lignin plants may have increased digestibility for cattle, the lignin biosynthesis-controlling technology may be useful for production of optimal feedstocks. The Chimeric REpressor Gene-Silencing Technology (CRES-T) is a newly developed transgenesis-based approach for functional analysis of transcription factors in plants (Mitsuda et al., 2011). A CRES-T transgenic construct was developed to target a zinc finger transcription factor gene, which is negatively related to salt tolerance (Cen et al., 2016). Transgenic perennial ryegrass plants with the CRES-T construct showed a higher tolerance to salt stress (up to 300 mM NaCl). Interestingly, the transgenic perennial ryegrass also showed a vigorous phenotype under non-salt stress condition, which may be useful for forage breeding purposes.

Perennial ryegrass plants with a high-energy trait have been also developed, using the transgenic technologies. A synthesized construct of perennial ryegrass 6-glucose fructosyltransferase (6G-FFT) and sucrose:sucrose 1-fructosyl-transferase (1SST) genes were introduced into the perennial ryegrass genome, aiming enhancement of fructan biosynthesis in the leaf blades (Panter et al., 2017). The transgenic plants showed a substantial increment in fructan accumulation in leaf blades, as well as enhanced biomass production. These traits could be beneficial for the livestock industry, as leaf blades are the major part as feed for grazing ruminants (Lee et al., 2003). The transgenic plants were generated through insertion of manipulated perennial ryegrass genes, and this cultivar can be regarded as a third generation transgenic.

Improvement of biotic stress tolerance in white Clover (*Trifolium repens* L.), an important pasture legume in temperate regions, has been also developed through the generation of plants immune to infection by Alfalfa mosaic virus (AMV) (Smith et al., 2007). Although, this AMV resistant clover has not been commercially released yet, different studies have demonstrated the expression and stability of the viral coat protein gene encoded, by the sub-genomic RNA4 of AMV in white clover, under glass house and field conditions (Panter et al., 2012; Smith and Spangenberg, 2016).

Forage crops improvements, *via* genetic modifications and genome editing, have the potential to play a key role in fulfilling the increasing demand for animal products. Therefore, risk assessment must ensure its safety for humans, animals, and the environment, in order to have an agricultural system economically and environmentally sustainable.

GM OR GE?

Genome edited (GE) plants are gaining popularity and are still classified as GMs in some jurisdictions, so the authors considered it necessary to address the issues on these crops as well in this manuscript. Both GM and GE crops and their products are required to be subjected to rigorous evaluations as a part of several regulatory requirements before their commercial release into the market. The legislation for GM and GE crops is based on the principle of assuring the safety of humans, animals, and the environment. Comprehensive studies on the risk assessment of GM food crops have been published previously (König et al., 2004; Delaney, 2015; Domingo, 2016; Gurău and Ranchhod, 2016; Dadgarnejad et al., 2017; Tutel'yan, 2017; De Santis et al., 2018; Kumar et al., 2018).

Some variation between regulatory frameworks of GM crops exists across jurisdictions. In the US, safety assessment of new GM crops is mainly performed though comparison with conventional counterparts, to provide scientific evidence that GM products can be considered as safe as a conventional product, if the characteristics and composition are substantially equivalent (FAO/WHO, 2000). The assessment of the Canadian authority also focuses on the products itself (Alexandrova et al., 2005). European regulation focuses more on the certification of the genetic modification process, instead of the products (European Parliament, 2001), as well as Argentina, Japan, and South Africa (Seyran and Craig, 2018). The Australian regulatory authority, the Office of the Gene Technology Regulator (OGTR), has followed a safety assessment model promoted by the World Trade Organization (WTO). Such model has provided as general guideline that safety assessment of GM plants should include consideration of the risk for humans, animals, and environment, and should be science-based (Dibden et al., 2013).

Similarly, there is variation in the regulation of GE products compare with GM across jurisdictions with much of the debate being polarized (Jones, 2015). Some regulatory bodies argue that genome editing techniques are very similar to those used to produce GMOs, so they should be regulated similarly (Seyran and Craig, 2018). However, the scientific community argues that unintended effects can always occur, regardless of the types of techniques used for crop development (Fernandez and Paoletti, 2018).

From a scientific point of view, the number of alleles changed in the process of traditional breeding, such as crossing and

selection of superior genotype combinations, should be typically higher than that of transgenesis or genome-editing approach. With other breeding techniques such as radiation and chemical-based mutagenesis, there is no established method for most crop species, to accurately assess the number of genes changed. In case of soybean, a combination of high-throughput DNA sequencing and molecular cytogenetics-based copy number variations assay, suggested that single nucleotide substitutions and structural variations generated during *Agrobacterium*-based transgenesis, was substantially less than those of radioactive-based mutagenesis (Anderson et al., 2016). The same approaches, also indicated that the frequencies of single nucleotide substitutions and structural variations generated through the transgenesis and mutagenesis, were considerably less than those found between existing soybean cultivars.

Different breeding techniques and their requirement for safety assessment before commercialization are described in **Figure S2**. Traditional breeding and mutagenesis, in general, change a high number of genes and mutations generally involve loss of function, while GM offers the advantage of knowing the actual gene(s) being inserted and usually involves a gain of function (Gepts, 2002). Nevertheless, the regulatory system assesses plants resulting from hybridization, radiation, or chemical induced mutagenesis, which may produce thousands of uncharacterized random mutations, as non-GM crops (Urnov et al., 2018). A recently published article that evaluates the impact of the risk assessment on public acceptance, concluded that the rigorous regulatory vigilance of modern biotechnology (transgenesis and gene editing), leads to public distrust and contribute to the idea that GM crops are unsafe. Therefore, a risk-disproportionate regulation of these technologies not only confuses the purpose of risk assessment, but also interferes with the delivery of beneficial technologies to the market (Herman et al., 2019).

As a result of such opinion polarization between the scientific community and regulatory bodies, the Court of Justice of the European Union (CJEU; Luxembourg) has decided to classify genome-editing technologies as genetic modification, submitting this plant-breeding approach to severe GM regulations and risk evaluation (Eriksson, 2019). In contrast, both the United States and Canada regulate new crop varieties according to their characteristics rather than by the method with which they are produced (Wolt, 2017). Therefore, gene-edited products without any transgene and with history of production and safe consumption, do not require special regulations (Hundleby and Harwood, 2019). The regulations implemented in the United States and Canada, is shared by a number of bodies including the UK's Biotechnology and Biological Sciences Research Council, the German Academies, the European Plant Science Organization, and the French High Council for Biotechnology (Laaninen, 2016).

Recently, the Australian regulator gene technology regulator (OGTR) has made amendments to make the legal position of genome editing clearer. These amendments clarify that organisms modified by SDN-1 (Site-Directed Nuclease 1) techniques, present no different risk than organisms carrying naturally occurring genetic changes, and do not require unnecessary regulation (OGTR, 2019). Although in many jurisdictions, a conclusive regulatory decision has not been provided, it is possible that the position regarding GE products of the United States, Canada, Australia, and some European national regulators, will affect regulatory decisions in other countries and regions.

Both GM and GE belong to the same or similar plant biotechnology applications. In some cases, plants are transformed using recombinant DNA to introduce the GE tools, and then, they are self-pollinated or crossed to remove the incorporated DNA, leaving only the intended mutation (Metje-Sprink et al., 2020). This ability of GE to produce changes without the integration of recombinant DNA can avoid GM regulations in some countries. The reduced risk of DNA integration into the genome provided by GE, along with the indirect human exposure to GM feed, can have great implications in the commercialization of new GE feeds.

LABELING POLICIES

Since the commercialization of the first GM crop in the mid-90s, some consumer groups have argued for more detailed and comprehensive labeling or extensive labeling to enable choice for the purchaser (Halle, 2008). Therefore, in response to the difficulty of maintaining zero tolerances, each jurisdiction has established a tolerance threshold for the involuntary or inevitable presence of GM material in non-GM products. If the amount of GM material in a product exceeds the tolerance threshold, the products should be labeled as containing GM material (Devos et al., 2009).

Labeling policies can include a ban on labeling, voluntary labeling indicating that a product is GM free, or mandatory labeling indicating that a product contains GM. When the latter is implemented, a legal tolerance threshold is set, and it also varies between countries, making trade and shipment of goods complex. In the US, Canada and Japan for instance, the legal tolerance threshold for conventional food and feed products has been set at 5%. Australia, New Zealand, South Africa, Brazil and China have tolerance thresholds at 1%, while in the European Union if a commercial product has more than 0.9% of GM material, it must be labeled as a GM product to inform consumers (Ramessar et al., 2010). Most countries have GM mandatory labeling laws, however, the US authority regards the nature of the product more critical than the process itself, so that GM labeling may be voluntary (Huffman and McCluskey, 2017).

To date, mandatory GM labeling laws, however, have largely excluded products from animals fed with GM feed (such as meat, milk, and eggs) as well as GM processing aids and enzymes (such as rennet for making cheese) (Van-Eenennaam and Young, 2017). In Europe, the labeling requirements (European Regulation 1830/2003; European Parliament, 2003) on GM food and feed, results in those agricultural products not requiring labeling. However, some EU member countries have established regulations and guidelines to label animal products voluntarily as non-GM, to allow consumers to choose products where no GM feed stuff were directly used in its production (Venus et al., 2018).

Even though food products derived from animals fed with GM feed crops do not require labeling, the safety assessment procedure for GM feed crops is the same than for GM food.

Regulatory bodies have based such decision on the fact that it is impossible to prevent the contact of GM feed material with humans, during cultivation, transport and storage of the crops (Food Standards Australia and New Zealand, 2014).

SAFETY ASSESSMENT

The scientific evidence that must be provided in the safety assessment of GM crops can vary among different legal jurisdictions (Alexandrova et al., 2005). However, a detailed molecular characterization of the transgene insertion, development of tracking and tracing methodologies to ensure legality and traceability, and environmental studies to enable coexistence frameworks, are common studies in the safety assessment of GM crops (Van Haver et al., 2003). Other studies, such as toxicological, allergenicity, nutritional, and horizontal transfer, have been performed following a case-by-case approach considering newly emerging scientific knowledge and technologies (König et al., 2004). The use of GM crops as a feed can reduce concerns around human safety and underline other features such as feeding value and nutritional equivalence (Flachowsky et al., 2002).

From an industrial point of view, the assessment required for the regulatory authorities could be divided into two groups; pre- and post-marketing issues. The former includes relatively standard technologies for all GM food and feed, such as molecular characterization and development of tracking and tracing tools for traceability purposes. Pre-marketing issues also involves technologies under development that varies on a case-by-case basis, including environmental, food/feed, toxicological, and allergenicity safety studies. Post-marketing issues are related with regulatory monitoring, which contemplate GM labeling and traceability (**Figure S3**).

Molecular Characterization

Molecular characterization of GM crops is a full description of the structural information of the transgene and stability of the trait (Li et al., 2017). It is the foundation of all GM product safety assessments before commercialization and also serves as a baseline for the development of detection and identification tools to satisfy traceability and labeling requirements (European Parliament, 2003). Stakeholders of both GM food and feed, must provide information on the genomic locus/loci modified, copy number of the inserted transgene, insertion site, and flanking regions (Guttikonda et al., 2016).

Selection of low insertion copy number DNA transformants is preferred for the subsequent safety assessment process as it facilitates risk and hazard characterization (Tiwari and Singh, 2018). The methods most commonly used to determinate the number of transgenes integrated have been Southern blot analysis and polymerase chain reaction (PCR), in its various formats such as real-time PCR (qPCR) (Li et al., 2017).

The Southern blot analysis involves a careful selection and broad screening of restriction enzymes and designing of probes, which in some cases dependent on prior sequence information of the transgene insertion (Urquiza and Silva, 2014). However, the approach is relatively time-consuming and laborious, and also includes a manual interpretation process. In addition, the result may not accurately reflect the copy number of a transgene, if sequence rearrangements have occurred, which have affected the position(s) of the restriction enzyme recognition site(s) in the inserted transgene(s) (Yang et al., 2005).

A qPCR-based assay can more accurately quantify the copy number of transgenes by comparing to an endogenous reference sequence (endogene), which has provided a simplified alternative to Southern blot analysis (Li et al., 2017). However, identification of a single copy reference gene is occasionally difficult in crop species, due to ancestral whole genome duplications or due to polyploidy, causing complex structures and genetic redundancy (Ren et al., 2018). To overcome the identification of a reference gene and dependency on DNA calibrations, droplet digital PCR (ddPCR), a method that identifies the absolute DNA copy number in a sample, has been proposed for determination of GM copy number (Głowacka et al., 2016; Dalmira et al., 2016).

Following identification of low-copy number transformants, the precise location(s) of the transgene(s) in the crop genome is required to be identified (Park et al., 2017). DNA sequencing approaches have been used for this purpose, and this process may also identify backbone sequence(s), which were not intended to be introduced from the transformation vector into the host genome (Kononov et al., 1997). The method traditionally used for this purpose was based on Sanger sequencing (Guttikonda et al., 2016). However, the second-generation sequencing (SGS) technologies have been proposed as a new tool for molecular characterization of GM crops, due to a larger sequencing capacity and potentially higher accuracy of the resulting assembled sequence (Kovalic et al., 2012; Yang et al., 2013; Pauwels et al., 2015; Arulandhu et al., 2016).

The SGS approaches can increase speed, scalability, and automation in the selection of potential valuable events on the basis of their molecular profile, facilitating post-transformation screening (Kovalic et al., 2012; Pauwels et al., 2015; Guttikonda et al., 2016). However, these technologies do not directly provide information about the position of the insertion(s) in native DNA, due to short read lengths (50–400 base pairs) (Goodwin et al., 2016), while transgenic constructs are typically thousands of base pairs. A computational process for alignment and/or assembly of the short sequencing reads is essential for the molecular characterization purposes, and repetitive elements commonly found in plant genomes can generate problems for the alignment/assembly procedure (Liang et al., 2014).

Recently, single-molecule sequencing, also termed third-generation sequencing (TGS) platforms, have been commercialized allowing a large increase in read length up to tens of thousands of bases per read (Loose et al., 2016). Read length is limited by the input DNA fragment size, but over 300 kb have been reported (Jain et al., 2016). The increment in read lengths up to tens of thousands can facilitate a more reliable GM characterization process, by extending the sequence reads of the flanking regions present in the captured fragments and potentially solving alignment problems.

The most common TGS platforms are products from Pacific Biosciences (PacBio, Menlo Park, CA, US) and Oxford Nanopore Technologies (ONT Oxford Science Park, Oxford). PacBio uses a sequencing-by-synthesis method to capture a single DNA molecule and a circular consensus sequence (CCS) to increase accuracy. The CCS uses a circular DNA template by ligating hairpin adaptors to both ends of target double-stranded DNA, so the DNA template is sequenced multiple times to generate a continuous long read (Weirather et al., 2017). Nanopore sequencing, uses nanopores to sequence native single-stranded DNA, by measuring the changes in an electric current passed across the pore as the DNA bases pass through, disrupting the current to different levels with different nucleotides (Giordano et al., 2017).

Nanopore sequencing offers potential benefits in molecular characterization of GM products compared with PacBio, since it delivers raw data in real-time, is relatively easy to manipulate, and has low setup costs. The MinION from ONT is a portable device that has been successfully assessed for detection of unauthorized GM products (Fraiture et al., 2018). Further assessment demonstrated the capability of the MinION to determine the full molecular characterization of three transgenic crops (ryegrass, canola and clover) within 48 hours.

Although, new guidelines are emerging from regulatory bodies to generate pre-market submission of data using whole genome sequencing (Health Canada, 2019) and NGS (UCD Centre for Food Safety et al., 2018). Sequencing approaches are typically being used in tandem, for example Sanger sequencing with SGS or SGS with TGS, for verification and validation purposes during risk assessment of GM crops (Boutigny et al., 2019).

In short, GM forage species including Alfalfa (Barros et al., 2019), Switchgrass (Dumitrache et al., 2017), Sorghum (Rooney et al., 2007) have been characterized adopting the same technologies used for GM food. However, the expression of introduced traits in plant parts not used for food, should be considered. For instance, a GM plant can be produced with specific production of the Bt toxin only in leaves, preventing insect attack and removing the exposure of humans to the compound when consuming its grains (OECD, 2003). However, in such cases those plant parts would substantially increase the exposure of animals to the GM toxin when consuming them, and this difference must be taken into account in the molecular characterization.

GM Traceability

GM traceability describes a system that enables tracking of GM food/feed products at all stages of the supply chain (Giraldo et al., 2019). Detection methods for GM products in different matrixes or substrates, such as grain, flour and forage, are not only important to ensure legality and traceability, but also to comply with GM labeling regulations (European Parliament, 2003).

Methods for GM detection and identification usually rely on certified reference materials that are in powdered form, however, routine detection must be performed in different agricultural and food products (Cankar et al., 2006). The selection of DNA extraction protocols is of crucial importance, since the DNA can be present in low amounts, carrying inhibitors or degraded (SanJuan-Badillo et al., 2014). Therefore, the extraction method should be evaluated for each agricultural product, guaranteeing high DNA yield and purity (Turkec et al., 2015).

The method chosen to comply to traceability and labeling requirements, should be sensitive enough to detect the transgene(s) at levels below the corresponding jurisdiction tolerance threshold (e.g., 5% in US, 1% AU, and 0.9% in EU) (Ramessar et al., 2010). Additionally, it should be able to detect the transgene(s) from raw agricultural commodities entering the feed production chain. For instance, fresh leaves, dry leaves (hay), pollen, seeds, tillers or stems, and forage that could enter the feed chain as unprocessed material (Ardizzone et al., 2018; Giraldo et al., 2019).

Currently, qPCR is the standard method used in national reference laboratories for detection and quantification of GM events (Dalmira et al., 2016). The requirement for reference material to be used as calibrants, which sometimes are not commercially available, limits its effectiveness (Dobnik et al., 2016; Dalmira et al., 2016). The GM product detection process followed by national reference laboratories consist of two consecutive steps; first, a qPCR screening of vectors commonly found in GM products, such as the 35S promoter from cauliflower mosaic virus, *Agrobacterium tumefaciens* (tNOS) and selectable markers. Then, the samples with a potential presence of GM materials, are tested using the corresponding GM event-specific method (Fraiture et al., 2017).

Droplet digital PCR (ddPCR) technologies use the same DNA amplification principles as qPCR, but the technologies can provide a higher quantification precision through partitioning PCR mix into thousands of nanoliter-sized droplets in which PCR amplification is carried out (Dobnik et al., 2016; Dalmira et al., 2016). Features such as absolute quantification, avoidance of using standard curves, and high resilience to inhibitors, makes ddPCR a promising alternative for GM event detection (Rački et al., 2014; Corbisier et al., 2015).

SGS technologies have also been proposed to comply with the requirements for GM traceability due to the ability to detect all target sequences in multiple samples without the development and validation of target-specific methods and reference material (Arulandhu et al., 2018). However, the requirement of bioinformatics knowledge for data analysis and more sophisticated devices limits its use in routine GM event detection (Park et al., 2017). **Figure S4** compares qPCR, ddPCR, and SGS in terms of GM identification and quantification, multiplexing capacity and ability to detect known and unknown sequences. It also underlines that the cost and processing time increase from qPCR to ddPCR and to SGS.

In recent years, significant effort has been performed to replace the time-consuming and expensive qPCR screening procedure (Holst-Jensen et al., 2016; Salisu et al., 2017). As a result, other technologies are been evaluated including ddPCR (Dalmira et al., 2016; Dobnik et al., 2015; Köppel et al., 2015; Demeke et al., 2016; Dobnik et al., 2016; Gerdes et al., 2016; Głowacka et al., 2016; Iwobi et al., 2016; Grelewska-Nowotko et al., 2018; Niu et al., 2018; Corbisier and Emons, 2019; Giraldo et al., 2019), SGS (Willems et al., 2016; Fraiture et al.,

2017; Arulandhu et al., 2018), DNA enrichment approaches (Arulandhu et al., 2016) and combined strategies of DNA walking and SGS (Fraiture et al., 2017).

However, all the above methods are lab-based and time consuming, so that the development of rapid and portable devices with more routine throughput screening methods are likely to continue into the immediate future. Recent research related to *on-site* detection of GM crops include; loop-mediated isothermal amplification (LAMP) (Hardinge et al., 2018; Singh et al., 2019; Loo et al., 2019), LAMP and a lateral flow biosensor (Cheng et al., 2017), PCR and a lateral flow biosensor (Gao et al., 2019), handheld field-portable qPCR systems (Nguyen et al., 2018; Russell et al., 2018), and nanopore sequencing (Fraiture et al., 2018; Russell et al., 2018).

Technologies to fulfil regulatory requirements for GM food traceability have been established. The qPCR-based method is suitable for a high-throughput screening of transgene(s), and the ddPCR-based approach can provide a higher accuracy of the measured GM product concentration, especially at a low level. Although, the same approaches of GM food may be used for GM feed to comply the regulatory requirements, for GM feedstuff special considerations should be given to the use of plant parts not used for human food and by-products from other industries using GM plants (OECD, 2003).

Environmental Safety Studies

Environmental risk assessments (ERA) aim to determine whether a new GM crop variety has direct effects on the natural environment (Hilbeck et al., 2011; Devos et al., 2016). Although a range of factors, such as effects on biodiversity, modification of soil and water quality, and disease and weed control, must be considered in this process, the major concern of ERA is gene flow (GF) of the transgene(s) to wild relatives (Warwick et al., 2009).

GF is a result of the movement of gametes or individuals from a specific population to another, which may generate a significant change in the allele frequency of the receiving population (Slatkin, 1987). This phenomenon not only has been observed between populations of the same species, but also between closely related species (Wilson and Manhart, 1993; Bartsch et al., 2002). In case of natural plant populations, such movement can happen *via* seeds, vegetative propagules or pollen and its importance varies among plant species (Tsatsakis et al, 2017). General approaches to quantify GF use foreign herbicide and antibiotic resistance genes to provide insight into the rates and importance of hybridization (Mallory-Smith et al., 2015). However, morphological and molecular markers are also required to assist with rapid identification or to identify/confirm hybrids.

To allow GM and non-GM crops to exits in mutual tolerance and minimize undesired GF, a concept defined by the term "coexistence" was introduced. Coexistence refers to the right that consumers have to choose between conventional, organic, and GM crop production, in compliance with the legal obligations for labeling defined in each jurisdiction (Devos et al., 2009). Although, different varieties of the same species have coexisted prior to appearance of GM crop variety, the need for the strategies to manage the consequence of inadvertent fertilization have become more outstanding with the commercial release of GM crops (Ramessar et al., 2010).

A potential risk of transgenes GF is cross-pollination between GM crops and native species, which has been intensively discussed since the commercial realize of GM plants (Messeguer, 2003; Kuparinen et al., 2007; Warwick et al., 2009; Tsatsakis et al., 2017). Pollen-mediated GF has been reported during production of commercial GM crops including maize, rapeseed, cotton, soybean, and creeping bentgrass (Reichman et al., 2006; Chifflet et al., 2011; Baltazar et al., 2015; Rizov and Rodriguez-Cerezo, 2015; Loureiro et al., 2016; respectively).

One of the most common coexistence measures to reduce pollen-mediated GF is isolation distances. It is defined as the minimum distance between GM and non-GM crop fields of the same species that should prevent a cross-pollination rate from reaching to threshold levels (Cunliffe et al., 2004). Multiple factors, such as population size, distance, and flowering synchrony between donor and receiver fields, as well as local wind conditions, all influence the determination of an appropriate isolation distances (Devos et al., 2009).

Different isolations distances have been determinate to avoid or minimize cross-fertilization between GM and non-GM fields. **Table 1** describes different isolation distances required for the four major GM crops (maize, canola, soybean, and cotton), to maintain cross-fertilization ratio below legal tolerance thresholds. For instance, to maintain cross-fertilization levels below 1% an isolation distance of 20 m is require for maize (Baltazar et al., 2015), 9 m or more for cotton (Baltazar et al., 2015), and 5 m for soybean (Rizov and Rodriguez-Cerezo, 2015).

In GM forage species, undesired pollination can be the major type of GF, since forage grass species typically exhibit a highly outcrossed nature and are wind-pollinated (Holme et al., 2013). Smith and Spangenberg (2016) reviewed the most important coexistence strategies in outcrossing forage species, highlighting that a coexistence framework for the dominant cross-pollinated grain crops (canola and maize) is already well established in Europe, as well as alfalfa in the US. Hence, existing principles such as development of detection techniques, segregation and agronomic management can be applied to other forage crops when developing coexistence frameworks.

TABLE 1 | Cross-fertilization level in different isolation distances for the major forage crops commercially available.

Crop	Isolation distance	Cross-fertilization level	Reference
Maize	50 m	<0.5%	Sanvido et al., 2008
	20 m	<1%	Baltazar et al., 2015
Canola	30 m	<0.03%	Staniland et al., 2000
	33–200 m	<0.015%	Cai et al., 2008
Soybean	5 m	0.9%	Rizov and Rodriguez-Cerezo, 2015
	10 m	0.1%	
Cotton	10 m	<0.9%	Loureiro et al., 2016
	>9 m	<0.1%	van Deynze et al., 2005
Alfalfa	150 m	1.39%	Fitzpatrick et al., 2003
	500 m	0.08%	

Compared with commercially available grain crops, studies on pollen-mediated GF in wind-pollinated forages is scarce. A study on transgenic alfalfa (*Medicago sativa* L.) using the *cp4 epsps* transgene as marker, showed that a 500-m isolation distance is required for maintenance of the cross-fertilization level below 0.1% (Fitzpatrick et al., 2003). Rigid ryegrass (*Lolium rigidum*) gene flow was reduced from 37.8% at 0 m and 0.93% at 100 m (Busi et al., 2008). In other species with high levels of self-compatibility such as barnyardgrass (*Echinochloa crus-galli*), cross-fertilization rates at 0-m distance was 12.5% (Bagavathiannan and Norsworthy, 2014) and for tall fescue (Festuca arundinacea) 5% were detected at 50 m and less than 1% at 150 m (Wang et al., 2004).

Transgenic GF of the commercial GM grains has been intensively studied and discussed as a main part of ERA, some of such information can be apply to GM forage crops, the potential risks may be depending on the nature of transgenic traits. For GM forages considerations such as life cycle, since forage crops are largely perennial not annual or biannual, can have repercussions on pollen flow and it can remove management of gene flow through farming systems temporally.

Different regulations and laws about GM plants create complexities for the movement of agricultural products between borders and has a significant impact on international trade. The Cartagena Protocol on Biosafety is an international treaty that aims to protect biodiversity from the potential risk of GMO resulting from modern biotechnology. Such Protocol dictates that GM plants should follow the precautionary principle, which states that "In order to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities" (Secretariat of the Convention on Biological Diversity, 2000). Therefore, standardized methods of ERA for GM crops has not been established and it is managed by individual laws in each country. Usually, a case-by-case assessment needs to be performed and the methods used for GM food and feed are the same.

Feed Safety Studies

The vast majority of forage crop products are fed to livestock; hence, any human consumption of GM feedstuff is an indirect effect and can easily be regulated and mitigated to ensure complete safety. Feed safety studies examine whether the genetic modification could unintentionally increase the potential toxicity or allergenicity of the transgenic plant for humans or animals, as well as changes in nutritional characteristics (Pauwels et al., 2015). As forage crop products are predominantly eaten by animals and human consumption of GM feedstuff represents an indirect effect, and this type of assessment should be unique to GM feed. Feeding studies focus on answering three main issues; substantial equivalence, the safety of the new crop for humans and animals, and the safety of the product derived from animals raised on transgenic feed. Those issues are comprehensively discussed in Aumaitre et al. (2002) and Ramessar et al. (2007).

According to The Organization for Economic Co-operation and Development (OECD), the substantial equivalence concept refers to the idea that existing food products can serve as a basis for comparison when assessing the nutritional value and safety of food modified by modern biotechnological methods (OECD, 1993). This comparison helps quantify the effect of the transgene as well as understanding the variation in the natural species for the trait being modified. The analysis of chemical composition would serve as the first step for the nutritional evaluation. Such comparison is, however, largely based on the premise that an existing cultivar with a history of safe use, can serve as a comparator when evaluating the safety of a GM food/feed (Flachowsky et al., 2012). The type of comparators, key characteristics selected, and interpretation of compositional data may vary change among different countries. The OECD, an intergovernmental organization in which representatives of 30 industrialized countries in North America, Europe, and the Pacific, have consensus documents about common GM plants, which are a valuable source to ensure a consistent assessment (OECD, 2000).

Some examples of GM products that passed the substantial equivalence test include a maize line with the sb401 gene incorporated to increase lysine content, the equivalent test with a conventional protein quality maize (Nongda 108) showed that the lysine-rich maize was safe (Tang et al., 2013). The compositional equivalence of a herbicide-tolerant rice (Bar 68-1) exhibited no significant differences, when compared with its isoline (D68) (Li et al., 2008). The evaluation of a *Bt* soybean (products Mon 87701 * Mon 89,788) that confers pest resistance and glyphosate resistance, concluded that *Bt* soybean was as safe as its traditional counterparts (Berman et al., 2009). Similarly, Bollgard II cotton (event 15985) with insect resistance properties was compared with traditional cotton varieties, and the results demonstrate that it was as safe and nutritious as conventional cotton for food and feed use (Hamilton et al., 2004).

When substantial equivalence of GM feed product to its traditional analogues cannot be concluded, a further assessment comprises of the following steps: *in silico*, or *in vitro* preliminary studies, the study of nutritional value of the products; quotas in the diets of animals; methods of use in nutrition, and during lactation; digestibility, evaluation of intake of individual components (if the expected intake is more than 15% of the daily requirement); impact on the intestinal microflora (if GM product contains live microorganisms) (Levitsky 2016). The safety assessment of each country can be different and in some jurisdiction animal experiments are not required. For instance, the OECD consider that GM plants where compositional analyses demonstrate no significant differences from the comparator, animal feeding studies with target species will add little to a safety assessment, so that nutritional equivalence can be assumed (OECD, 2003).

As a part of assessment for animal safety issues, the *in silico*, or *in vitro* studies also can provide an estimation of the impact of GM feed in the nutrition of target animals, prior to the feeding studies involving animals. *In silico* evaluation may serve to identify changes in key nutritional components and *in vitro* simulated gastric and intestinal fluids to study the digestive stability of novel proteins (ILSI, 2007; DBT, 2008). All laboratory studies should be conducted according to the internationally recognized guidelines (OECD, 1998). The information obtained from these studies help to determine the need for future *in vivo* studies with target animals (EFSA, 2008). The OECD has

developed guidelines for different animal and *in vitro* testing (OECD, 1995: Guidelines 401, 407, 408, 414, 415, 416, 420, 421, 423, 425, 451, 452, 453, 474, 475, 478, 486).

Tudisco and Infascelli (2014) used an *in vitro* gas production technique to compare the nutritional value of GM corn and soybean with their traditional counterparts. They found that the fermentation kinetics of GM corn and gas production of GM soybean were respectively faster and lower compared with their counterparts. Another *in vitro* experiment using batch fermentation models assessed the possibility of transfer of cry1Ab transgene to porcine jejunal microbiota, concluding that the transfer of transgene was not detected (Buzoianu et al., 2012a).

Swiatkiewicz et al. (2014), comprehensively reviewed the published information on health status, blood parameters, immunological characteristics, histopathological examination of cattle, poultry and fish. Concluding that the quality and potential risks for human consumption of livestock products such as meat, milk, and eggs and the metabolic parameters were not significantly affected when livestock were fed with commercialized GM crops. Although, there is a large proportion of studies on livestock animals, most of them focuses on the possibility of horizontal gene transfer (HGT) from GM crops to animal tissues.

Feeding test, using model animals has been conducted for a safety assessment of GM food crops. The main animal species, which would consume the GM feed products are, however, livestock, such as cattle, sheep, swine, and poultry (Nadal et al., 2018). Cattle (dairy and beef) and sheep belong to the ruminant category, with a unique digestive system that gives them the ability to obtain nutrients from forage crops by breaking down its cellulose content in a special stomach compartment using microbial actions. On the other hand, swine have a monogastric digestive system with an enzymatic stomach very similar to humans, and poultry have an avian digestive system without teeth to chew the feed (France et al., 2006). Cattle and sheep are herbivores, whereby their diet is mainly forage-based, while swine, poultry, and humans are omnivores, so they eat mostly grains and some plants (Flanders and Gillespie, 2015).

Until now the safety assessment of GM plants has focused in human exposure, animal safety while not ignored, has received less importance (Aumaitre et al., 2002). Pigs have been widely used as models for humans, because of their similar gut anatomy and physiology (especially mucosal immunity) and nutritional requirements (Ladics et al., 2010). Rodent tests have received the greatest importance from regulatory bodies, especially for toxicological and allergenicity studies of the products of introduced genes. However, these animals are not usually feed with the entire GM plant or their by-products, while livestock animals have greater exposure to GM feedstuff, compromising a high percentage of their diet on a daily basis and often for their complete lifespan (Aumaitre et al., 2002). Additionally, variations in the livestock system can determine the evaluation profile, for instance, forage grasses will be relevant to ruminant species and have little to no value to monogastric species.

Due to their anatomical and eating habits differences, it is impossible to assess the safety of a transgenic GM feed with a single and unified test. A more specialized case-by-case approach is essential for the safety assessment of GM feed. Development of standardized *in silico* and *in vitro* approaches, can reduce time and cost for this process and avoid the use of animals. When required, *in vivo* feed studies offer the possibility of conducting feeding trials in the target species, something not possible with GM food in humans.

Toxicological Studies

The purpose of toxicological studies is to characterize intended changes and detect active substances or compounds that could have unexpected toxic effects for non-targeted organisms (Van Haver et al., 2003). All toxicity assessment for GM material should be performed based on a case-by-case approach, considering the toxicological profile of new introduced substances (Domingo, 2007).

The methods to assess the toxicity of a specific compound in the body, usually compromise the use of animal studies, considering the target species and the critical effects (Levitsky, 2016). However, new strategies to identify GM feed anti-nutrient or toxicants include research on the in-planta metabolism pathway, such as "-omics" techniques that may generate a better understanding of the complex pleotropic effects of new plant cultivars (Fernandez and Paoletti, 2018). Additionally, *in vitro* assays with gastric enzymes, cultured cell lines, receptor proteins, and *in vivo* animal studies can be performed (Van Haver et al., 2003).

High-throughput "-omics" profiling techniques, which involve the use of metabolomics, transcriptomics, and proteomics, have been suggested as a nontargeted approach to detect unintended effects in GM crops (Ricroch, 2013). Profiling studies using omics techniques include GM glyphosate-tolerant soybean, where some specific metabolites were different compared with the isogenic line and the results were explained by modifications in the regulation of the shikimate pathway (Garcia-Villalba et al., 2008). Nevertheless, a GM stacked rice carrying the herbicide-resistant gene *bar* and insect-resistance *cry*, was found substantially equivalent to its conventional genetic breeding and natural genetic cultivars, when their proteome profiles were compared (Gong et al., 2012). A review of the safety assessment of GM crops using omics techniques, indicated that transgenesis has less unintended impacts than conventional breeding (Ricroch, 2013). Another study showed that there were more transcriptomic alterations in mutagenized plants than transgenic plants (Batista et al., 2008).

Several toxicological studies in GM feed using omics techniques, involve the analysis of fungus or their secondary metabolites. For instance, mycotoxins, which are undesired substances produced by crop-related fungus. In hybrids *Bt* maize, one of the principal components of feeding formulas for livestock, plants experienced less fumonisin concentration compare with its isoline (Bowers et al., 2014). It was hypothesized that the reduction of fumonisins was due to the pest reduction in the GM maize, since the fungus spore migration and colonization may be facilitated with damages from insects. Therefore, it could be concluded that GM corn can provide reductions in the risk of fumonisins contamination, but not increment of toxicological risk for animals.

Similarly, in the transgenic high-energy perennial ryegrass, an evaluation of alkaloids, secondary metabolites produced by endophytic fungus, found that the alkaloids concentration in transgenic plants was same or lower compare with the isogenic line. The lower alkaloid concentration could be partly attributed to higher growth of transgenic plants, which could generate a dilution effect in the modulation of fungal biomass (Giraldo et al., 2018).

When performing *in vivo* studies, toxicology acute (14 days studies), subacute (28 days studies), chronic (90 days studies), or specific toxicity (reproductive, mutagenicity, etc.) assessment can be considered (Levitsky, 2016). In a chronic study feeding mice with crushed *Bt* cotton seeds, *cry* genes and *tnos* promoter were detected only in intestinal tissue, while they were not found in stomach, blood, liver, kidney, heart, and brain (Sajjad et al., 2014). In long term studies (>100 days), no toxic effects were found in cattle and chickens fed with *Bt* maize. They concluded that short fragments of plant chloroplasts (<200 base pairs) can be detected in blood lymphocytes of cattle, but DNA fragments were not detected in other organs investigated (muscle, liver, spleen and kidney) (Einspanier et al., 2001). Similarly, some small changes in the metabolic profile of sheep fed with *Bt*176-maize were found when compared with non-GE maize, according to the authors such changes did not represent a health hazard (Snell et al., 2012). In pigs fed with *Bt* maize (MON810 event), although all serum biochemistry parameters were within the normal reference interval for pigs, small differences were reported. The authors concluded that the differences were the result of a lower enzyme-resistant starch in the GM compare with the non-GM control (Buzoianu et al., 2012b).

Safety assessments of GM feeds should consider the maximum level present in any plant part consumed by animals or in any by-product used as a feed ingredient, since the introduced traits can express differently in the plant parts, affecting the concentration of novel proteins. This can have implications in the level of exposure, selection of comparators and determination of the novel protein concentration used in acute/sub-chronic toxicity studies (OECD, 2003).

In short, a case-by-case approach is also required for toxicological assessment of GM crops, and the assessment procedure has not been standardized. A comparison with conventional counterparts has been a common approach for GM food products, and a similar approach may be used for forage products. A more cautious and stringent examination may be required for GM feed, due to that a wider range of plant organs may be used for animal consumption than those for human consumption, and storage conditions of the GM forage products may be less uniformed and controlled than GM food.

Allergenicity Studies

Allergenicity and toxicological studies may be assessed at once, since both are designed to detect newly expressed substances. Allergenic reactions can cause more severe symptoms, but usually to only some individuals, while toxicity is predictable and reproducible between individuals as it affects the majority of exposed individuals with only minor differences in susceptibility (De Santis et al., 2018).

In US, concerns about potential risks for allergy have arisen from GM food crops, including one under development for several times. A 2S albumins gene from Brazil nut was introduced into a soybean cultivar, for a purpose of nutritional enhancement. The transgene products, however, were identified to have potential allergic risks for human, especially those with allergy to the Brazil nut, and development of the GM soybean cultivar was suspended (Moreno and Clemente, 2008; Delaney, 2015). Concerns for the Cry9C protein, a type of insect pest resistance protein from *bacillus* also arose, due to a higher stability to heat and possible prolong time for digestion (Wiedinmyer et al., 2000). As a consequence, the StarLink maize, an unauthorized maize containing the Cry9C transgene, was not approved for human consumption by the US authority (Zhang et al., 2016).

In case of a GM feed safety assessment, both human and animals may need to be included in an allergenicity study as test subjects. An allergenicity assessment for animals could be performed with a similar approach to that for human. There is, however, currently no standardized procedure to predict allergenic reactions to non-endogenous proteins even in humans. The European Food Safety Authority (EFSA) has recommended using animal models to evaluate the sensitizing potential of novel proteins on a case-by-case basis (Marsteller et al., 2015). The most common species to assess GM allergenicity are rodents, also referred to as a rat 90-day evaluation, which is now compulsory in the EU for new GM crops (Hong et al., 2017). However, the published studies on rats, mice, and pigs, aimed to assess the allergic risk of humans (Ladics et al., 2010), using animals mostly as food allergy models.

The most common approaches to assess allergenicity include amino-acid sequence homology, *in vitro* digestibility tests, serum screening and animal models (Van Haver et al., 2003). Amino acid sequence homology or similarity uses bioinformatic methods to determine the possibility, that a novel protein can be closely similar to a known allergen that can create a risk of cross-reactions (Naegeli et al., 2017). However, such bioinformatic methods cannot predict the likelihood that the novel protein might become a *de novo* allergen, so other methods like *in vitro* digestibility tests, serum screening, and animal models may need to be used (Ladics et al., 2010). The *in vitro* pepsin resistance assay is the most commonly used protein digestion test, which provide information about the susceptibility of a novel protein to digestion. This assay can be used as an additional evidence of possible adverse reactions to GM food/feed, since gastrointestinal digestion can affect the immunogenicity of dietary proteins related to both IgE and non-IgE reactions (Naegeli et al., 2017).

Serum screening and immunoassays are alternative ways to assess endogenous allergens, using sera from individuals with relevant allergies. Although, these types of assays are the current reference method for *in vitro* detection and definition of an allergenic proteins, detection of allergic animals pose limitations for the applicability in the safety assessment of GM feed. Alternatively, there are other analytical and molecular profiling techniques, which can serve as alternative tests for

the comparative assessment of the endogenous allergenicity between the GM plant and its non-GM comparator (Fernandez et al., 2013).

Information on immunological responses, and particularly allergenic reactions on livestock fed with GM products is scarce and is likely to need more extensive research. Nevertheless, allergenicity assessment of GM feed offers the advantage of direct evaluations in target species, something not possible with GM food in humans.

Horizontal Gene Transfer

HGT refers to the movement of a genetic material to a living cell or organism across boundaries between species. In the case of GM organisms, movement of transgene(s) into other species, especially microorganism, or natural population of the taxonomically related species have been concerned, as such transfer may have impacts on human/animal health, and natural environments. (EFSA, 2007; EFSA, 2009; ADAS, 2013; Nicolia et al., 2014; Van Eenennaam and Young, 2014; Giacomo et al., 2016). This section relates to environmental, feed safety, toxicological, and allergenicity studies, since the transfer of recombinant DNA into other organisms can affect the health of humans, animals, and the environment.

Although assessment of the digestion fate of recombinant DNA or its new proteins can be performed using *in vitro* gastric or intestinal fluid-based digestion systems to assess HGT in bacteria (Fuchs et al., 1993; Wehrman et al., 1996; Entransfood 2004; Sharma et al., 2004; Bertrand et al., 2005), studies using cattle species have been also commonly conducted evaluating the digestive process of recombinant DNA and proteins from feedstuff (Faust and Miller, 1997; Ash et al., 2000; Aulrich et al., 2001; Jennings et al., 2003; Aumaitre, 2004; Phipps et al., 2005; FASS, 2006; Rizzi et al., 2012; Swiatkiewicz et al., 2014; Van Eenennaam and Young, 2014; Levitsky, 2016; Van Eenennaam and Young, 2017; Nadal et al., 2018). For example, recombinant DNA has been found in ruminal solid phase and duodenal digesta of cattle, but the DNA was not detected in liquid ruminal and duodenal phase, as well as milk, blood, and faeces. Those results indicate a rapid degradation of the transgene in the first digestive stages (Phipps et al., 2003).

In studies using cows fed with transgenic maize, recombinant DNA was not detected from milk (Faust and Miller, 1997; Giacomo et al., 2016), blood, muscle, kidneys, liver, or spleen (Einspanier et al., 2001). Also, recombinant DNA was not detected from milk of cows grown with feed including up to 26% of transgenic glyphosate-tolerant soybean (Phipps et al., 2002). Similarly, in studies on poultry fed with transgenic maize, recombinant DNA was not detected from muscle, kidneys, liver, and spleen, as well as eggs (Einspanier et al., 2001). Only a little possibility of incorporation of recombinant DNA into the genomes of human or animal of digestive organs has been suggested from some of these studies, and the majority of the studies concluded that such risk of horizontal transfer of transgene is insignificant (Chambers et al., 2002; Ramessar, et al., 2007; Levitsky, 2016). As the risk of incorporation of recombinant DNA into germ cells should be even lower, the possibility of inheritance of the recombinant DNA into the following generation should be insignificant.

In case of GM feed products, possible risks on human health may need to be also considered, as the products are indirectly consumed by human *via* cattle. The rapid digestion process of recombinant DNA, evidenced by the studies described above, suggests that the indirect risks on human health are low. The safety of food products produced from animals feed on transgenic crops has also been widely studied, and in the majority of studies, any recombinant DNA was not found in animal products. However, in a couple cases, short fragments of the recombinant DNA were detected in milk (Phipps et al., 2002; Agodi et al., 2006). The authors, however, interpreted their presence as a contamination of faecal or airborne material with feed particles (Agodi et al., 2006).

Most of the scientific finding till the date has not found significant risk directly related with the consumption of GM crops and these findings can be extrapolated to forage species. In general, proteins derived from recombinant DNA, as any protein, are degraded in the gastro-intestinal tract, while dietary DNA is not totally degraded and, in some cases, small fragments can be found into animal tissues (Nadal et al., 2018).

CONCLUSION

The use of molecular breeding technologies such as genetic modification and genome editing in forage crop species can help farmers address the challenges of climate change, sustainability, and global food security. Information about the safety assessment of GM forage crops intended only for animal feeding is scarce, even though most of GM products and its biomass is destined for livestock animals feeding. The regulatory assessment scheme is designed for GM food and a similar approach can be used for the assessment of forage crops considering the differences in risk profile of the contrasting outcomes.

The same techniques used for molecular characterization, GM traceability, environmental safety studies and HGT can be used for both GM food and feed. However, specific adjustments to the techniques may be required, considering that parts of the GM plant used to feed livestock may have different concentrations of the novel proteins, changing its level of exposure. Feed, toxicological, and allergenicity studies for GM feed only destined for animal consumption are not well defined. The design of specific strategies to cover GM feed safety can be more targeted for the safety of species that are going to consume the crop, while also potentially having a lower regulatory cost.

A new framework for the risk assessment procedure, for both GM food and feed, is necessary in order to make a more efficient use of resources and avoid unnecessary evaluation. The final aim should be to assess GM novel crops in a more effective way, to increase the commercialization of products with potential to provide economic and health benefits to consumers and producers.

AUTHOR CONTRIBUTIONS

PG has written the manuscript under the supervision and drafting of HS, NC, and KS. The review was finally edited by HS, GS, NC, and KS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01592/full#supplementary-material

FIGURE S1 | Difference between GM food and feed.

FIGURE S2 | Different breeding techniques and its requirement for safety assessment before commercialisation.

FIGURE S3 | Pre and pos-marketing issues to be solve before GM crops commercialisation.

FIGURE S4 | Comparison of qPCR, ddPCR and NGS.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

Figure S1: Difference between GM food and feed.

Figure S2: Different breeding techniques and its requirement for safety assessment before commercialisation.

Figure S3: Pre and pos-marketing issues to be solve before GM crops commercialisation.

Figure S1: Difference between GM food and feed.

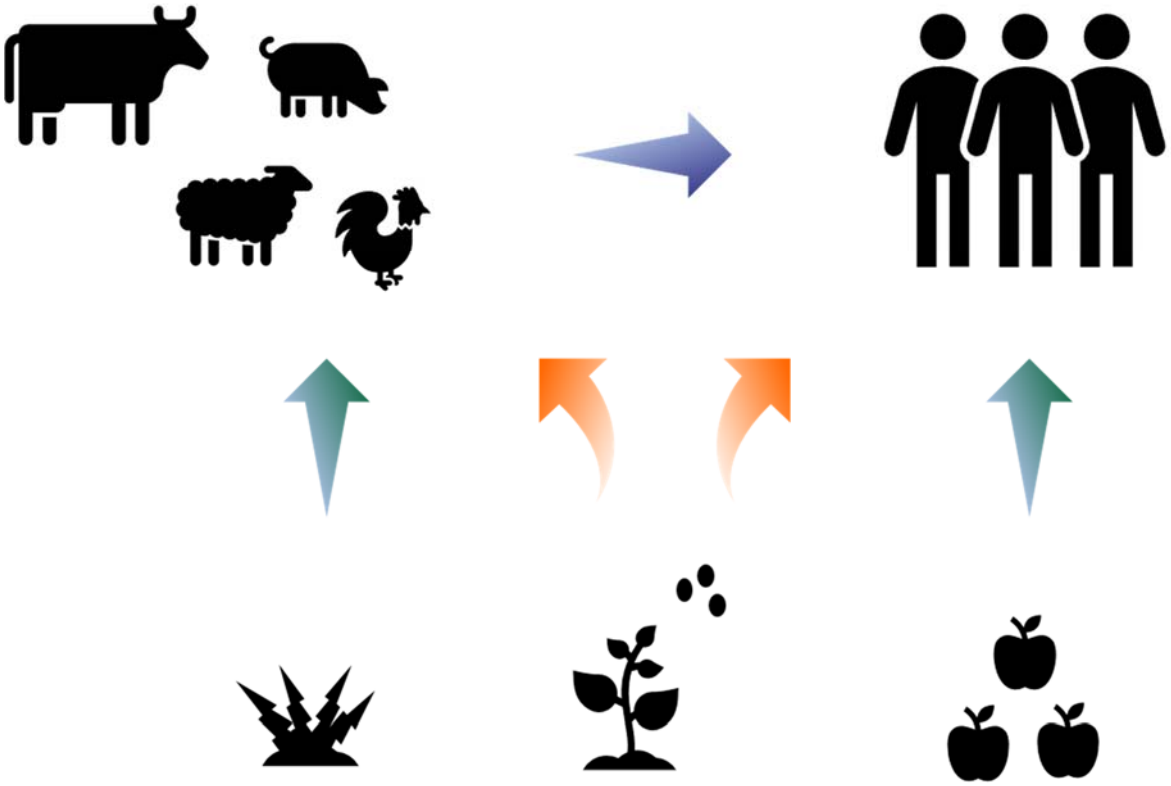


Figure S2: Different breeding techniques and its requirement for safety assessment before commercialisation.

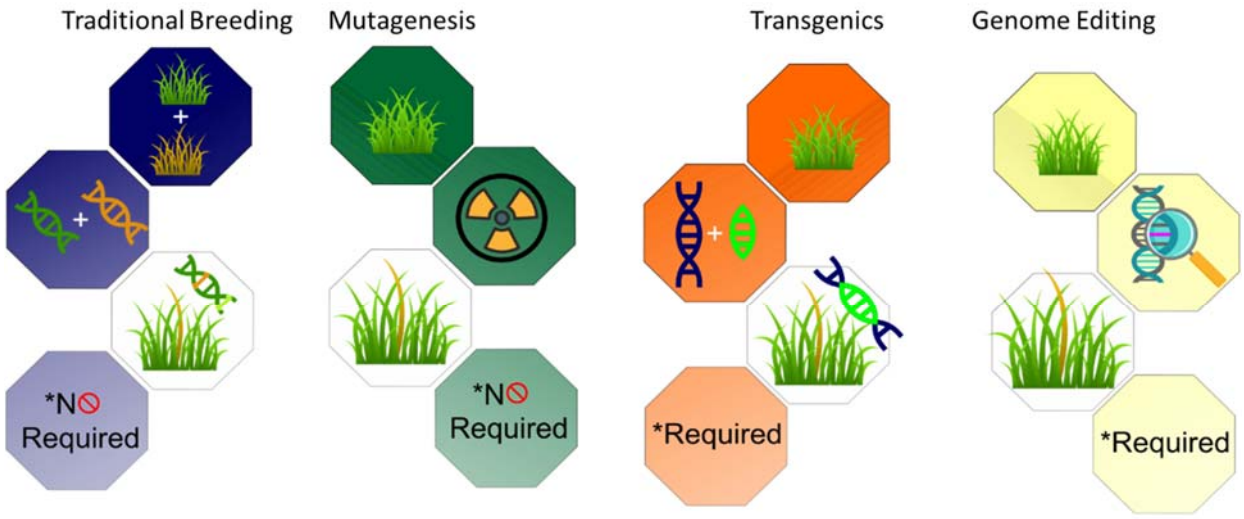


Figure S3: Pre and pos-marketing issues to be solve before GM crops commercialisation.

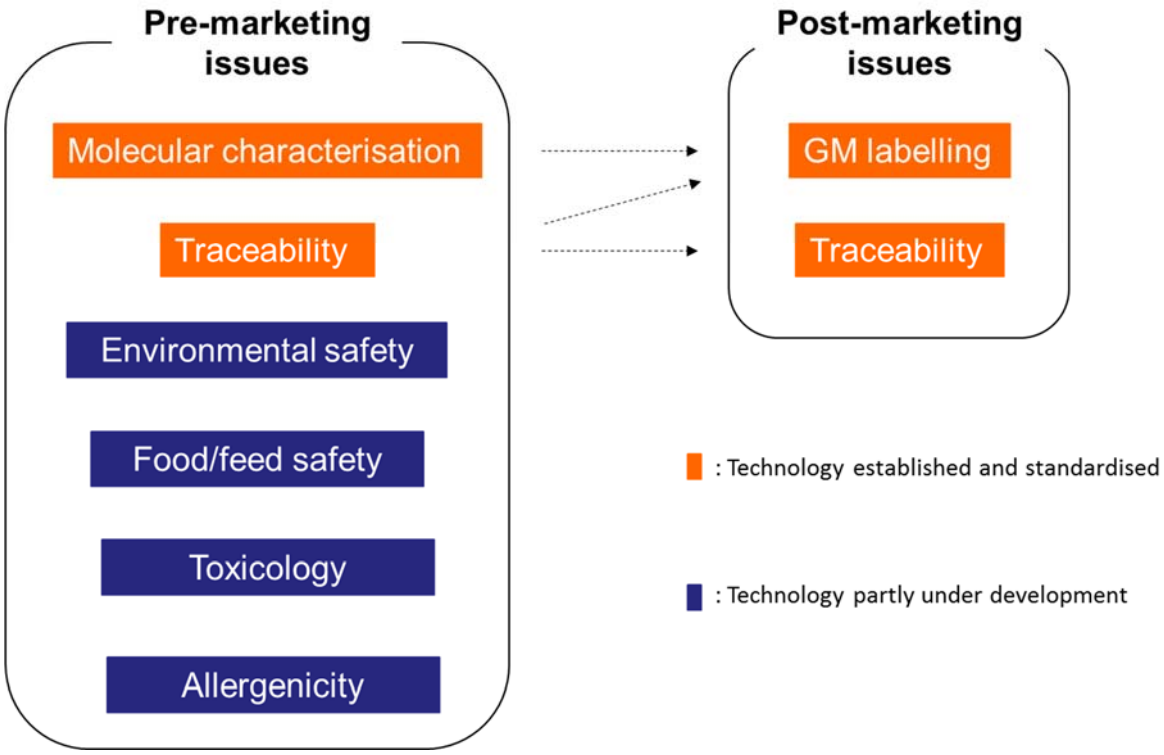
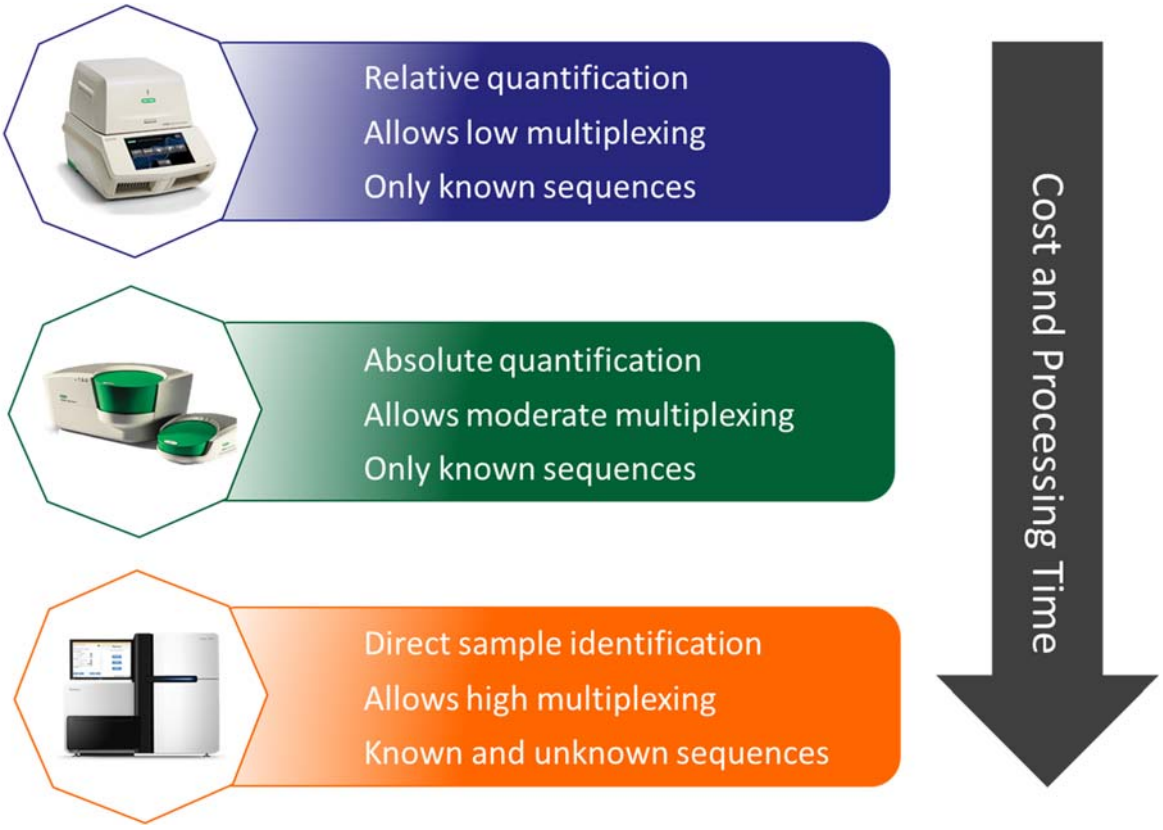


Figure S4: Comparison of qPCR, ddPCR and NGS.



Chapter 3

Rapid and Detailed Characterisation of Transgene Insertion Sites in Genetically Modified Plants Via Nanopore Sequencing

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Abstract

Molecular characterisation of genetically modified plants can provide crucial information for the development of detection and identification methods, to comply with traceability, and labelling requirements prior to commercialisation. Detailed description of the genetic modification was previously a challenging step in the safety assessment, since it required the use of laborious and time-consuming techniques. In this study an accurate, simple, and fast method was developed for molecular characterisation of genetically modified (GM) plants, following a user-friendly workflow for researchers with limited bioinformatic capabilities. Three GM events from a diverse array of crop species; perennial ryegrass, white clover, and canola, were used to test the approach that exploits long-read sequencing by the MinION device, from Oxford Nanopore Technologies. The method delivered a higher degree of resolution of the transgenic events within the host genome, than has previously been possible with the standard illumina short-range sequencing strategies. The flanking sequences, copy number, and presence of backbone sequences, and overall transgene insertion structure were determined for each of the plant genomes, with the additional identification of moderate sized secondary insertions that would have previously been missed. The proposed workflow takes only c. 1 week from DNA extraction to analysed result, and the method will complement the existing approaches for molecular characterisation of GM plants, since it makes the process cost-effective, rapid, and simpler.

Introduction

Implementation of plant biotechnology has enabled targeted alterations of crop traits through genetic transformation. The insertion of a specific transgenic element, containing a desired gene, into the host genome is the most common application of this technology to date (Fraiture, et al., 2017). Safety assessments of new transgenic plants are essential in order to identify potential threats to humans, animals, and the environment. The first step in any risk evaluation of genetically modified (GM) plants, prior to their approval for release into the market, is the molecular characterisation of the transgene(s). Such characterisation must include identification of the locus/loci of the genetic modification, detailing the flanking genomic regions, copy number of the inserted transgene expression cassette, and endogenous host gene interruptions by the transgenic DNA (Schouten, et al., 2017).

Cultivation of biotech (GM) crops is expanding rapidly, triggering the need for accurate and cost-effective molecular characterisation methods (Li, et al., 2017). Copy number determination of the transgenic event is an initial task that must be performed following their generation, as no transformation method can fully control the number of transgene insertions into the host genome (Tiwari and Singh, 2018). The most universally accepted techniques for molecular characterisation of new transgenic events have been DNA blot analysis, along with polymerase chain reaction (PCR) (Li, et al., 2017). These methods can attempt to determine transgene copy number, but fail to provide a detailed structure of the insertion.

DNA sequencing approaches have been used to determine the insertion site, flanking regions, and any possible endogenous gene interruption caused by the transgenic insert. The traditional and gold standard method for this purpose has been Sanger sequencing. Despite the robustness of Sanger sequencing, it is a time consuming and expensive method that can struggle to accurately sequence complex regions of the genomes

(Guttikonda, et al., 2016). Hence, during the last decade second generation sequencing (SGS, also called next generation sequencing) has been proposed as a cost-effective and rapid option for routine sequencing (Arulandhu et al., 2016, Pauwels, et al., 2015, Yang et al., 2013, and Kovalic et al., 2012). Although SGS, offers high-throughput, scalability, and time effectiveness (Guttikonda, et al. 2016, Wahler, et al., 2013, Yang, et al., 2013, Kovalic, et al., 2012), its short-read length nature makes the bioinformatic analysis highly challenging, due to the high percentage of ambiguous or incorrectly mapped reads (Park, et al., 2017). A combination of PCR-based genome walking methods, to enrich the target DNA region, with sequencing strategies is gaining popularity for routine molecular characterisation of transgenic events (Li, et al., 2017). However, such enrichment methods are also associated with short down- and up-stream DNA fragments, failing to characterise unintended insertions. Such a drawback is particularly problematic in plants with complex insertions, including endogenous genes or promoters, GC rich, and/or genomes with a high content of repetitive regions.

To overcome the assembly issues with SGS, recently third-generation sequencing (TGS also called single-molecule sequencing) has emerged. TGS has increased read lengths up to hundreds of kilobases, as well as reducing the sequencing time (Jain et al., 2016). The ability to increase read lengths can facilitate GM molecular characterisations, potentially solving alignment problems caused by repetitive and low complexity regions of a genome (Istace, et al., 2017). Currently, there are two TGS platforms commercially available, Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT). The potential benefits of the MinION ONT include long reads that may exceed 200 kbp, real-time data delivery, low cost, and portability (Magi et al., 2017).

Recently, ONT sequencing has been coupled with DNA walking methods to detect un-authorised GM organisms (Fraiture et al., 2018) and to identify transgenic alleles in soybean, when breeding to introgress them into other cultivars (Li, et al., 2019). However, this approach can fail to determine features such as copy number or unintended insertions, and the use of target-specific primers limits the size of DNA fragments. The reduction in sequencing cost and incremental advances of data yields and accuracy of the ONT flowcells, provides low coverage whole-genome shotgun sequencing, as the fastest way to identify transgenic insertions (Michael, et al., 2018) from across the genome in an

unbiased manner. This approach is particularly effective when the transgene integrates into complex repetitive regions of the genome, or where the transgene is cisgenic in nature and an endogenous copy of the same sequence is already present in the target genome, making walking strategies highly challenging. The major challenge in nanopore sequencing is the error rate (5-15%, Luo, et al., 2019), which is particularly important when detecting single-nucleotide polymorphisms (SNPs). However, improvements in the flowcells chemistry (R9 replacing the previous R7 version and the release of the R10 pore) and sequence analysis tools are constantly providing higher accuracy (Senol-Cali, et al., 2019). Another challenge to successful use of TGS technologies in the molecular characterisation of transgenic plants is the data analysis step, which requires bioinformatics pipelines and fit-for-purpose bioinformatic libraries (Li, et al., 2017).

In this article, we demonstrate how one can rapidly and inexpensively complete the full molecular characterisation of a transgenic event, by using a single ONT MinION flowcell. Three transgenic crops, spanning mono- and dicotyledonous species, with different genome sizes and complexity, were used as examples to show the utility of this methodology. The purpose of the present shotgun (random) whole genome sequencing, is to identify reads that encompass the genome-transgene boundary, which can describe the transgene integration site on the genome and copy number. Due to the evenness of genome coverage from the ONT technology (Malmberg, et al., 2019), an overall lower coverage (5-10x) could be sufficient to achieve satisfactory resolution of the transgene, as long as the DNA extracted is of sufficient length and generates the corresponding long sequence reads to provide the resolution required. The major contribution of this method is the user-friendly data analysis workflow, which is particularly useful for researchers with limited bioinformatic resources. To the best of our knowledge, this is the first time that ONT has been applied as a GM analysis strategy, to fully identify and completely characterise plant GM events at the molecular level in an unbiased untargeted manner, which will set the basis for transgene event sequencing into the future.

Materials and methods

Three GM crops; canola, white clover and perennial ryegrass were used as examples for the approach using the MinION, from ONT, for molecular characterisation of GM crops using a common analytical process. Graphic description of the transgenic vectors detected and identified in this study is shown in Figure S1.

Plant material and transformation

Canola, an allopolyploid crop with a 1,130 Mb genome size (Chalhoub, et al., 2014), was transformed with the ETIP vector using non-canonical zinc finger nucleases (ZFNs) for targeted modification of plant genomes (Figure S1a), and its transformation is described in Cogan, et al., (2014). Additionally, white clover, an allotetraploid crop with 1,093 mbp genome size (Bennett and Leitch, 2011), was transformed using *Agrobacterium tumefaciens* (denominated pCLV000032 vector) containing three transgenic vectors for delayed leaf senescence, aluminium tolerance and resistant to AMV (Alfalfa mosaic virus) and the *hph* gene (pDOI000080 vector) as a selectable marker, was also used (Figure S1b). Detailed description of the transformation with the triple stacked and selectable marker transgenes is reported in Narancio (2018). Finally, perennial ryegrass which has a diploid genome with 2,700 Mb in size (Kopecky, et al., 2017) was bombarded via biolistics with LpOs6g0680500 RNAi (glutamate receptor pGRA000120) construct (Figure S1c). The glutamate receptor is a potential male candidate gene for the S locus that partly controls the ryegrass self-incompatibility process and pollen recognition (Spangenberg, et al., 2019).

DNA extraction, concentration and purity

DNA was extracted using a modified version of the high molecular weight genomic DNA extraction from plant leaves (ONT). In summary, after disruption, plant samples were digested and genomic DNA was purified in three separate steps; G2 lysis buffer (QIAGEN,

Hilden, Germany), RNase and proteinase K. Then the samples were transferred into equilibrated QIAGEN Genomic-tip 100/G columns following the manufacturer's instructions (QIAGEN, Hilden, Germany), washed and the DNA eluted. Finally, the DNA was washed with isopropanol and ethanol and resuspended in TE buffer.

Each sample concentration was measured using the Qubit dsDNA HS assay kits and a Qubit 3.0 Fluorometer (Invitrogen™, Thermo Fisher Brand, Carlsbad, USA), according to the manufacturer's instructions. DNA quality was measured with the NanoDrop 1000 UV-vis spectrophotometer (ThermoFisher) using the A260/A280 and A260/A230 ratios, and the DNA size was analysed using the Genomic DNA ScreenTape, through the Agilent 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions. Samples with a minimum amount of 1.5 µg of genomic DNA in 47 µl of distilled water, A260/A280 ratio of 1.8 ± 0.1, A260/A230 ratio of 2.0 ± 0.1, and DNA length above 60 kbp were used for sequencing purposes (Figure 2).

Library preparation and sequencing

Each sequencing library was prepared from 1.5 µg of high molecular weight genomic DNA using the 1D Genomic DNA by ligation protocol (SQK-LSK109; ONT, Oxford, UK), according to the manufacturer's instructions. DNA fragmentation was not performed, to maintain the integrity of the high molecular weight DNA. Briefly, the DNA was repaired and blunt-ended followed by an AMPure XP bead clean-up. Then, the DNA was adapter-ligated and cleaned again, to finalise with a library priming and loading. The prepared library was loaded into a MinION Mk1B device using 106 R9 flowcells (ONT, Oxford, UK). In total, three flowcells were used, one for each crop and each sequencing run was performed for 48 hours using live base calling in the MinKNOW software version 1.4-1.13.1 (ONT). The quality control assessment of the raw generated data, in the fastq format, was analysed using python (version 3.6.4) and albacore (version 2.3.4). Albacore generated interactive quality control metrics and plots from base called nanopore reads or summary files. The benefit of MinION QC is that it works directly with the sequencing_summary.txt files produced by ONT's Albacore, which makes MinIONQC a lot quicker and allows the quick-and-easy comparison of data from multiple flowcells.

Sequencing data analysis

Analysis of the sequence dataset was performed using BLASTn (version 2.2.26). All nanopore sequencing reads for each sample were converted into a BLAST database, and each transgenic vector sequence was used as the query in the analysis. Selected reads were retained and isolated, using seqtk (subseq; available from https://github.com/lh3/seqtk) tools. Reads were manually mapped and visually checked using the software Sequencher (version 5.4.6; Gene Codes, Ann Arbour, MI, USA).

To identify the exact location of the transgenic vector within the host genome, a further sequence homology analysis using BLAST was performed. In this instance, the database was generated from each crop reference genome (full, relative or draft genome), and the query sequences were all reads that hit each transgene from the previous step. Due to the cisgenic nature of several elements present in the transgenic vectors, sequence reads matching only endogenous elements with endogenous flanking regions were discarded. The analysis was also filtered by e-value, so only reads with e-value threshold 0 were selected. In order to ensure that none of the identified sequence reads were endogenous sequences, the genome sequence at each chromosomal location was compared against the vector to confirm there was no match.

The reference genome used for *Brassica napus* (canola) is reported in Chalhoub, et al. (2014) and in the absence of a *Trifolium repens* (white clover) reference, the *Trifolium pratense* genome published in De Vega, et al. (2015) was used. For Perennial ryegrass there is not a full reference genome available, so three drafts were used (Shinozuka, et al., 2016, Velmurugan, et al., 2016 and Byrne, et al., 2015).

Results

The MinION device from ONT, was tested for molecular characterisation of GM crops using a common analytical process. Three crops contrasting in genome size, complexity of integration, available genome resources, and transformation method were selected. Canola (*Brassica napus* L.) has a full reference genome publicly available, that is 1,130 mbp in size, and was transformed with a transgenic vector of 10,648 bp with a GC content of 46.5%. White clover (*Trifolium repens* L.) has a 1,093 mbp genome and was transformed with two vectors; a triple stacked vector of 8,604 bp in size, 55.19% of which were endogenous elements (Figure S1b) and 45.8% GC content, and a selectable marker vector containing hygromycin phosphotransferase gene (*hph*) of 3,348 bp in size and 53.4% of GC content. Although, white clover does not have a reference genome, *Trifolium pratense*, a close relative, does have a genome sequence, which was used. In contrast, perennial ryegrass (*Lolium perenne* L.) had the largest genome size of 2,700 mbp amongst the three species studied, and it does not have any reference genome or close relative genome to be used as a proxy, so three published drafts were used in this study. Perennial ryegrass was transformed using a biolistic method with a transgenic cassette of 6,401 bp in size, where 15.12% of the genomic elements are endogenous (Figure S1c) and has 47.6% of GC content. Graphic description of the entire workflow is shown in Figure 1.

The extraction of purified high molecular weight DNA is critical in the proposed workflow (Figure 1). The method followed a modified version of the recommended protocol from ONT, for high molecular weight genomic DNA extraction from plant leaves. Sufficient yield for library preparation was achieved from all samples. DNA purity, assessed by absorbance ratios 260/280 nm and 260/230 nm, was measured with values obtained of 1,81 and 2,12 for canola; 1,85 and 2,15 for clover, and 1,87 and 2,08 for ryegrass respectively. DNA fragment length of all samples was assessed by microfluidic electrophoresis, and all samples presented an average fragment length of c. 50 kbp (Figure 2). For each library preparation a total of 1.5 µg of genomic DNA was used as input and the final sequencing libraries, ranged in mass from 559 to 696 ng. Before

priming and loading the library into each MinION flowcell, a platform-QC protocol was executed, the number of pores available were 1402, 1531, and 1581 for canola, clover, and ryegrass flow cells respectively. Finally, each library was primed, loaded into each flow-cell, and the sequencing run was started using the MinKNOW software.

Sequencing data generation

Each sample was independently prepared and sequenced on a single MinION flowcell. One hour after sequencing had been initiated, each experiment was visually checked and the MinKNOW metrics were exported. The number of pores sequencing were 368, 274, and 395 for canola, white clover, and perennial ryegrass respectively. The overall read length was longer for canola and ryegrass libraries (10.6 and 11.9 kbp), compared to that of clover (1.6 kbp) (Figure S2).

Once each sequencing run finished (after 48 hours), the quality of all raw data was analysed using the NanoPlot software (Figure S3). The total gigabases generated for canola, clover, and ryegrass were 10.4, 7.3, and 8.7, while the total reads generated were 986,303, 4,600,714, and 728,000 respectively. The N50 read length was 24.2, 4.5, and 22.1 kbp, and the maximum read lengths were 196, 130, and 173 kbp for canola, clover, and ryegrass respectively. Overall, canola and ryegrass samples provided large numbers of long reads with a median read length of 7.3 and 4.9 kbp, while clover only presented a median of 0.5 kbp. However, the ryegrass and canola libraries only provided around 15% and 20% of the read numbers compared to that of clover, whilst still delivering overall more sequenced nucleotides (Table 1 and Figure S3).

Detection of transgenic insertions

The canola sample was transformed by a single engineered transgene integration platform (ETIP) vector (Figure S1a), and during the BLAST search, 34 sequence reads with identified similarity lengths to the vector ranging from 471 to 11,591 bases were selected. The sequence homology analysis with BLAST for the triple stacked vector in clover, identified 80 sequence reads with sequence similarity with lengths between 710 and 6,876 bases, whilst there were 14 sequences for *hph* with read lengths between 997

and 3,686 bases. Ryegrass presented 8 sequences with sequence similarity to the relevant vector of between 487 and 2667 bases.

Location of transgenic vectors

Sequence reads of significant length can contain many elements, including the transgene as well as the surrounding host genome sequence, so the present of repetitive elements is also to be expected within the sequence analysis results. Therefore, it was necessary to carefully examine each output of the analysis with specific attention to the length of the alignment as well as the location, looking for commonality in the chromosomal position of the longest sequence matches, whilst discounting small alignments to the array of repeats across the genome.

After a detailed check of all the reads, 18 canola reads were discarded because the read length was too short (less than 1.000 bp), leaving only 16 reads of the initial 34. The longest 12 reads also had endogenous flanking sequence that aligned to chromosome A06 random, ranging from 1,675 to 1,689 kbp. Examination of the BLAST results also identified, as expected, the homologous region within the allotetraploid species as C07 at c. 32.1 mbp. The sequence similarity at this location had a higher degree of interruptions, and as a result had a lower contiguity that was reflected with significantly lower score values in the analysis. Sequence matches at the C07 homologous region did not exceed 5 kbp, whilst the longest contiguous sequence match at A06 random exceeded 15 kbp. The insertion of the ETIP transgene into the chromosome location contained no additional unintended DNA sequences in the 5' side, where the engineered landing pad (ELP) finished. However, in the 3' side there was 457 bp of backbone between the canola endogenous sequence and the AtUbi10 promoter. The transgene was located in an intergenic region between the genes BnaA06g39700D and BnaA06g39710D, and did not disrupt either of them (Figure 3a). Additionally, the remaining 4 reads that did not align to chromosome A06 random, were all located in chromosome C01 from 3,897 to 3,900 kbp. These reads identified an insertion of 680 bp of the transgenic cassette, that corresponded to a fragment of the AtORF1 terminator and the intron 2 (figure S1a).

For clover relating to the inserted triple stacked transgene, 59 from the initial 80 reads were discarded because the read length was too short, leaving 21 reads containing the transgene that matched the *Trifolium pratense* genome. All reads matched chromosome 5 from 13,124 to 13,140 kbp, which corresponded to the transgene integration (full transgenic cassette including backbone and double integration of two T-DNA elements). The transgene was located in an intron of the Tp57577_TGAC_v2_gene7933, which encodes for a 50S ribosomal protein l30-like (Figure 3b). Related to the *hph* transgene, there were 14 reads that matched the hygromycin resistance gene (*hph*), however only the 2 longest reads also had endogenous sequence that matched chromosome 5 from 7,600 to 7,601 kbp, while the remaining 12 smaller reads failed to align to the *Trifolium pratense* genome. The *hph* transgene was located at an intergeneric region between the Tp57577_TGAC_v2_gene37565 and gene37463, without disrupting either of these genes (Figure 3c).

For ryegrass there is not a reference genome published, and neither a close relative that can be used as a reference, so the exact chromosomal location of the transgene was unable to be elucidated with the available resources. From the initial 8 reads found in the transgenic insertion BLAST, 3 were discarded after checking read lengths and chromosomal locations. Then using the BLAST analysis to a collection of draft genome sequences, it is believed that the transgene had only inserted at a single scaffold, as from the 5 selected reads, 4 of them matched the same scaffold from one of the draft genomes. The scaffold identified is 22,641 bp in length and it is likely to be located at a repetitive region, since a BLAST search identified some small fragments of *Brachypodium distachyon* and *Triticum aestivum*.

Insertion site and flanking regions confirmation

The subset of sequence reads obtained from the initial BLAST to each vector, that were too short to hit the reference genome in the second BLAST, were used to map both transgenic insertions and flanking regions and confirm the accuracy of the previous steps. Once all transgenic insertions and chromosomal locations were confirmed, the longest read that hit each vector was selected for schematic purposes. In canola a 68.3 kbp-read was selected as the longest sequence with the ETIP vector centrally located, presenting a

mismatch rate between the read and vector reference of 3.47%. The selection of such a read, allowed the identification of 36.8 kbp of canola endogenous sequence in the 5' flanking side and 18.7 kbp in the 3' (Figure 4a).

The *hph* transgene identified in the clover genome, was detected in the middle of a 10.7 kbp-read with 1.8% mismatch rate between the read and vector reference, leaving 5.1 kbp of clover endogenous sequence in the 5' and 3.6 kbp in the 3' flanking side (Figure 4b). A single sequence read with the complete triple stacked vector (9.4 kbp) on chromosome 5, was not generated. All sequence reads identified were then used to reconstruct the insertion. From the detailed analysis and reconstruction of the insertion, it was identified that the entire transgene cassette, including backbone sequence had inserted along with a second copy of the OCS terminator and TrPT1 promoter, extending the transgenic insertion to 18.6 kbp. The three longest reads with 9.2, 15.3, and 17.7 kbp in length were mapped from 5' to 3', with 3.4 and 1.1 kbp overlap respectably to illustrate the insertion (Figure 4b). In ryegrass the transgene was found in the middle of a 31.3 kbp read with 4.2% mismatch rate between the read and vector reference, leaving 8.5 kbp of endogenous sequence in the 5' and 13.9 kbp in the 3' flaking side (Figure 4c).

Discussion

Molecular characterisation of new transgenic plants is a prerequisite before commercialisation, as recombinant DNA can be randomly inserted into the host genome. Identification of genomic features generated by transgene integrations, including insertion site, presence of backbone sequences, and copy number are crucial from scientific and regulatory perspectives, to deliver understanding of the changes made to the plant and the likely consequences (Jupe, et al., 2019). The presence of random insertion(s), can cause host gene interruptions that could have unintended results, or the integration of multiple transgenic sequences in one or more chromosomal locations, which affects the stability of the trait triggering transgene silencing (Tiwari and Singh, 2018).

In this study, nanopore reads between 10.7 kbp to 68.3 kbp in length, allowed the unequivocal characterisation of the transgene insertion sites, flanking regions, and location in the host genome without the requirement of a de novo assembly step. The perceived high error rates in ONT sequence reads, have proved not to be a hinderance to correctly identify the specific transgene location in any of the species tested, including the highly duplicated complex canola genome. The error rate of ONT and the similarity of the transgenes used in this study to each reference genome, make it difficult to provide fixed guidelines on the level of resolution of a minimum detection threshold. Malmberg, et al. (2019) identified in canola, due to the close genome similarity between the A and C subgenomes, that many illumina SGS sequence reads misaligned in the genome in small localised areas. However, when long ONT reads were generated, they correctly aligned to the specific sub-genome within the allotetraploid, due to the presence of multiple variants within the larger area characterised. ONT sequence reads, despite the higher error rate compared to SGS, are more able to be accurately resolved than SGS, even within highly complex polyploids with highly similar sub-genomes.

The characterisation of three contrasting GM plants, was achieved using a single MinION flowcell per plant in the present study. The workflow described here, is broadly applicable for the vast majority of plant species and all transgene insertions. However, for crops with large genome sizes, such as wheat (genome size estimated at c.14.5 Gb; Appels, et al., 2018), more than one MinION flowcell may be required or the use of higher output devices such as the PromethION (also from ONT) could be more effective. With the requirement of increased confidence for some transgenic events, increased sequence read depths may be required, to exclude the possibility of missing an insertion. However, the work presented has extensively characterised the known transgene events and identified additional insertions and complexity that was previously unknown, supporting the recommendation of 5-10x genome coverage for initial screening purposes.

Hitherto, the combination of different existing techniques allowed the identification of transgene copy number and insertion site, but molecular tools failed to comprehensively describe the "whole picture". The lack of comprehensive characterisation was due to the techniques available that targeted parts of the transgene (amplicons between 200 bp and 4 kbp), usually short in length, to deduce the presence or absent of the entire vector. The optimal transformation method would integrate the gene of interest into the host genome in a single copy. However, during the transformation process, transgenic DNA can be inserted into the host genome at random (biolistic transformation) or induced locations of double strand breaks (Agrobacterium transformation) (Tzfira, et al., 2003). Additionally, backbone sequences are frequently integrated along with the transgenic sequence, resulting in chromosomal rearrangement. Therefore, a high percentage of transformed plants deviate from the ideal, containing only part or concatenated fragments of transgenic DNA, which is often underestimated (Gelvin, 2017).

Several publications using different molecular tools have reflected such phenomena. For instance, the initial molecular characterisation of soybean event GTS40-3-2 using PCR, found a single copy of the cassette (Padgette et al., 1995). However, later research identified the insertion of two unintended segments of the transgene (Product Safety Centre, 2000) and unexpected rearrangements of endogenous DNA, causing the amendment of its molecular characterisation (Windels et al., 2001). Recently, Nicholls et al. (2019), used the nanopore sequencing technology to detect the site of integration of a

fluorescent reporter gene in mouse germ lines, which also allowed the identification of E. coli DNA contamination integrated within the transgene. Similarly, the isolation and comparison of the longer reads that mapped to each of the transgenic insertions evaluated in this study, allowed the identification of transgenic DNA rearrangements and backbone sequences, which are important features for long term stability of transgene expression (Tiwari and Singh, 2018).

The identification of an unintended insertion of 680 bp transgenic cassette in canola chromosome C01, as well as endogenous elements presents in cisgenic cassettes were attained. Unexpected insertions of backbone sequences, usually associated with the integration of transgenic DNA in the 3' end of A. tumefaciens transformants (Hwang, et al., 2017) such as canola, were also identified in this study. In clover, the presence of large amounts of phenols, which suffer a rapid oxidation and form reactive quinones that irreversibly bind to proteins affecting their solubility (Whitlock, et al., 2008), may have affected the quality of genomic DNA generating shorter reads. Despite these issues with DNA quality, the read length was sufficient to characterise a complex integration of the stacked transgene, which was initially thought to be 9.4 kbp. However, following full characterisation of the insertion the transgene was identified to be 18.6 kbp in length, due to a linear concatenated insertion. The generation of longer sequence reads greater than the transgenic insertion is highly important for the description of an event and for any application of the approach described here, DNA quality and integrity is of the highest value.

In complex GM crop genomes, such as the examples used in the present study, both walking methods coupled with SGS and whole genome sequencing approaches are ineffective to fully characterise the transgenic insertion and flanking sequences. The size limitation of enrichment methods and assembly issues of short-read sequencing would make the characterisation of plants used in this study highly challenging, due to difficulties enriching cisgenic cassettes and assembling large repeat structures with high sequence identity. In ryegrass for instance, repetitive sequences are usually longer than the length of an SGS read, so a single short read will not completely span a repetitive region (Senol-Cali, et al., 2019). Additionally, the cisgenic nature, size, and complexity of

stacked transgenes such as GM clover, makes its molecular characterisation with PCR enrichment and/or short-read sequencing approaches practically impossible.

The use of nanopore sequencing allowed us to obtain flanking endogenous sequences between 3.6 and 38.6 kbp and identify their exact chromosomal location and/or gene interruptions, when a reference genome was available. A successful application of the proposed workflow would depend on the availability of full genomes to identify the location of the insertion site, something that still represents a bottleneck. However, when a reference genome is not available, close relatives are extremely valuable. In the instances where only draft scaffolds are available, it is still possible to draw conclusions from the data generated. With the continual improvement of TGS technology, there are also an increasing number of species with significant reference genomic sequence resources.

Additionally, the application of ONT sequencing for molecular characterisation of GM plants, would depend on the successful establishment of bioinformatic pipelines. Fraiture, et al. (2018), found challenges in the analysis of raw nanopore sequencing data to detect unauthorised GMOs, possibly as the study integrated DNA walking strategies with nanopore sequencing, which required the development of adequate pipelines, reliable bioinformatic tools, and limited the sequence length they obtained around the transgene. An advantage of the workflow proposed in this study, is that there is no need of assembly, since assemblies create unavoidable assumptions made on the overlapping sequences. Although, nanopore sequencing basecalling has significantly improved, generating highly accurate consensus sequences (Krehenwinkel, et al., 2019), the bioinformatic step represents a great challenge, especially for researchers with little or no bioinformatic capability. In this study, the data analysis uses a simple strategy based on common BLAST tools, which can be used by researchers without extensive bioinformatics expertise.

Conclusion

The aim of this study was to develop a simple, fast, and cost-effective method for molecular characterisation of transgenic plants, with a simple and robust bioinformatic pipeline, and strategies that would be broadly applicable and accessible. The proposed workflow can be performed in one week, using a single nanopore flowcell per transgenic event, and has been initially exemplified across a range of crop genome sizes up to 2.7 gbp. The method described is broadly applicable for the vast majority of plant species and transgene insertions. The methodology can be used to prioritize transgenic events in the molecular characterization assessment, as it provides the insertion description in a short period of time. Additionally, it could potentially be used for traceability purposes, using a custom database to screen for vectors and common transgenic elements. To the best of our knowledge, the present study is the first assessment and full molecular characterisation of transgenic plants using only nanopore sequencing. The approach described here will complement the range of methods for molecular characterisation of GM plants, since it can make the process more cost-effective, rapid, and simple.

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Author Contributions Statement

PG performed all laboratory experimental work with guidance from HS. Data analysis was performed by PG with assistance by NC. PG prepared the manuscript under the supervision and drafting of NC, HS and KFS. The manuscript was finally edited by NC, KFS, HS, and GS.

Conflict of Interest Statement

The authors declare no conflict of interest.

Contribution to the Field Statement

This manuscript describes a simple, fast and cost/effective method for molecular characterisation of transgenic plants, with a simple robust bioinformatic pipeline and strategies that would be broadly applicable and accessible. In this study, the long-read sequencing nature of Oxford Nanopore Technology (ONT) allowed the unequivocal characterisation of three complex and contrasting genetically modified (GM) plants, without the requirement of PCR enrichment approaches. The identification of endogenous elements present in cisgenic insertions and any unintended integrations were also attained without the need of a de novo assembly step. The pipeline described is broadly applicable for the vast majority of plant species and all transgene insertions. To the best of our knowledge, the present study is the first assessment of only Nanopore sequencing for full molecular characterisation of GM plants.

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Figure and Table Legends

Figure 1: Workflow for the molecular characterisation of genetically modified plants, using the MinION device of ONT. Graphic illustration from DNA extraction (left), library preparation and sequencing (middle) and data analysis (right).

Figure 2: Sample comparison of each extracted genomic DNA from canola, clover and ryegrass, using the function of the 2200 TapeStation software with a ladder of 60 kbp maximum measurement.

Figure 3: Schematic representation of the genomic location of the genetically modified plants. (a) Genomic location of the ETIP transgene on canola's chromosome 6 random. (b) Predicted genomic location of the triple stacked transgene on clover's chromosome 5. (c) Predicted genomic location of the hygromycin resistance gene (*hph*) on clover's chromosome 2. *T-cassette: transgenic cassette.

Figure 4: Schematic diagram of the longest nanopore sequence reads describing the complete insertion site, flanking sequences, transgene rearrangements and GC content in the selected genetically modified plants. (a) ETIP transgene inserted into canola's genome. (b) triple stacked transgene and the hygromycin resistance gene (*hph*) inserted into clover's genome. (c) Glutamate transgene inserted into ryegrass's genome. All numeric values are in base pairs (bp).

Table 1: Nanopore sequence read lengths and metrics for the three sequencing experiments.

Figure 1

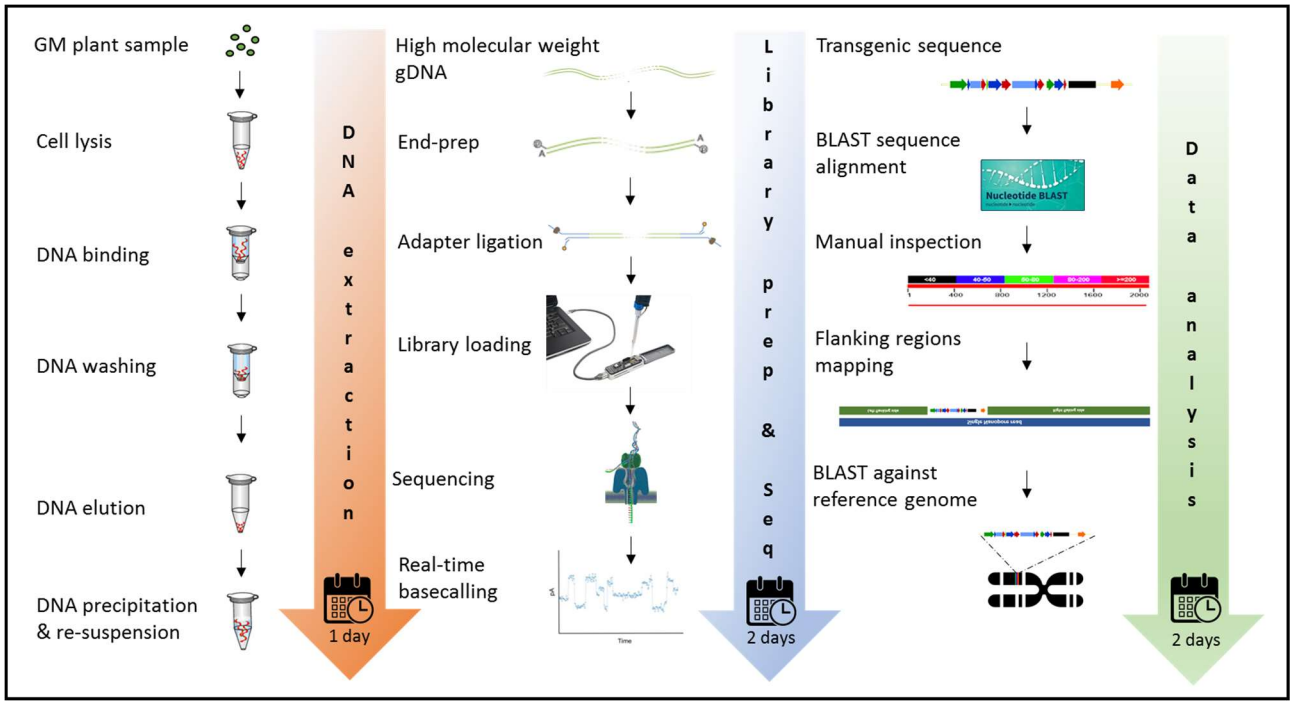


Figure 2

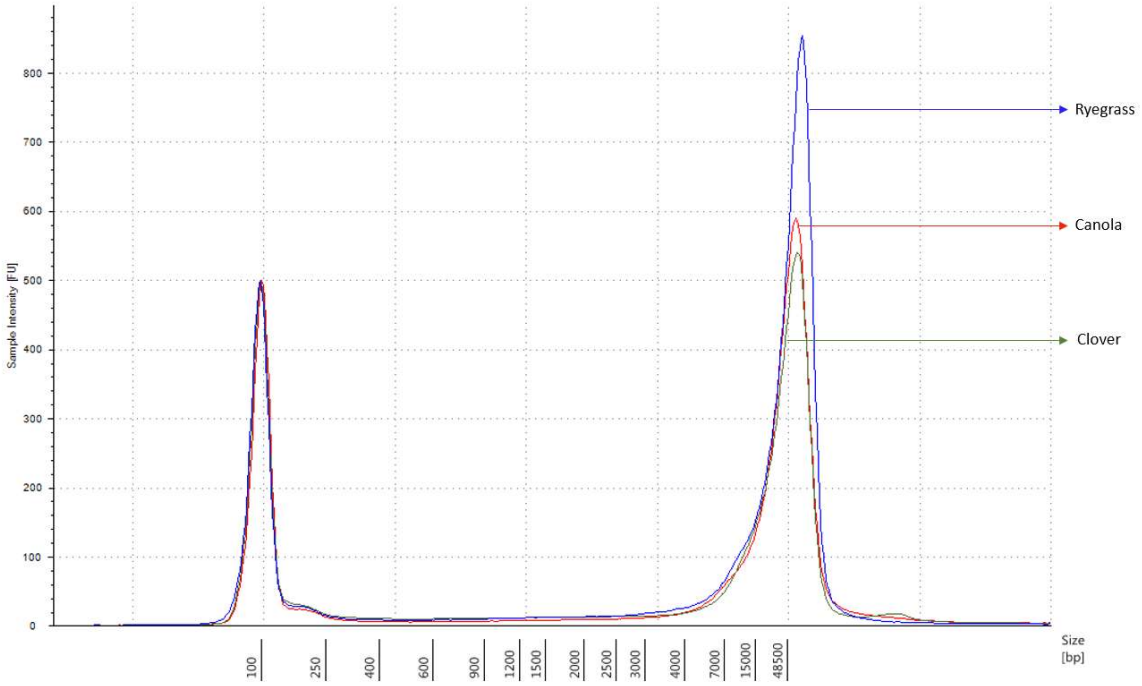


Figure 3

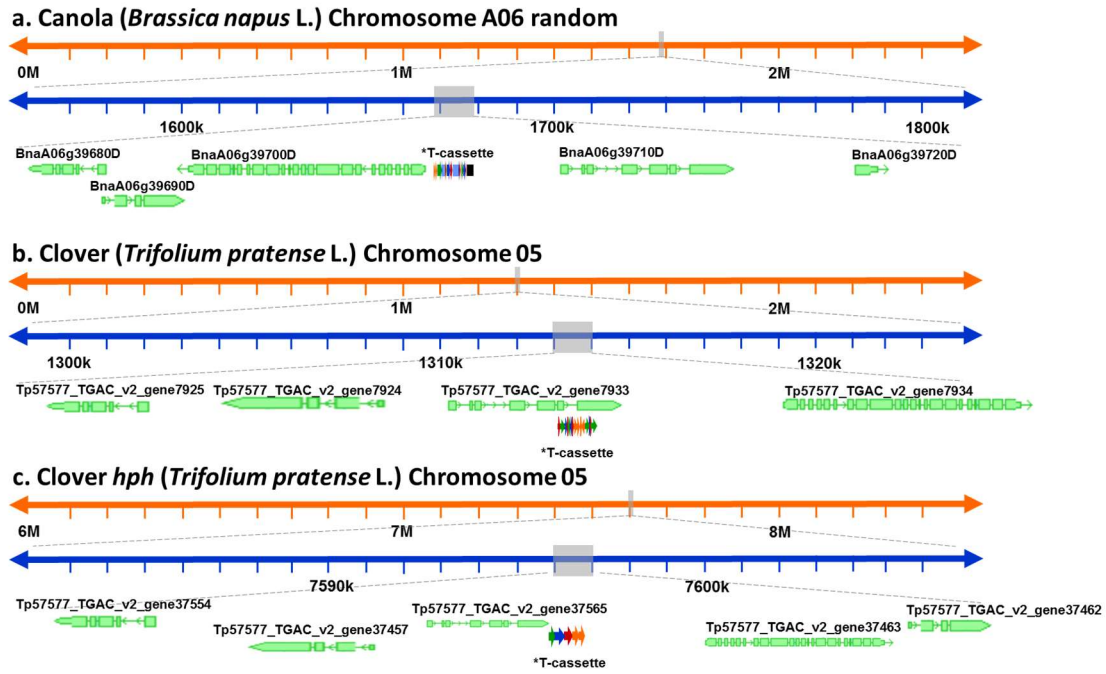


Figure 4

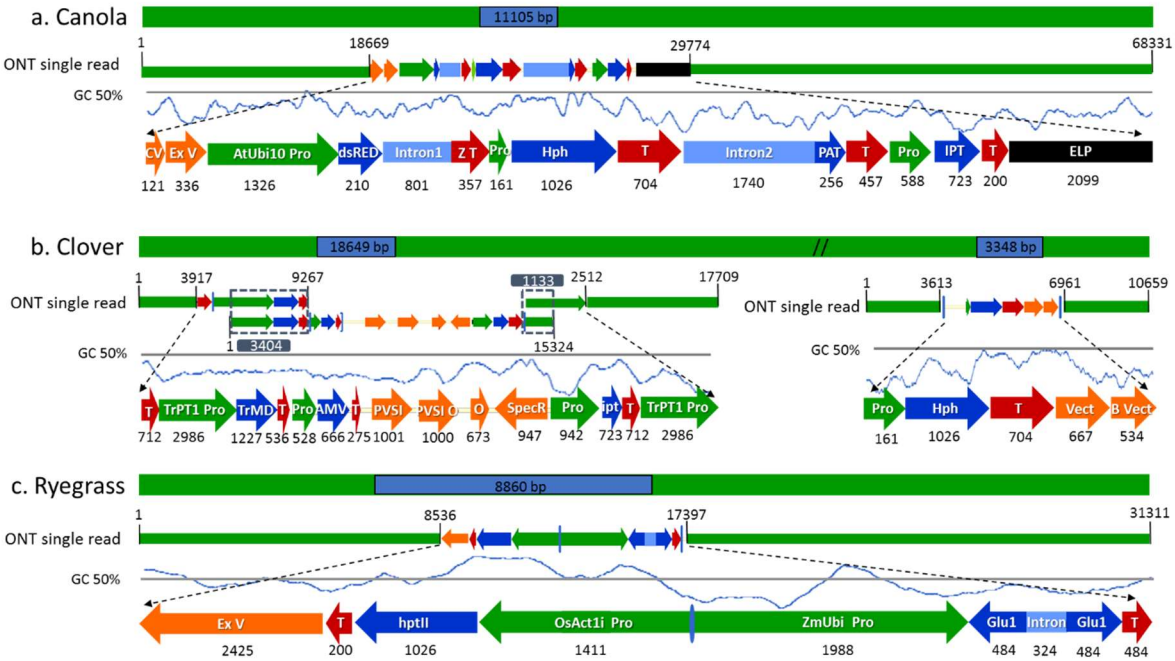


Table 1

QC parameters	Canola	Clover	Ryegrass
Mean read length (base)	10,577.7	1,584.6	11,923.0
Mean read quality (Q score)	10.0	9.7	9.8
Median read length (base)	4,942.0	577.0	7,321.0
Median real quality (Q score)	10.1	9.7	9.9
Number of reads	986,303.0	4,600,714.0	728,000.0
Read length N50 (base)	24,209.0	4,535.0	22,105.0
Total bases	10,432,863,768.0	7,290,150,516.0	8,679,914,588.0
Maximum length (base)	196,540.0	129,999.0	172,891.0

Supplementary Material

Figure S1: Schematic diagram of vectors used to construct the transgenic lines. (a) Engineered transgene integration platform (ETIP) cassette for expression in canola. (b) pCLV000032 cassette with a triple stacked gene in the left and the pDOI000080 cassette for hygromycin phosphotransferase gene (*hph*) as a selectable marker in the right for expression in clover. (c) pGRA000120 cassette with the glutamate receptor for expression in ryegrass.

Figure 2: MinION real-time basecalling evaluation after 24 hours of sequencing, using the MinKNOW software. Data acquisition, real-time analysis and feedback for (a) canola, (b) clover, and (c) ryegrass.

Figure S3: Nanoplot illustration of the read lengths vs average read quality of (a) canola, (b) clover, and (c) ryegrass.Figure S1: Schematic diagram of vectors used to construct the transgenic lines. (a) Engineered transgene integration platform (ETIP) cassette for expression in canola. (b) pCLV000032 cassette with a triple stacked gene in the left and the pDOI000080 cassette for hygromycin phosphotransferase gene (*hph*) as a selectable marker in the right for expression in clover. (c) pGRA000120 cassette with the glutamate receptor for expression in ryegrass.

Figure S1

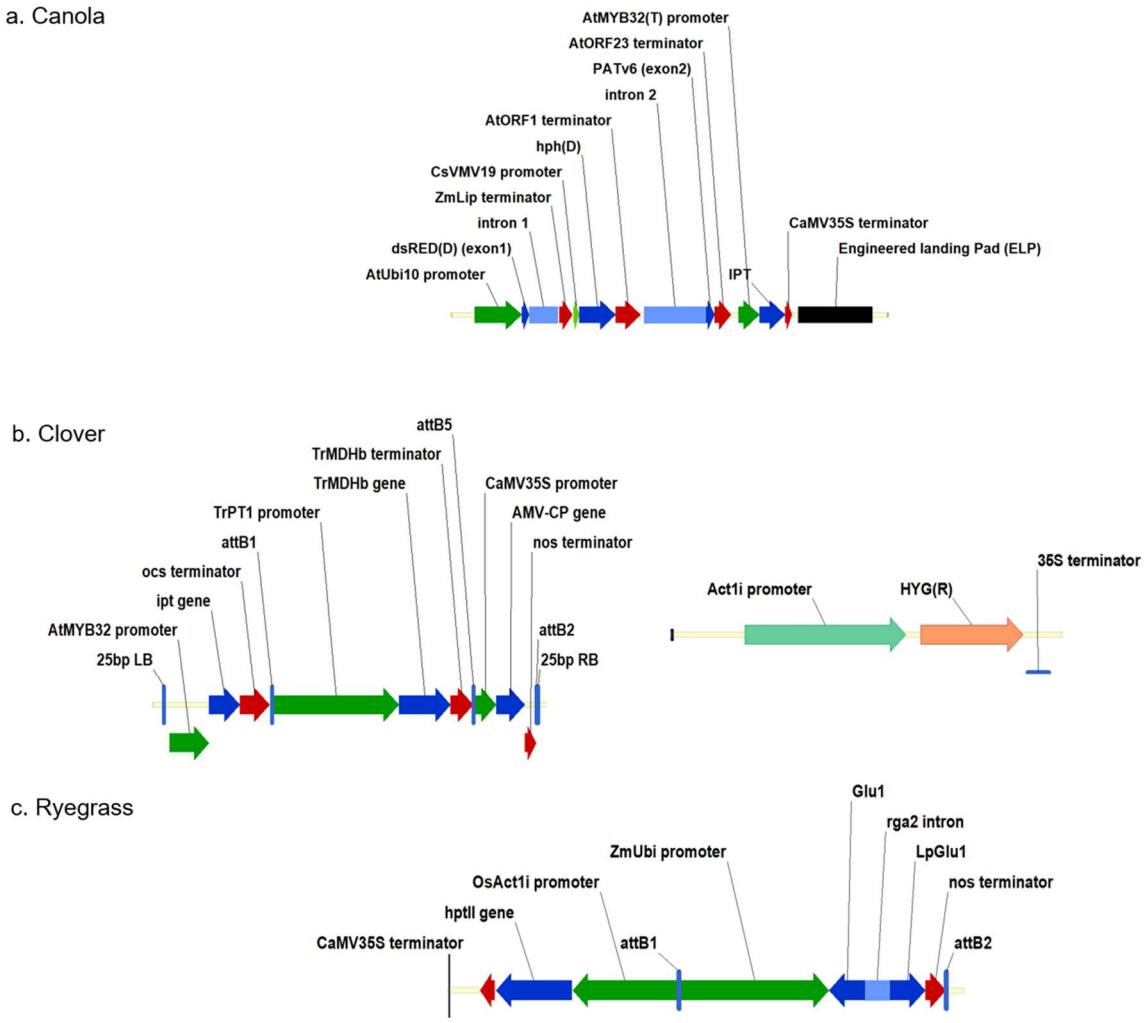

a. Canola
AtMYB32(T) promoter
AtORF23 terminator
PATv6 (exon2)
intron 2
AtORF1 terminator
hph(D)
CsVMV19 promoter
ZmLip terminator
intron 1
dsRED(D) (exon1)
AtUbi10 promoter
IPT
CaMV35S terminator
Engineered landing Pad (ELP)
b. Clover
attB5
TrMDHb terminator
TrMDHb gene
CaMV35S promoter
TrPT1 promoter
AMV-CP gene
attB1
nos terminator
ocs terminator
ipt gene
AtMYB32 promoter
25bp LB
attB2
25bp RB
Act1i promoter
HYG(R)
35S terminator
c. Ryegrass
Glu1
rga2 intron
ZmUbi promoter
LpGlu1
OsAct1i promoter
nos terminator
hptII gene
CaMV35S terminator
attB1
attB2

Figure S2

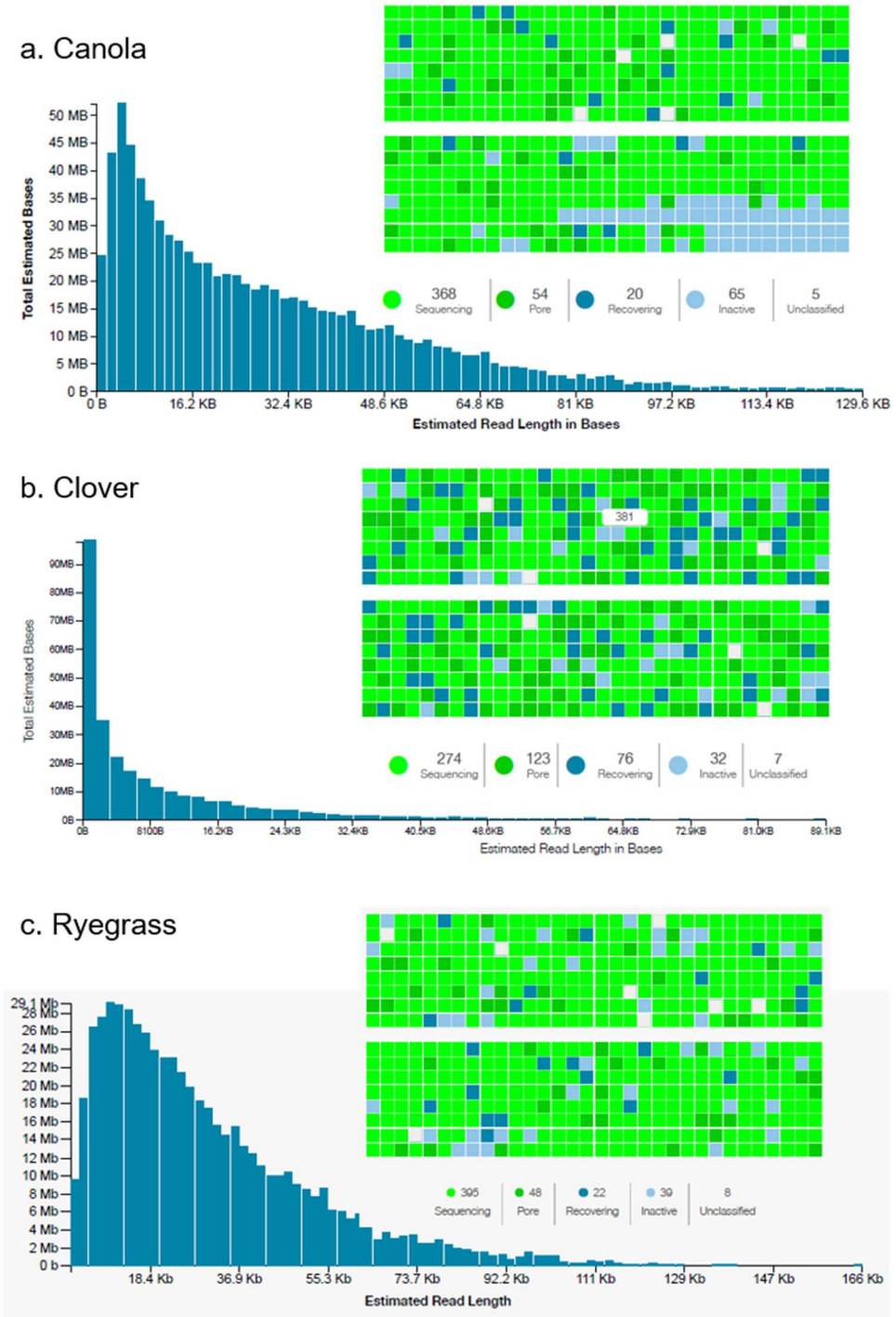
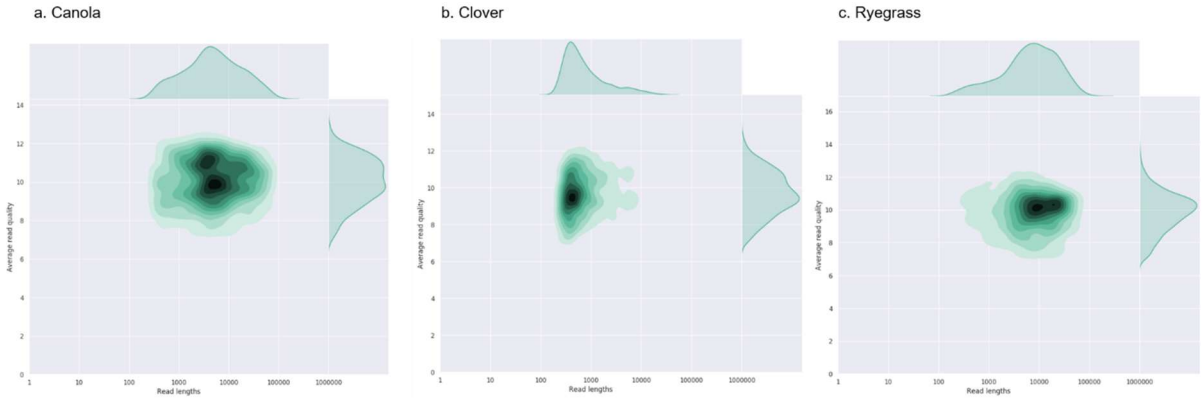

a. Canola
Total Estimated Bases
50 MB
45 MB
40 MB
35 MB
30 MB
25 MB
20 MB
15 MB
10 MB
5 MB
0 B
0 B
16.2 KB
32.4 KB
48.6 KB
64.8 KB
81 KB
97.2 KB
113.4 KB
129.6 KB
Estimated Read Length in Bases
368 Sequencing
54 Pore
20 Recovering
65 Inactive
5 Unclassified
b. Clover
Total Estimated Bases
90MB
80MB
70MB
60MB
50MB
40MB
30MB
20MB
10MB
0B
0B
8100B
16.2KB
24.3KB
32.4KB
40.5KB
48.6KB
56.7KB
64.8KB
72.9KB
81.0KB
89.1KB
Estimated Read Length in Bases
381
274 Sequencing
123 Pore
76 Recovering
32 Inactive
7 Unclassified
c. Ryegrass
29.1 Mb
28 Mb
26 Mb
24 Mb
22 Mb
20 Mb
18 Mb
16 Mb
14 Mb
12 Mb
10 Mb
8 Mb
6 Mb
4 Mb
2 Mb
0 b
18.4 Kb
36.9 Kb
55.3 Kb
73.7 Kb
92.2 Kb
111 Kb
129 Kb
147 Kb
166 Kb
Estimated Read Length
395 Sequencing
48 Pore
22 Recovering
39 Inactive
8 Unclassified

Figure S3


a. Canola
b. Clover
c. Ryegrass
Average read quality
Read lengths
1
10
100
1000
10000
100000
1000000

Chapter 4

frontiers in Plant Science

ORIGINAL RESEARCH
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Development and Application of Droplet Digital PCR Tools for the Detection of Transgenes in Pastures and Pasture-Based Products

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Implementation of molecular biotechnology, such as transgenic technologies, in forage species can improve agricultural profitability through achievement of higher productivity, better use of resources such as soil nutrients, water, or light, and reduced environmental impact. Development of detection and quantification techniques for genetically modified plants are necessary to comply with traceability and labeling requirements prior to regulatory approval for release. Real-time PCR has been the standard method used for detection and quantification of genetically modified events, and droplet digital PCR is a recent alternative technology that offers a higher accuracy. Evaluation of both technologies was performed using a transgenic high-energy forage grass as a case study. Two methods for detection and quantification of the transgenic cassette, containing modified fructan biosynthesis genes, and a selectable marker gene, hygromycin B phosphotransferase used for transformation, were developed. Real-time PCR was assessed using two detection techniques, SYBR Green I and fluorescent probe-based methods. A range of different agricultural commodities were tested including fresh leaves, tillers, seeds, pollen, silage and hay, simulating a broad range of processed agricultural commodities that are relevant in the commercial use of genetically modified pastures. The real-time and droplet digital PCR methods were able to detect both exogenous constructs in all agricultural products. However, a higher sensitivity and repeatability in transgene detection was observed with the droplet digital PCR technology. Taking these results more broadly, it can be concluded that the droplet digital PCR technology provides the necessary resolution for quantitative analysis and detection, allowing absolute quantification of the target sequence at the required limits of detection across all jurisdictions globally. The information presented here provides guidance and resources for pasture-based biotechnology applications that are required to comply with traceability requirements.

Keywords: genetically modified (GM), forage, real-time PCR (qPRC), droplet digital PCR (ddPCR), TaqMan-probe, SYBR Green I

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INTRODUCTION

Grasslands are among the largest ecosystems on earth, compromising 35% of the global land area, compared with 12% used for cultivation of agricultural crops (Dubois, 2011). Implementation of transgenic technologies in forage species can improve agriculture through higher productivity, better use of resources and reduced environmental impact. Genetic solutions for forage quality limitations, pest and disease resistance, nutrient acquisition efficiency, tolerance to abiotic stresses and the targeted modification of growth and development, can be achieved by introducing novel high impact traits into forage breeding programs (Smith et al., 2007).

All new genetically modified (GM) cultivars are required to be assessed for regulatory requirement purposes prior to commercial release, which aims to provide an evaluation of their potential impacts on human, animal and environmental health. Establishment of tracking and tracing tools for the transgene insertion is an essential part of the deregulation process. Detection methods for GM identification and quantification, are not only important to ensure legality and traceability, but also to comply with GM labeling regulations (European Parliament, 2003).

To date assessment of GM crops has focused on the plant product that is going to be used for human consumption. For instance, grains of GM maize, beans of GM soybean, and seeds of GM rapeseed and cotton. However, between 70 and 90% of all GM crops and their biomass are used in farm as animal feed (Flachowsky et al., 2012). The European Food Safety Authority (EFSA) recently have acknowledged the need for clarification on the safety assessment of GM feed of plant origin and published an explanatory note providing a forage definition for the major GM commercial crops (maize, soybean, sugarbeet, rapeseed, and cotton) (European Food Safety Authority [EFSA] et al., 2018).

Currently, most GM event detection and quantification methods used by national reference laboratories are developed and optimized for a real-time PCR (qPCR) platform. Nevertheless, qPCR has some notable drawbacks, such as the negative impact of inhibitors in the amplification efficiency, which represent challenges for applicants during the GM de-regulations process. Additionally, the requirement of reference material to use in calibrations, which is rarely commercially available, especially for niche transgenic cassettes or sequences and unauthorized events is another downside.

Emerging PCR-based technologies, such that droplet digital PCR (ddPCR), can overcome those obstacles. This technology relies on the same DNA amplification principles as the standard PCR and qPCR, but works through partitioning PCR mix into 20,000 nanoliter-sized droplets. Features such as absolute quantification, avoidance of using standard curves, high resilience to inhibitors leading to a less restrictive amplification efficiency, make ddPCR a promising alternative for GM event detection (Rački et al., 2014; Corbisier et al., 2015).

In temperate forage species that are being studied for potential GM-based improvement, qPCR could be the preferred technique to deliver the necessary GM traceability. The most relevant forage species in temperate areas have been the subject of active research into the development of transgenic cultivars including grasses such as perennial ryegrass (*Lolium perenne* L.) (Badenhorst et al., 2018), and tall fescue (*Festuca arundinacea* Schreb.) and legumes such as white clover (*Trifolium repens L.*) (Panter et al., 2012) and red clover (*Trifolium pratense* L.) (Wang and Brummer, 2012). The "Roundup Ready" cultivar of alfalfa (*Medicago sativa* L.) is the first commercially available transgenic forage, and issues relating to the detection of the transgene and market co-existence have been addressed (Putnam et al., 2016).

A transgenic high-energy perennial ryegrass was selected as case study for the evaluation and comparison of qPCR and ddPCR. A targeted up-regulation of fructan biosynthesis in the leaf blades of perennial ryegrass was obtained by re-programming the expression of fructan biosynthesis genes through the transgenic manipulation of 6-glucose fructosyltransferase (6G-FFT) and sucrose:sucrose 1-fructosyl-transferase (1SST) (Panter et al., 2017). During the transformation of high-energy ryegrass an antibiotic resistance factor, hygromycin B phosphotransferase (*hph*) gene, was introduced as a selectable marker.

For detection of GM sequences from plants, a sensitive and reliable endogenous reference gene is required as an experimental control. Such reference gene should be ideally single-copy within the genome, and non-variant in copy number across the cultivars and species (Xue et al., 2014). Due to several whole genome duplications in the evolutionary history of flowering plants (Angiosperm), crop genomes typically exhibit complex structure and genetic redundancy making the identification of the optimal reference gene more complex (Ren et al., 2018). Plant Cullin4 (Cul4) genes are potential candidates, because they are relatively highly conserved between species, and an *in silico* analysis suggested the single copy status of the gene in a range of flowering plants (Marín, 2009).

Additionally, GM detection must not only identify the presence of the transgenic sequence in a low concentration, but also be accurately identified in all different agricultural commodities the species in question generates across the agricultural supply chain (Cankar et al., 2006). For instance, in forage legumes these include fresh leaves, dry leaves, pollen, seeds, stems, hay and honey (Panter et al., 2015). In wind pollinated grass species used for grazing fresh leaves, dry leaves, pollen, seeds, stems, hay and silage are target products based on their role in agricultural production systems or relevance in a co-existence framework (Smith and Spangenberg, 2016). Adequate sampling protocols must be developed in conjunction with appropriate and validated methods of extraction, amplification and detection of the possible exogenous GM sequences.

In the present study, evaluation of the common qPCR and new ddPCR-based transgene detection techniques in relevant agricultural commodities of GM forage crops is discussed. Transgenic high-energy ryegrass was selected because it exemplifies one of the most complex scenarios for GM crops tracking and tracing purposes; the transgene is composed of endogenous genes (cisgene) and the sequence composition is highly skewed toward to guanine and cytosine (GC rich). Additionally, *Lp*Cul4, a single copy endogenous gene of perennial ryegrass, is reported for first time.

MATERIALS AND METHODS

Experimental Materials

All plant materials were maintained at the Agriculture Victoria Research Hamilton centre (Department of Economic Development, Jobs Transport and Resources). For the transformation, perennial ryegrass variety FLP418-20, was selected for use as donor material, based on the observed shoot regeneration from embryogenic callus (EC) derived from mature seeds of FLP418 (PGG Wrightson Seeds, Christchurch, New Zealand). Clonal replicates of the genotype FLp418-20, were subjected to transformation using biolistic-mediated DNA delivery. A detailed description of the transgenic ryegrass plants generation is published in Panter et al. (2017). The transgenic event 10 used in this experiment was hemizygous for the transgene, and the non-transgenic material was its null segregants (a genotype from perennial ryegrass plant FLP418-20). All transgenic and non-transgenic plants were grown under physical containment level 2 glasshouse conditions.

Raw Material

Fresh leaves, tillers, seeds and pollen (ca. 0.5 g) of transgenic perennial ryegrass and its null segregants (negative control), were harvested into 50 mL falcon tubes. Pollen grains were isolated from ryegrass inflorescences using a method adapted from Becker et al. (2003). Briefly, mature ryegrass inflorescences in which the terminal florets had not yet opened were collected into a 50 mL plastic falcon tube, and 2 mL of distilled water per inflorescence was added to the tube, which was then agitated to release the pollen from the anthers. Aliquots of 1 mL of each sample were transferred to 1.5 mL microcentrifuge tubes and pollen grains were precipitated by centrifugation.

Conserved Material

To produce hay or air-dried mature herbage, approximately 10 g of fresh material between head emerge but prior to flowering was harvested into paper bags, distributed evenly through the bags and dried in a horizontal position for 72 h on raised wire racks in a growth chamber, with 16 h at 25°C (day) and 8 h at 18°C (night). To produce silage, approximately 100 g of fresh plant material were harvested, ensiled in vacuum bags, and storage in a dark place at room temperature (22°C ± 2°C). After 4 weeks of fermentation, vacuum bags were unsealed and stored at −80°C.

Experimental Methods

DNA Extraction

DNA was extracted from 20 biological replicates of the untransformed perennial ryegrass genotype (FLP 418-20), and event 10 plants. Six different agricultural products were analyzed for a total of 240 DNA extractions. All sample materials were freeze-dried for 48 h with the freeze-dry system, FreeZone 4.5 Liter Benchtop Freeze Dry System instrument (Labconco, Kansas City, MO, United States) and ground to a fine powder using the TissueLyser II instrument (Qiagen, Hilden, Germany). Genomic DNA was extracted using the DNeasy™ Plant Mini Kit (Qiagen), following manufactures' instructions. DNA concentrations were measured using the NanoDrop 1000 UV-vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and DNA concentrations were normalized to 10 ng/μl.

Assay Design

Manual sequence analysis and primer design was performed using Sequencher version 5.0.1 (GeneCodes, Ann Arbor, MI, United States) and Primer3.[1] PCR primers and probes for the perennial ryegrass Cullin 4 gene, *Lp*Cul4 (Marín, 2009), the transgenic cassette containing modified fructan biosynthesis genes (1SST-6G-FFT; Panter et al., 2017), and the *hph* gene (Blochlinger and Diggelmann, 1984) were designed in this study and tested to generate amplicons shorter than 201 base pairs in length (**Supplementary Table S3**). The same primer and probe sets were used for both qPCR (SYBR Green I-based and probe-based) and ddPCR assays. The vector, possible and actual location of the designed construct-specific primer pairs and probe targeting the transgene are shown in **Figure 1A**, as well as GC content distribution of the transgenic cassette (**Figure 1B**). All primers and probes were synthesized at Integrated DNA Technologies Pte. (Singapore Science Park II, Singapore). Before detection and quantification of endo and exogenous genes, amplification efficiency and reproducibility for each primer set were examined through a standard curve assay, using DNA dilutions of plasmid DNA in the case of the exogenous genes, and genomic DNA for the reference gene. For ddPCR, assays were optimized using a thermal protocol with a range of annealing temperatures (55–65°C). Each PCR-based assay was performed in four technical replicates.

Real-Time PCR

Reactions were prepared using the SsoAdvanced™ Universal Supermix kit (Bio-Rad Laboratories, Hercules, CA, United States) for probes and SYBR® Green I. PCR reaction (total volume: 20 μl) consisted of 20 ng (in 4 μl) DNA template, 1 μl each of the forward and reverse primer adjusted to 10 μM, 10 μl SsoAdvanced™ Universal Supermix (2x), and for the probe-base assay 0.5 μl target-gene probe adjusted to 10 μM. All reactions were performed using a CFX connect qPCR instrument (Bio-Rad Laboratories) using the following program; initial denaturation of template DNA at 95°C for 30 s, followed by 40 cycles of amplification reaction (20 s 95°C; 30 s 60°C). Genomic DNA fragments and DNA plasmids (described above) were used as positive control (PC) templates for amplification, along with no-template controls (NTC). For each tissue (6 in total), 20 biological replicates of the isogenic control (FLP 481-20) and 20 of event10 were analyzed within four technical replicates.

Digital-Droplet PCR

The ddPCR Supermix for Probes (no UTP) kit (Bio-Rad Laboratories) was used as the basis for all reactions, the primers and probes was adjusted to a concentration of 100 μM. Following manufacturer's instruction, a total volume of 22 μl

[1] http://bioinfo.ut.ee/primer3-0.4.0/

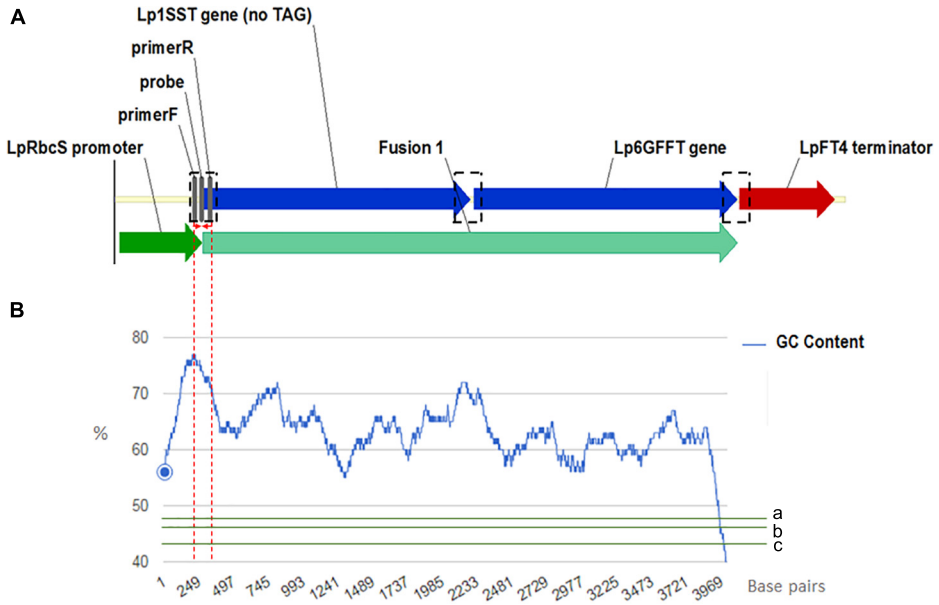

FIGURE 1 | Frame of transgenic elements in the event 10 ryegrass genome **(A)**. Location of the designed construct-specific primer pairs and probe targeting the transgene are in gray color. The transcriptional direction is indicated with arrow, and the perennial ryegrass rubisco promoter, 1SST-6G-FFT fusion protein gene, and FT4 terminator sequences are shown with dark green, blue, and red arrows, respectively. in black dotted rectangles possible location for the construct specific primers and probe. For designing the SST-FFT fusion protein gene, the terminal codon (TAG) of the perennial ryegrass SST gene was removed. GC content distribution **(B)** of 1SST-6G-FFT fusion protein gene in blue, compare with rice genic (a), rice genome average (b) and rice intergenic (c) in green. Red dotted lines showed a 200 base-pairs window where the set of primers and probe are located.

was prepared, containing; 0.12 μl target-gene forward primer, 0.12 μl target-gene reverse primer, 0.024 μl target-gene probe, 0.12 μl reference-gene forward primer, 0.12 μl reference-gene reverse primer, 0.024 μl reference-gene probe, 12 μl ddPCR Supermix for Probes (2x), and 9.472 μl distilled water. To each solution, 20 ng DNA template was added, nanoliter-sized droplets were generated on the AutoDG^{TM} Instrument (Bio-Rad Laboratories), following manufacturer's instruction. PCR amplification was performed with a T100 PCR Thermal Cycler (Bio-Rad Laboratories), with the following temperature profile: 10 min at 95°C for initial denaturation, 40 cycles of 95°C for 30 s, and 60°C for 60 s, followed by 98°C for 10 min. After PCR cycling was complete, the reactions were placed in a QX200 instrument (Bio-Rad Laboratories) and droplets were analyzed according to manufacturer's instructions. For each tissue (6 in total), 3 biological replicates of the isogenic control (FLP 481-20) and 3 of event10 were analyzed within 3 technical replicates.

Data Analysis

qPCR raw data were processed using BioRad CFX Manager 3.1. The cycle threshold (Ct) value denotes the cycle at which the fluorescent signal first showed significant difference with respect to the background. All biological and technical replicates were used to calculate the average Ct value. Relative copy number of the target gene (1SST-6G-FFT) was calculated using the comparative ΔΔCt method with *Lp*Cul4 as reference gene. ddPCR data was analyzed with the QuantaSoft software versions 1.3.2.0 (Bio-Rad Laboratories).

RESULTS

DNA Extraction

At the end of the extraction procedure, purified DNA was eluted with 100 μl of the AE buffer, and a subsequent measurement indicated that the concentrations varied between 4.8 and 83.25 ng/μl, and absorbance ratio (260/280) were between 1.66 and 1.85. On average, DNA concentrations from fresh leaves, tiller, seeds, pollen, silage and hay were 83.2, 23.8, 30.47, 4.8, 48.3, and 79.27 ng/μl, respectively. The lowest yield was obtained from pollen samples (4.8 ng/μl), which required an additional purification and concentration step to reach an acceptable concentration. A relatively low DNA purity was observed from the silage sample on NanoDrop system (260/280 = 1.66).

Assay Designing and Validation

Standard curve assays were performed for each primer pair and probe sets, in both probe and SYBR Green I based assay. Amplification efficiencies using SYBR Green I fluorescence were between 95 and 107% (**Supplementary Figure S1**) and those of probe-based assay were between 104 and 81% for *Lp*Cul4 and 1SST-6G-FFT and 91 and 88% for *Lp*Cul4 and *hph*, respectively (**Supplementary Figure S2**). Additionally, melting curve assays were performed when using SYBR Green I fluorescence (**Supplementary Figure S8**). A gradient PCR was performed to identify that the optimal range of annealing temperatures was between 59°C and 61.2°C for all *Lp*Cul4, 1SST-6G-FFT and *hph* primers and probes (**Supplementary**

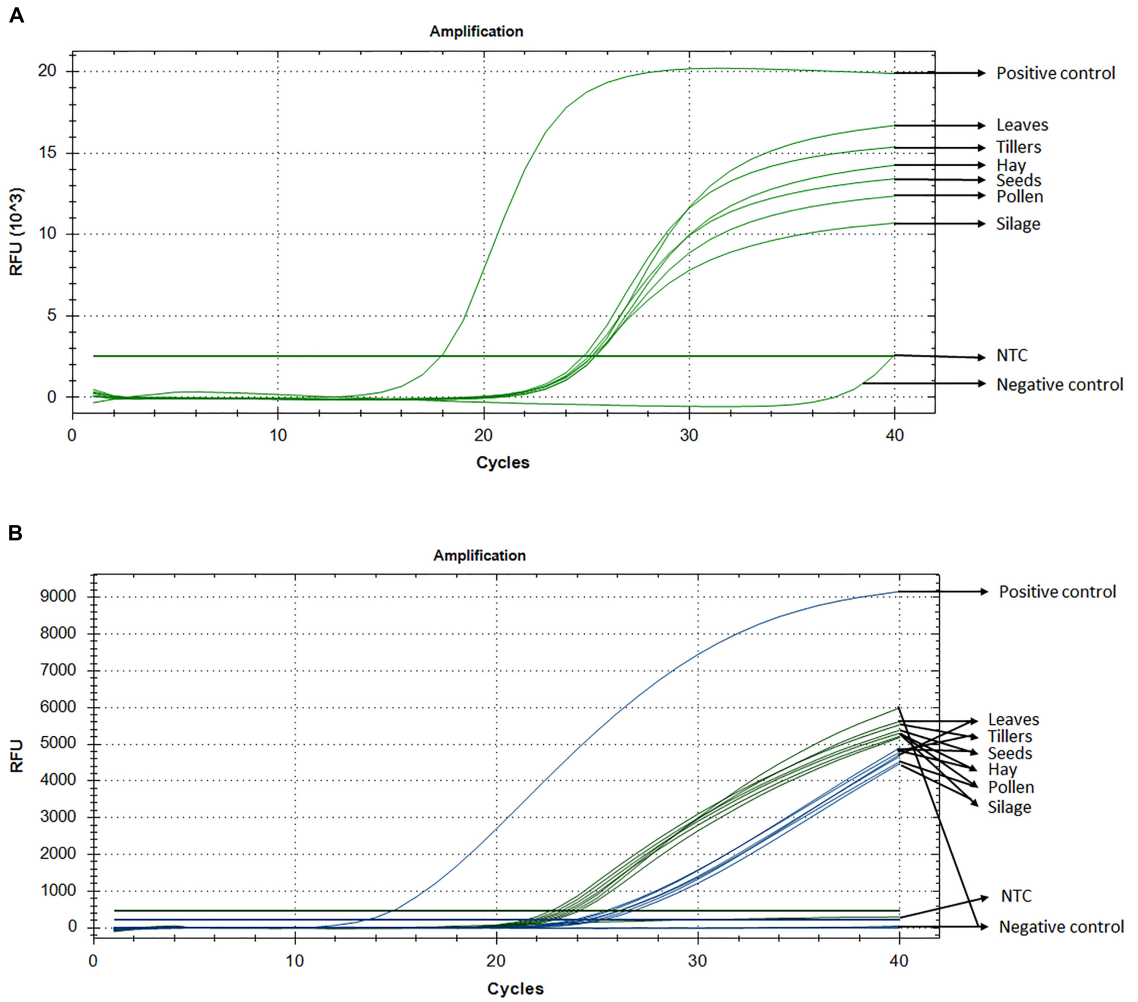

FIGURE 2 | Detection and quantification of 1SST-6G-FFT construct, using qPCR with SYBR Green I **(A)** and fluorescent probe **(B)**. In probe-base assay the target construct (1SST-6G-FFT, FAM) is shown in blue and the reference gene (*Lp*Cul4, HEX) in green. *Y*-axis shows relative fluorescence unit (RFU), and the *X*-axis denotes PCR cycle number.

Figure S3). Therefore, an optimized annealing temperature of 60°C was chosen for the subsequent experiments. Due to the similarity of the complete data set derived from the qPCR assays (both SYBR Green I and fluorescent probe-based), a single representative sample is presented (**Figure 2**). And, Ct means, standard deviation and coefficient of variation for all tissues are presented in **Supplementary Table S1**.

SYBR Green I-Based qPCR

Detection of the transgenic insertion 1SST-6G-FFT with SYBR Green I fluorescence was successfully achieved from all transgenic plant-derived DNA samples. The target sequence was amplified from PC (plasmid DNA) after 17 cycles, while amplification from transgenic plant-derived DNA samples started at around 25 cycles. Amplification from non-transgenic plant-derived DNA samples was observed only after 39 cycles. All Ct values (the cycle numbers in which fluorescent signals reached the threshold) from transgenic tissue were between 24.8 and 25.4 with the lowest value observed from hay and the highest from silage. Transgenic plant-derived DNA samples were differentiated from those of non-transgenic plants, with around 15 cycles (**Figure 2A**). Detection of the selectable marker, *hph*, was also performed, and similar results were obtained. PC amplified earlier (Ct = 19.7), while transgenic tissues fluctuated between 24 and 25 cycle and non-transgenic tissue (negative control) amplified after 38 cycles (**Supplementary Figure S4A**). No significant amplification was observed from NTC, within 40 cycles.

Probe-Based qPCR Assay

Probe and primer set for 1SST-6G-FFT insertion cassette (FAM fluorescence) and *Lp*Cul4 reference gene (HEX fluorescence), was tested with the PC (plasmid DNA containing the 1SST-6G-FFT sequence), transgenic tissue samples, and negative control (FLP418-20 genomic DNA). As expected, in the PC amplification was detected for 1SST-6G-FFT (Ct = 13.02) but not for *Lp*Cul4,

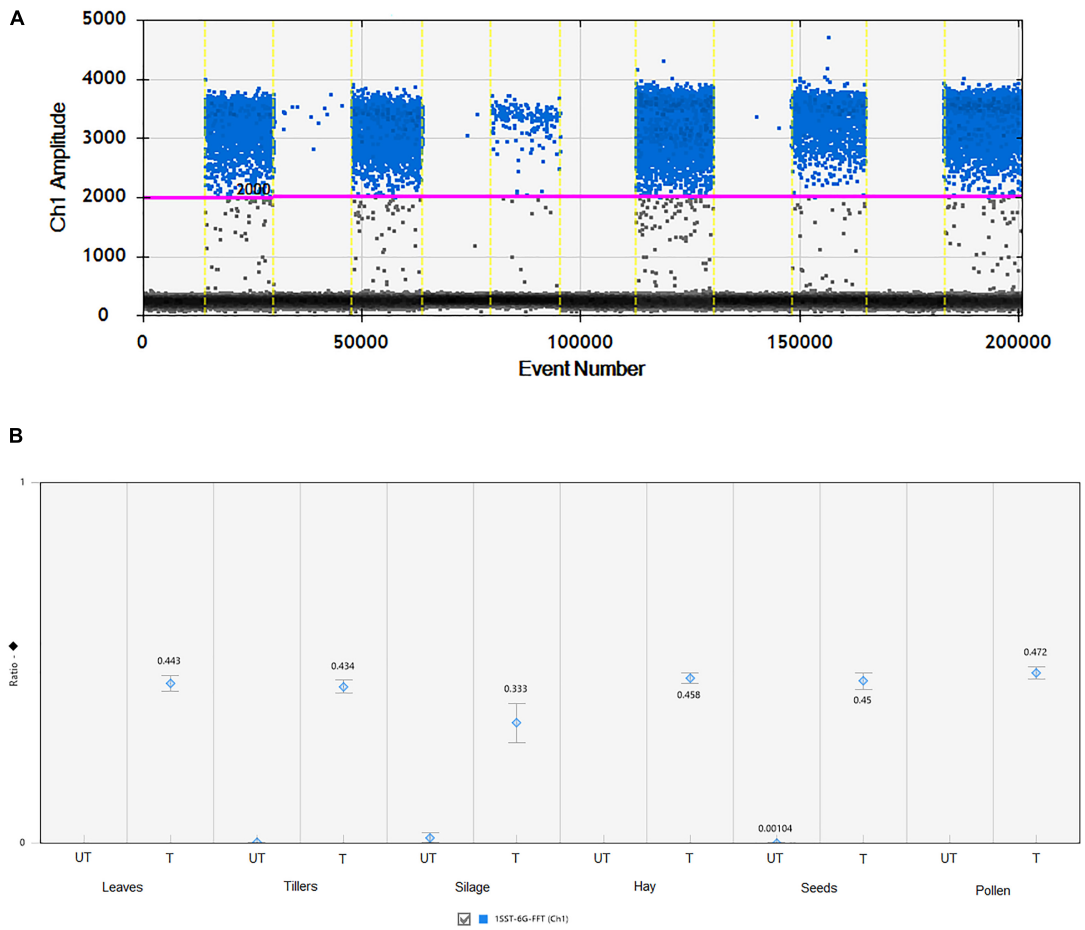

FIGURE 3 | Detection and quantification of 1SST-6G-FFT construct (FAM in blue) using ddPCR. **(A)** 1D fluorescence amplitude plot, where set threshold is shown with a pink line, blue dots indicate presence of the 1SST-6G-FFT sequence in the droplet, and gray dots indicate absence of the sequence. **(B)** Ratio of 1SST-6G-FFT construct and *Lp*Cul4. UT and T stand for untransformed and transformed, respectively. Error bars indicate the Poisson 95% confidence intervals for each measurement.

whilst the negative control showed the opposite, amplification for *Lp*Cul4 (Ct = 22.16) and none for 1SST-6G-FFT (**Figure 2B**). Amplification from transgenic event 10 samples was detected with both fluorescence channels, presenting Ct values between 23.1 to 28.2 for *Lp*Cul4 (HEX) and 24.2 to 30.7 for 1SST-6G-FFT (FAM) (**Figure 2B** and **Supplementary Table S1**). There was a difference in 1SST-6G-FFT (FAM) Ct values between transgenic and non-transgenic plant-derived samples This trend was also observed with highly processed samples, such us silage, in which 4 cycle difference in Ct values were observed between transgenic and non-transgenic plants. qPCR probe-based assay could effectively detect event 10 transgenic material in all agricultural commodities evaluated. The same probe assay with six tissue samples was evaluated with *hph* and *Lp*Cul4 genes, obtaining similar results (**Supplementary Figure S4B**).

ddPCR Assay

All agricultural commodities were analyzed using the ddPCR technologies and results are presented in **Figure 3** and **Supplementary Table S2**. Results showed that the 1SST-6G-FFT transgenic insertion is detected in the relevant samples, for instance when the sample was non-transgenic it presented only between 7 and 11 droplets, while transgenic samples showed between 161 and 1223 positive droplets (**Figure 3** and **Supplementary Table S2**). Although, all DNA concentrations were normalized to 10 ng/μl, differences in the droplet counts for the transgenic insertion and reference gene were observable among tissues. From the DNA purity and quality evaluation the silage samples were identified as more degraded relative to the other tissues, but still there was a difference in count numbers between transgenic and non-transgenic silage. The ratio between the target genes (1SST-6G-FFT) and the endogenous gene (*Lp*Cul4) was relatively close to 0.5 for most of the tissue samples (0.434–0.472), except for silage (0.333), this was expected since the event 10 samples were hemizygous, and the *Lp*Cul4 probe was designed to detect both alleles of *Lp*Cul4 (two copies in the diploid perennial ryegrass genome). The same ddPCR assay was performed for the *hph* gene as the target gene and results are comparable with the 1SST-6G-FFT gene (**Supplementary Figure S5**).

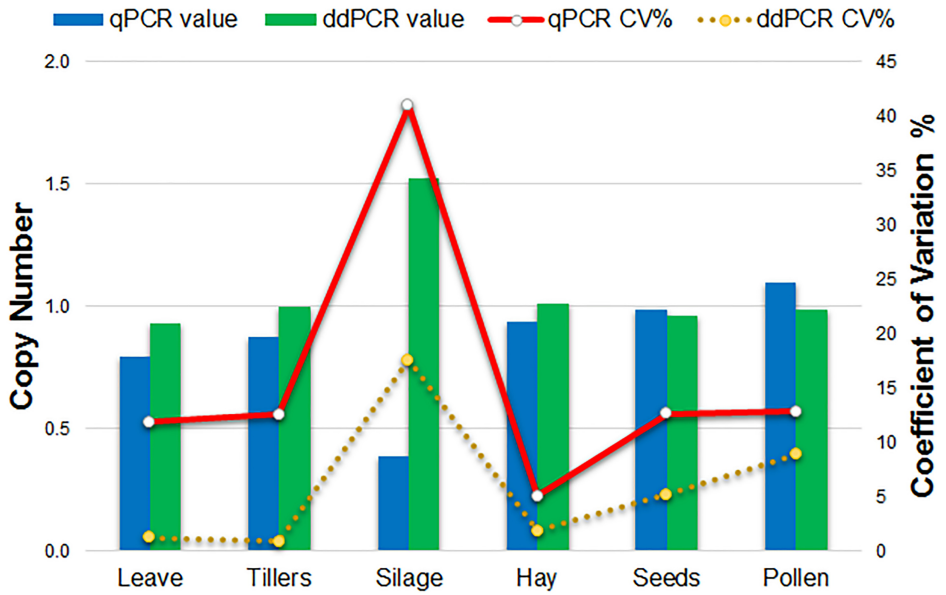

FIGURE 4 | Comparison of qPCR and ddPCR in all agricultural commodities. Histograms indicate the average relative copy number in each tissue **(left scale)**. Lines show the trend of the variation (CV%) of the qPCR and ddPCR assays **(right scale)**.

Copy Number Assay

Comparison of qPCR and ddPCR copy number assay results for all agricultural commodities with 1SST-6G-FFT as target, and *Lp*Cul4 as endogenous reference is presented in **Figure 4**. For the majority of commodities, copy number averages in qPCR and ddPCR were around one. However, the silage result for qPCR was three times higher than that of ddPCR (0.4 and 1.5 copies respectively). Reproducibility of the qPCR and ddPCR assays was evaluated using the coefficient of variance (CV) in lines (**Figure 4**). ddPCR revealed improved reproducibility (CVs between 0.9 and 17.6%) compare with those of qPCR (CVs between 5.1 and 41%).

LoD and LoQ

A DNA dilution test was performed with ddPCR and qPCR-based technologies for the 1SST-6G-FFT and *hph* sequences, using the *Lp*Cul4 probe as reference (**Figure 5** and **Supplementary Figures S6, S7**). Genomic DNA from transgenic leaves (event 10) and non-transgenic leaves (FLP 418-20) were adjusted to 10, 5, 1, and 0.5 ng for the 20 µl PCR mixture. In qPCR, using primers and probes for 1SST-6G-FFT reliable detection was achieved at 10 and 5% dilution, while for *hph* detections up to 1% was obtained. Both exogenous constructs showed similar results in the ddPCR assay, the number of positive droplets for the target gene decreased relatively linearly, compared with the reference. When analyzing 1SST-6G-FFT with Cul4,

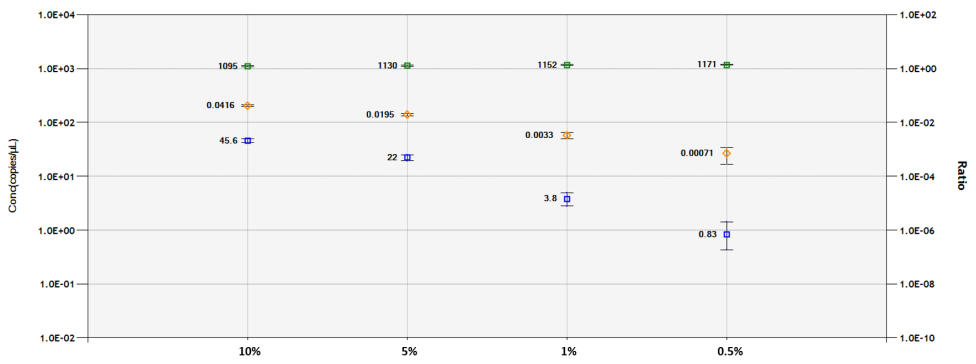

FIGURE 5 | Limit of detection and limit of quantification of 1SST-6G-FFT construct (FAM in blue), and *Lp*Cul4 (HEX in green) as reference gene using droplet digital PCR. Blue and green plots indicate the concentration of positive droplets (counts/µL; *Y*-axis on the left side) for the 1SST-6G-FFT and *Lp*Cul4 sequences, respectively, and the average concentration is shown the left side of the plot. Orange plots show the copy number ratio of 1SST-6G-FFT (*Y*-axis on the right side), which was calculated through the average concentration of FAM-positive droplets divided by HEX-positive droplets. Error bars indicate the Poisson 95% confidence intervals for each measurement.

concentration of the reference gene was 1095, 1130, 1152, and 1171 copies/μl, while for the target gene was 45.6, 22.0, 3.8 and 0.83 copies/μl for 10, 5, 1 and 0.5% respectively (**Figure 5**). Similarly, when analyzing *hph* with *Lp*Cul4, concentration of the reference gene was 1096, 1097, 1117, and 1132 copies/μl, while for the target gene was 47.3, 22.5, 3.9, and 1.5 copies/μl for 10, 5, 1 and 0.5% respectively (**Supplementary Figure S7**). The ratios (target/reference) for 1SST-6G-FFT were 0.0416, 0.0195, 0.0033, and 0.00071 and those for *hph* were 0.0432, 0.0205, 0.003, and 0.00013 with 10, 5, 1, and 0.5% of DNA respectively.

DISCUSSION

Development of detection techniques for new transgenic events are a prerequisite to comply with traceability and labeling requirement of GM plants for commercial realize (European Parliament, 2003) and in the management of co-existence frameworks in agricultural production systems (Putnam et al., 2016; Smith and Spangenberg, 2016). Therefore, development and validation of techniques capable of detecting, and quantifying the presence of GM forage crops at the farm gate, the processor, and the retailer level are necessary. In forage species intended for animal feeding, where human consumption is indirect, studies have focused on the digestive fate of recombinant DNA and proteins. Most of the studies concluded that transgenic DNA was broken down in the digestive system of animals (Flachowsky and Reuter, 2017). Therefore, efforts in GM detection for feedstuff species should not focus on milk or meat, but rather on other agricultural products (Smith and Spangenberg, 2016).

While all applicants for commercial release of transgenic products are required to provide a reliable method to detect the transgene below the tolerant threshold of each legislation, not all consider evaluating the method in different agricultural products. Determination of sample matrixes to be tested should be assessed in a case-by-case basis. For transgenic high-energy ryegrass, the most relevant tissues are fresh leaves, dry-leaves (hay), pollen, seeds, tillers and silage. The detection method of the transgene in herbage (fresh leaves and tillers) is essential, since it is the diet of gazing animals. Other factors such as traded material for sowing new pastures (seeds), gene flow (pollen) and the potential use of preservation and storage methods (hay and silage) are also needed to be considered. In the current study, a suitable DNA extraction method has been provided, using a commercially available kit.

Two main factors were evaluated during the sample preparation process, DNA quantity and quality. The first aspect refers to that sufficient DNA must be available to guarantee reliable detection of the transgene, and the second concerns the presence of undesired substances co-extracted with DNA, that may affect the accuracy of the detection method (Nadal et al., 2018). In the case study of high-energy ryegrass, DNA yield from pollen was relatively low, partially due to that pollen grains have a hard-outer shell, called exine (Lalhmangaihi et al., 2014). The presence of such tough coating in this matrix required additional DNA purification steps to obtain sufficient quality DNA. The DNA extraction kit used in this experiment was designed for plant tissue, and DNA extraction from fresh leaves, tillers, and even hay (dry leaves) was successfully performed. However, DNA from silage obtained after a 28-day fermentation process of the herbage showed the lowest level of quality, presumably due to endonucleotic enzyme activity during the fermentation (Tremblay et al., 2008).

Methods for detection of commercial transgenic forage crops are usually validated based on certified reference materials provided by the respective applicants. Transgenic and control reference material should be provided at the time of deposition of the dossier (Grelewska-Nowotko et al., 2018). For high-energy ryegrass, plasmid DNA with the exogenous cassette (1SST-6G-FFT and *hph*) was used as transgenic reference material. Due to the requirement for the endogenous reference gene to be quantitatively stable in all possible host genetic backgrounds (Marmiroli et al., 2008), the *Lp*Cul4 (Marín, 2009) single copy gene was selected.

Marín (2009) established the pattern of emergence and diversification of Cullin proteins in eukaryotes. It reveals that Cullin-RING ubiquitin ligases in animal, plant and fungi genomes, are ancient complex highly conserved and are likely to have a single copy status. In the present study, primers and probes for the Cul4 gene of perennial ryegrass, were designed, tested and the results corroborated that Cul4 is a suitable reference in ryegrass. In the same way a primer/probe set for the selectable marker (*hph*) were in house designed and tested. Although well-functioning primer/probe set for *hph* have been published previously (Collier et al., 2017), transgenic vectors may have small variations (Day et al., 2000).

Designing a set of primers and probe specific to the 1SST-6G-FFT sequence with acceptable amplification efficiency required an additional effort to be made, due to two main reasons. The first was that the coding sequence is constituted by two ryegrass endogenous genes (1SST and 6G-FFT) involved in the metabolic production of sucrose and fructan. Therefore, the only possible location for primers specific to the transgene was across junctions between two elements within the construct to avoid PCR amplification from the endogenous 1SST and 6G-FFT genes. The second, is that the nucleotide composition of insertion cassette was highly skewed toward guanine and cytosine.

Despite these limitations, the standard curve assays for the exogenous constructs and endogenous reference gene were reasonable. The low efficiency obtained with 1SST-6G-FFT in the probe-based assay (81.5%), is possibly related with formation of a complex secondary structure at GC-rich regions of the target amplicon, since GC content influences both optimal annealing temperatures and primer specificity (Mamedov et al., 2008). The optimal annealing temperature for the primer set, analyzed with a thermal gradient in ddPCR (**Supplementary Figure S1**), showed that to ensure GC-rich primers anneal stably to the template, higher annealing temperatures were necessary.

GM detection methods has depended on qPCR technologies, using either SYBR Green I or probe based fluorescence (Gao et al., 2016). The SYBR Green I assay showed ability to detect

both 1SST-6G-FFT and *hph* in a range of agricultural products (**Figure 2A** and **Supplementary Figure S4A**). Even though this fluorescent detection method is less specific because it binds any double-stranded DNA, including undesired non-specific amplicons and primer dimers. SYBR Green I methods are gaining popularity as it enables adding an existing method to the already available screening, which can be run in a single 96-well plate (Broeders et al., 2015). Additionally, this technology is more cost-effective as no dye-labeled oligonucleotide probes are required.

Fluorescent probe-based qPCR is the preferred method when a quantitative analysis is required, since it reflects high accuracy, specificity and sensitivity (Gao et al., 2016). The evaluation of different agricultural products in this study, allowed to distinguish between different amplification products in the same reaction. Therefore, with this approach the differences in PCR efficiency and cycle threshold between reference gene and exogenous fragment amplicons were well defined. Although, the CV values in the qPCR results (**Supplementary Table S1**) were higher, compared to those obtained using ddPCR (**Supplementary Table S2**), in the first assay approach, the CVs were calculated from the Ct values, while in the second assay approach those were calculated based on the concentration of the positive droplets (copies/μL), so that the variability in the results was typically higher in qPCR than in ddPCR (**Figure 4**). Compared with ddPCR, fluorescent probe-based qPCR requires reference material to calibrate the results, which adds more variation due to factors such as inhibitors and inherent measurement uncertainty (Köppel et al., 2015).

ddPCR can overcome those specificity issues in qPCR, through compartmentalization of a regular PCR mixture into millions of fractions (Lievens et al., 2016). A high sensitivity was observable in the evaluation of transgenic and non-transgenic agricultural products, and although the difference in DNA quality obtained from different commodities was still evident, results were clear and reliable (**Figure 3**). The high GC content of 1SST-6G-FFT, did not seem to have affected the amplification with this technique. However, the DNA quality of silage which is a product of partial degradation, was shown to affect the results. Similarly, Fearing et al. (1997) did not detect the CryIA protein from Bt maize silage, and they indicated that the ensiling process breaks down protein and fiber, rendering nutrients readily digestible to the ruminant animal. Hupfer et al. (1999) also demonstrated the effect of maize DNA degradation during the ensilage on the detectability of target sequences using qPCR, mainly due to the release of endogenous nucleases of the plant and/or exogenous nucleases of the microflora.

Transgenic DNA copy number assays using ddPCR have been reported in the major GM crops commercially available, such as maize (Dalmira et al., 2015; Xu et al., 2016; Collier et al., 2017; Grelewska-Nowotko et al., 2018), canola (Demeke and Eng, 2018), and soybean (Köppel et al., 2015; Iwobi et al., 2016; Wan et al., 2016). In the present study, all plants were previously selected to have a single copy of the transgene (**Figure 4**). However, the number of transgenic copies in other studies varies depending on the type of transformation used. For instance, a study on maize to determinate copy number of *T-nos*, using *hmg* as the reference gene, found that the number of copies in different varieties were between 88.22 and 0.88, with a coefficient of variance from 14.8 to 2.3% (Dalmira et al., 2015). These results agree with the ddPCR results obtained in this study, which despite having predetermined single copy samples, CV varies between 0.9 and 17.6%.

The minimum labeling threshold on GMO content in feed and foodstuffs are 0.9% in European Union (EU), 1% in New Zealand and Australia, 3% in Korea, and 5% in the United States (US) and Japan (Gao et al., 2016). Although, qPCR showed to be effective, ddPCR achieved a reliable detection of both exogenous constructs below the threshold of all jurisdictions. Similarly, ddPCR studies have showed reliable transgene detection of maize, soybean and canola at 1% (Dalmira et al., 2015; Demeke et al., 2016; Wan et al., 2016 respectively). qPCR can be a convenient method for qualitative detection with lower cost, in terms of instruments and reagents compared with ddPCR, but an accurate detection in qPCR can be limited when the target is present at low concentrations.

CONCLUSION

Detection and quantification of all transgenic pasture-based feed products should be assessed in all relevant agricultural commodities, since there is a high variability in DNA quantity and quality extracted from them, which affects its subsequent quantification. Determination of agricultural commodities to test, should consider factors such as storage, and/or processing. qPCR may be more suited for routine screening as it is very cost-efficient, while ddPCR may be more suitable for quantitative analysis as it allows an absolute quantification of the target sequence.

For high-energy ryegrass, detection of associated selectable marker genes such as *hph* would be advisable if using qPCR to avoid the GC-rich nature of the specific transgene, and both targets (1SST-6G-FFT and *hph*) can be used in ddPCR. To comply with labeling requirements in Europe as well as set global standards, ddPCR should be used to guarantee a reliable detection below the minimal threshold. However, in all other legislations with feed traded at a national level, qPCR with SYBR Green I can be used for general screening of a small number of targets common to numerous events (such as *hph*), Fluorescent probe-base qPCR to quantify event copy number and ddPCR can be used to support the results if need. For feed products intended to be traded globally, a standard method proven to be sensitive and reliable such us ddPCR should be considered.

This document provides guidance to Plant biotechnologists working on pasture based crops to assess GM crops in different agricultural commodities and with complex transgene sequences, such as cisgenic sequences (endogenous gene) that are also GC rich. However, studies must be carried out following a case-by-case approach for the evaluation of GM feed.

AUTHOR CONTRIBUTIONS

PG and HS conducted the experimental work and data analysis. NC, KS, and GS provided overall project leadership. PG, NC, and HS prepared the primary drafts of the manuscript and contributed to finalization of the text. PG, HS, KS, and GS co-developed interim and final drafts of the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01923/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplement legends

Table S1: Cycle threshold mean (Ct), standard deviation (SD), and coefficient of variation (CV%) using qPCR with probe-based fluorescent. GOI refers to gene of interest, and plus (+) and minus (-) signs indicate positive (+) and negative (-) for GOI.

Table S2: Concentration (Conc, copies/uL), standard deviation (SD), and CV using ddPCR with fluorescent probe-based. GOI refers to gene of interest, and plus (+) and minus (-) signs indicate positive (+) and negative (-) for GOI.

Table S3: Oligonucleotide primer and probe sequences of the exogenous constructs and endogenous reference gene.

Figure S4: Standard Curve assay for *Lp*Cul4 (**a**), 1SST-6G-FFT (**b**), and *hph* (**c**), using qPCR with SYBR Green I fluorescence.

Figure S5: Standard curve assay for 1SST-6G-FFT construct (FAM in blue) and *Lp*Cul4 (HEX in green) (**a**), and *hph* (FAM in blue) and *Lp*Cul4 (HEX in green) (**b**) using qPCR with fluorescent probes.

Figure S6: 1D fluorescence amplitude plot of the ddPCR assay using a gradient of temperature (65-55°C) for 1SST-6G-FFT construct (FAM in blue) and *Lp*Cul4 (HEX in green) (**a**), and *hph* (FAM in blue) and *Lp*Cul4 (HEX in green) (**b**).

Figure S7: Detection and quantification of *hph* construct, using qPCR with SYBR Green I fluorescence (**a**), and fluorescent probes (**b**). In the probe-base assay, the target construct (*hph,* FAM) is shown in blue and the reference gene (*Lp*Cul4, HEX) in green. The Y-axis shows relative fluorescence unit (RFU), and the X-axis denotes PCR cycle number.

Figure S8: Detection and quantification of *hph* construct (FAM in blue) using ddPCR. (**a**) 1D fluorescence amplitude plot, where set threshold is shown with a pink line, blue plots indicate presence of the *hph* sequence in the droplet, and grey plots indicate absence of the sequence. (**b**) Ratio of *hph* construct and *Lp*Cul4 (HEX). UT and T stand for untransformed and transformed, respectively. Error bars indicate the Poisson 95% confidence intervals for each measurement.

Figure S9: Limit of detection and limit of quantification of 1SST-6G-FFT construct (FAM in blue) (**a**) and *hph* (FAM in blue) (**b**) using *Lp*Cul4 (HEX in green) as the reference gene in a qPCR probe-based assay.

Figure S10: Limit of detection and limit of quantification of *hph* construct (FAM in blue) and *Lp*Cul4 (HEX in green) as reference gene, using droplet digital PCR. Blue and green plots indicate the concentration of positive droplets (counts/µL; Y-axis on the left side) for the *hph* and *Lp*Cul4 sequences, respectively, and the average concentration is shown the left side of each plot. Orange plots show the copy number ratio of *hph* (Y-axis on the right side), which was calculated through the average concentration of FAM-positive droplets divided with that of HEX-positive droplets. Error bars indicate the Poisson 95% confidence intervals for each measurement.

Figure S11: Melting curve assay and TapeStation of qPCR products for 1SST-6G-FFT (a and b respectively), and *hph* (c and d) and using SYBR Green I fluorescence.

Table S1: Cycle threshold mean (Ct), standard deviation (SD), and coefficient of variation (CV%) using qPCR with probe-based fluorescent. GOI refers to gene of interest, and plus (+) and minus (-) signs indicate positive (+) and negative (-) for GOI.

Tissue	GOI	Primers	Mean Ct	SD	CV (%)
Leave	-	1SST-6G-FFT	35.0	0.0	0.0
		LpCull4	24.0	0.4	1.7
Leave	+	1SST-6G-FFT	25.7	0.7	2.6
		LpCull4	24.2	0.6	2.5
Tiller	-	1SST-6G-FFT	34.9	0.3	0.8
		LpCull4	24.5	0.6	2.6
Tiller	+	1SST-6G-FFT	25.6	0.8	3.2
		LpCull4	24.2	0.7	2.9
Silage	-	1SST-6G-FFT	34.4	0.8	2.3
		LpCull4	27.7	1.6	5.7
Silage	+	1SST-6G-FFT	30.7	1.0	3.3
		LpCull4	28.2	0.8	2.8
Hay	-	1SST-6G-FFT	35.0	0.0	0.0
		LpCull4	23.1	0.6	2.8
Hay	+	1SST-6G-FFT	24.2	0.6	2.7
		LpCull4	23.1	0.6	2.6
Seed	-	1SST-6G-FFT	35.0	0.0	0.0
		LpCull4	24.9	0.2	1.0
Seed	+	1SST-6G-FFT	29.0	1.3	4.5
		LpCull4	25.0	0.4	1.6
Pollen	-	1SST-6G-FFT	35.0	0.0	0.0
		LpCull4	23.4	1.2	5.1
Pollen	+	1SST-6G-FFT	27.0	0.5	1.7
		LpCull4	24.6	1.6	6.5

Table S2: Concentration (Conc, copies/uL), standard deviation (SD), and CV using ddPCR with fluorescent probe-based. GOI refers to gene of interest, and plus (+) and minus (-) signs indicate positive (+) and negative (-) for GOI.

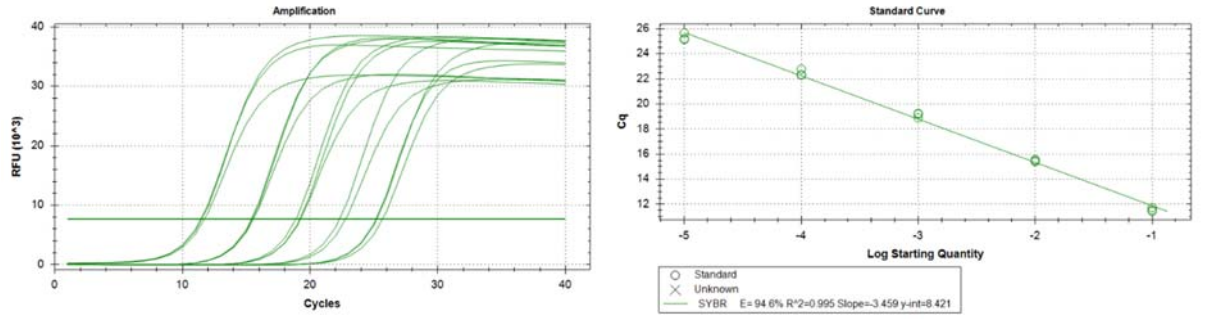
Tissue	GOI	Primers	Conc(copies/uL)	SD	CV (%)
Leave	-	1SST-6GFFT	3.8	0.1	1.8
		LpCull4	474.6	14.7	3.1
Leave	+	1SST-6GFFT	287.8	5.8	2.0
		LpCull4	463.4	22.9	4.9
Tiller	-	1SST-6GFFT	0.3	0.0	5.8
		LpCull4	495.8	8.9	1.8
Tiller	+	1SST-6GFFT	305.3	2.2	0.7
		LpCull4	547.6	8.0	1.5
Silage	-	1SST-6GFFT	3.8	0.2	4.0
		LpCull4	25.9	0.5	2.1
Silage	+	1SST-6GFFT	26.4	0.5	1.9
		LpCull4	22.0	1.0	4.5
Hay	-	1SST-6GFFT	0.4	0.0	8.5
		LpCull4	774.2	3.7	0.5
Hay	+	1SST-6GFFT	381.6	5.7	1.5
		LpCull4	754.2	25.8	3.4
Seed	-	1SST-6GFFT	2.6	0.2	7.1
		LpCull4	303.6	5.8	1.9
Seed	+	1SST-6GFFT	108.0	1.7	1.6
		LpCull4	316.3	5.8	1.8
Pollen	-	1SST-6GFFT	71.3	1.0	1.4
		LpCull4	162.5	4.0	2.5
Pollen	+	1SST-6GFFT	42.0	2.3	5.4
		LpCull4	57.6	3.0	5.2

Table S3: Oligonucleotide primer and probe sequences of the exogenous constructs and endogenous reference gene.

Target	Primer	Sequence (5′–3′)	Product (bp)
Endogene	*Lp*Cul4-f	CCGATTAAGCCAGCGGATA	122
	*Lp*Cul4-r	CATCTCTTTCATGGCTGTCA	
	*Lp*Cul4-P (HEX)	ACCTGGAGAGAGACCGAAGTA	
Exogene	*Lp*1SST-6G-FFT -f	AGGCATAGCCCAGCTAGTTA	137
	*Lp*1SST-6G-FFT -r	CGCGTACGCATAAGGAAGCA	
	*Lp*1SST-6G-FFT P(FAM)	- CCCGCGGTGAATTCATGGAGTCCC	
Exogene	*Hph*-f	ATTTCGGCTCCAACAATGTC	105
	Hph-r	AGATGTTGGCGACCTCGTAT	
	Hph-P (FAM)	TTGACTGGAGCGAGGCGATGTTC	

Figure S4: Standard Curve assay for *Lp*Cul4 (**a**), 1SST-6G-FFT (**b**), and *hph* (**c**), using qPCR with SYBR Green I fluorescence.

a



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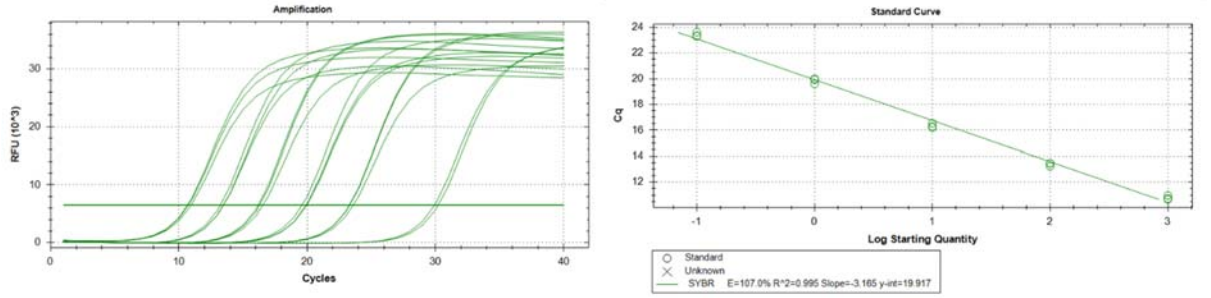

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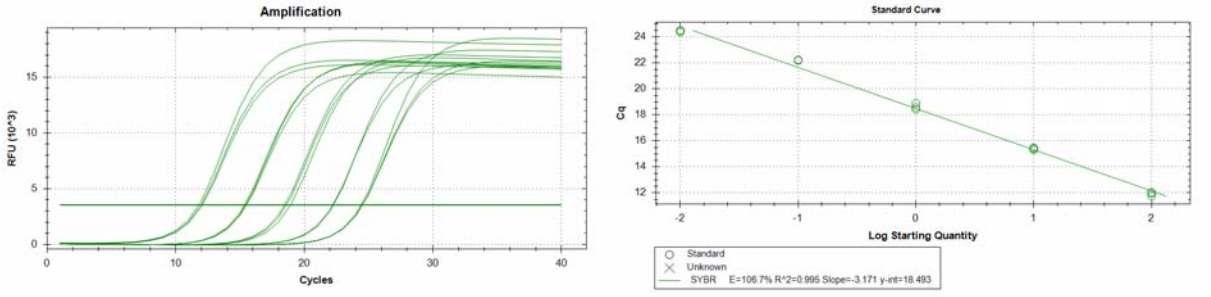

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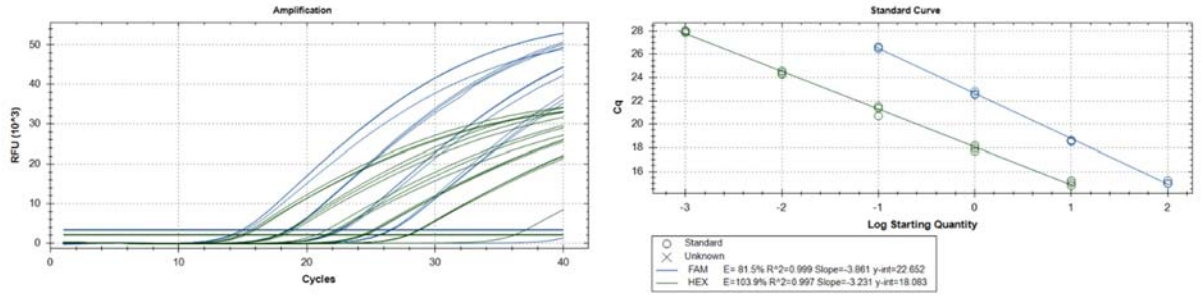
a



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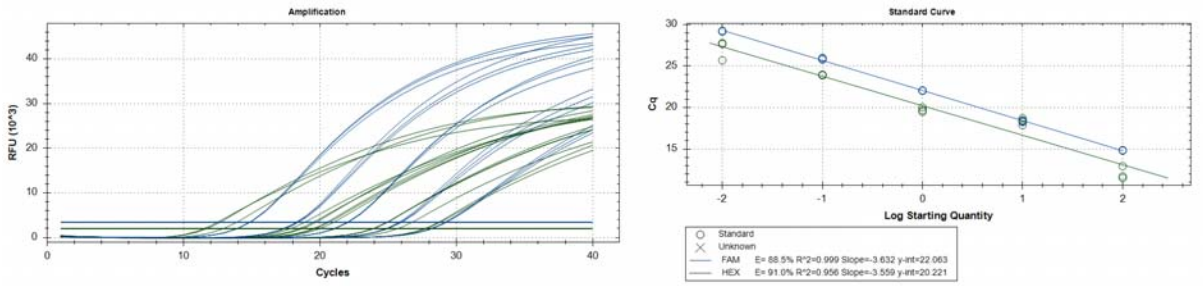
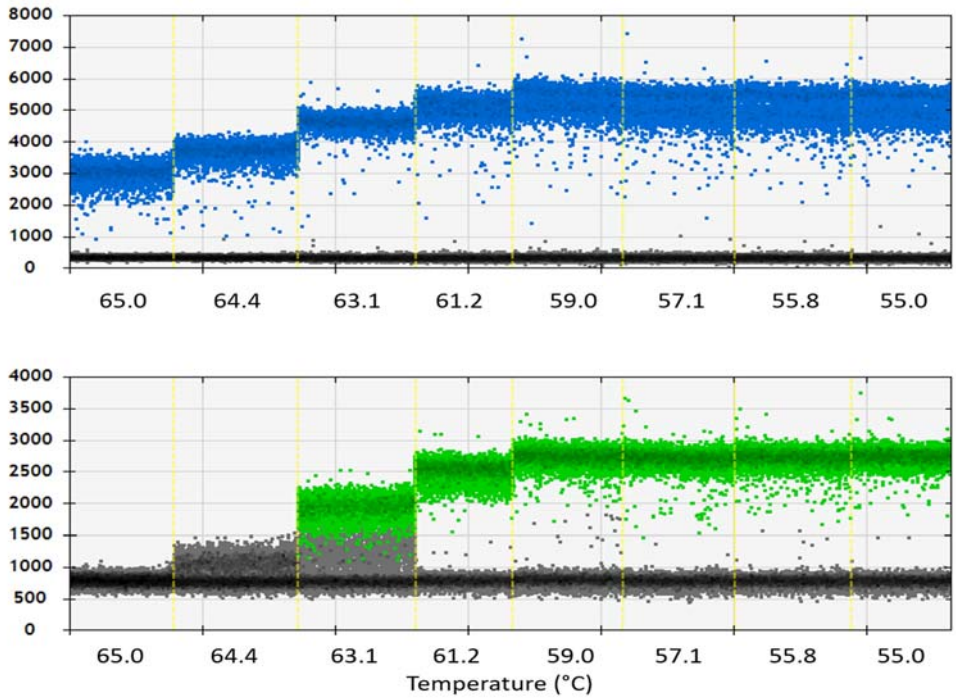


Figure S6: 1D fluorescence amplitude plot of the ddPCR assay using a gradient of temperature (65-55°C) for 1SST-6G-FFT construct (FAM in blue) and *Lp*Cul4 (HEX in green) (**a**), and *hph* (FAM in blue) and *Lp*Cul4 (HEX in green) (**b**).

a



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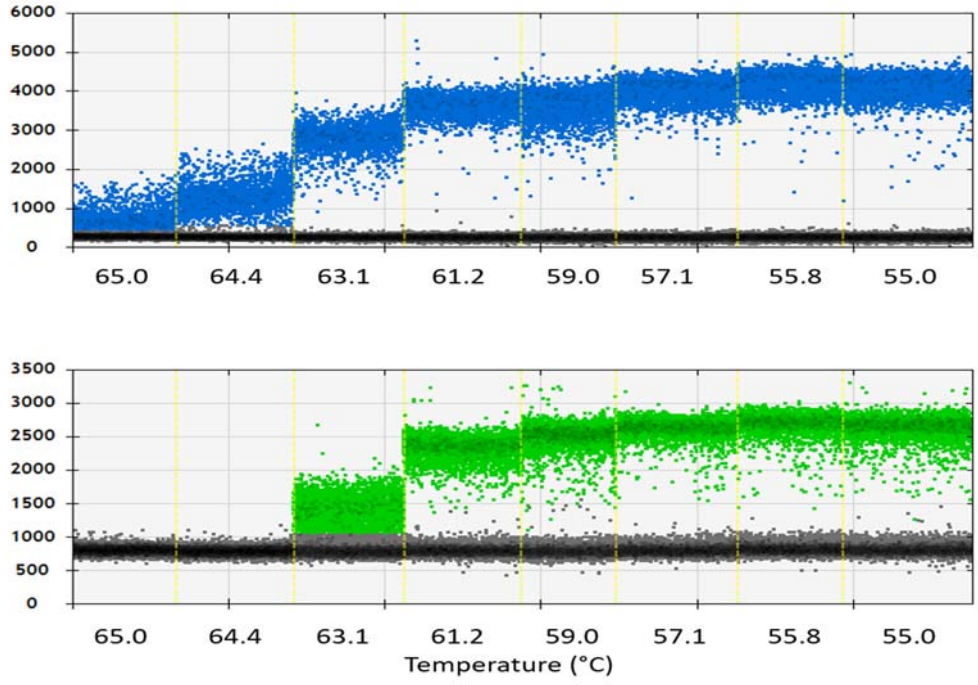
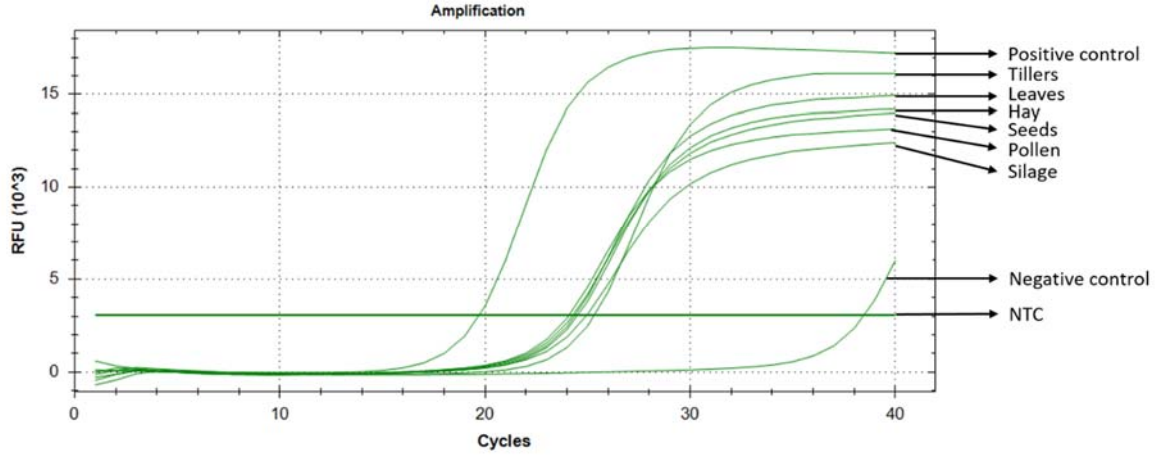


Figure S7: Detection and quantification of *hph* construct, using qPCR with SYBR Green I fluorescence (**a**), and fluorescent probes (**b**). In the probe-base assay, the target construct (*hph,* FAM) is shown in blue and the reference gene (*Lp*Cul4, HEX) in green. The Y-axis shows relative fluorescence unit (RFU), and the X-axis denotes PCR cycle number.

a



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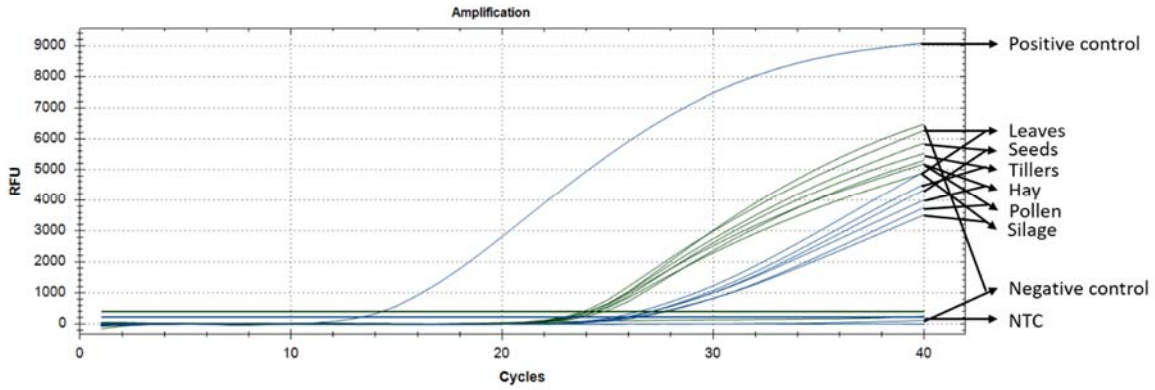

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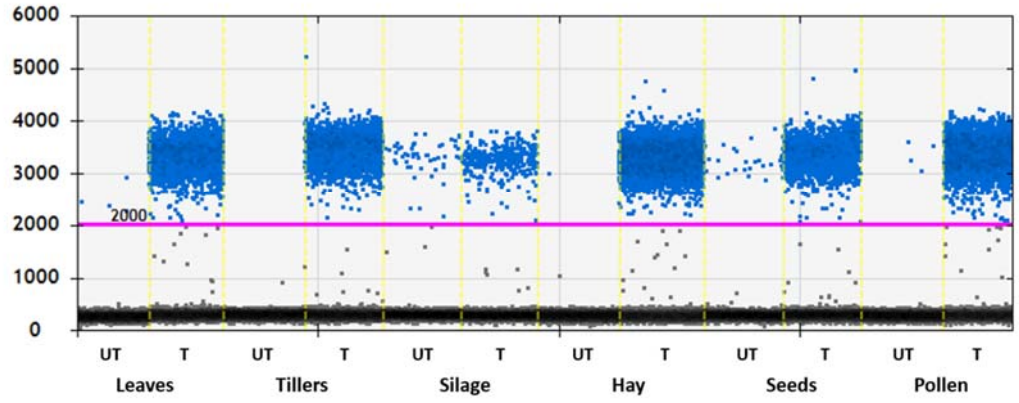
a



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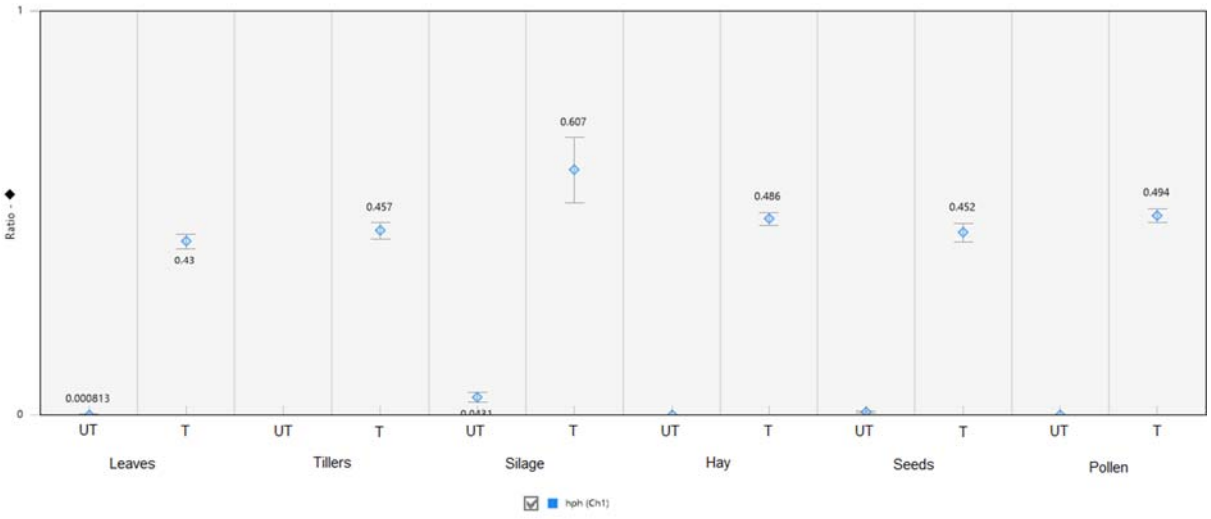
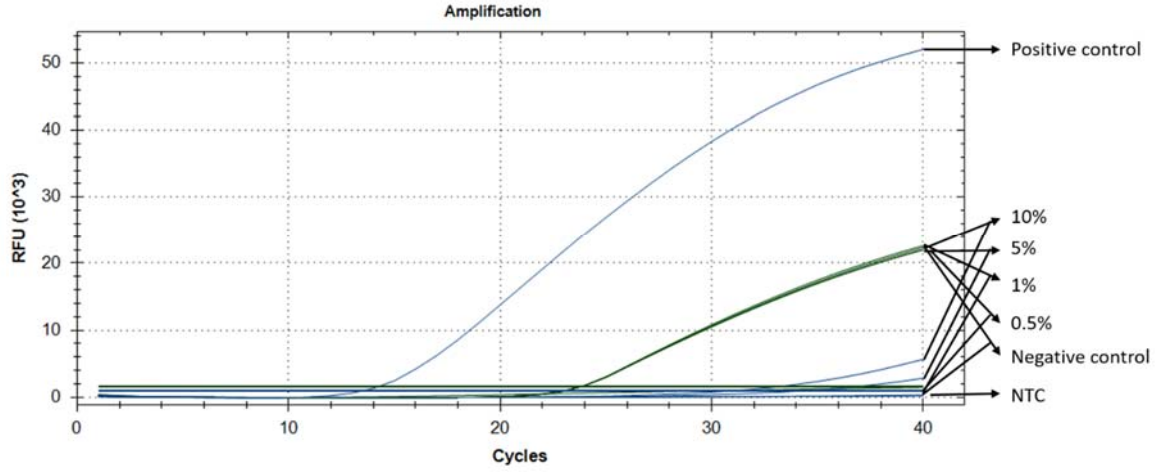


Figure S9: Limit of detection and limit of quantification of 1SST-6G-FFT construct (FAM in blue) (**a**) and *hph* (FAM in blue) (**b**) using *Lp*Cul4 (HEX in green) as the reference gene in a qPCR probe-based assay.

a



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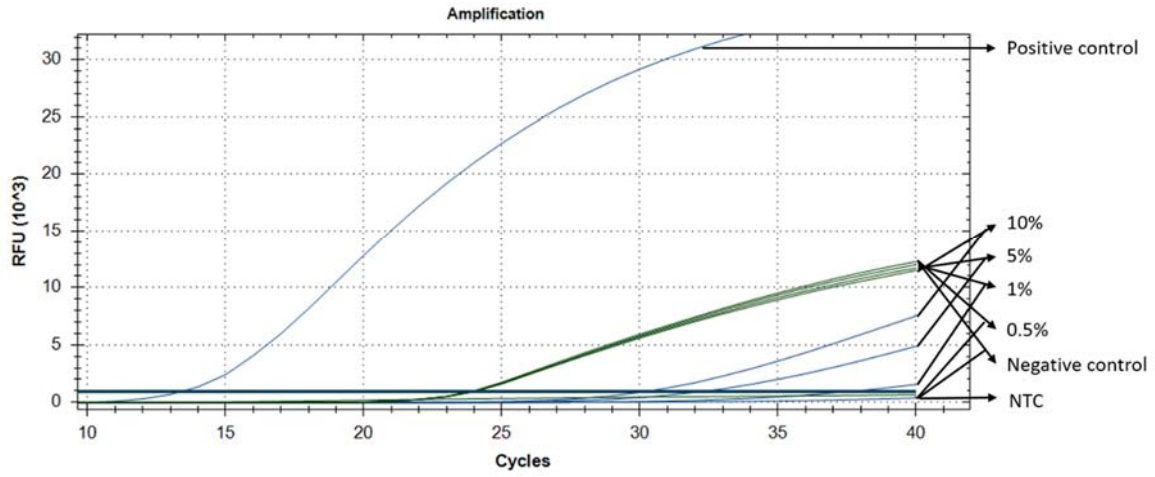

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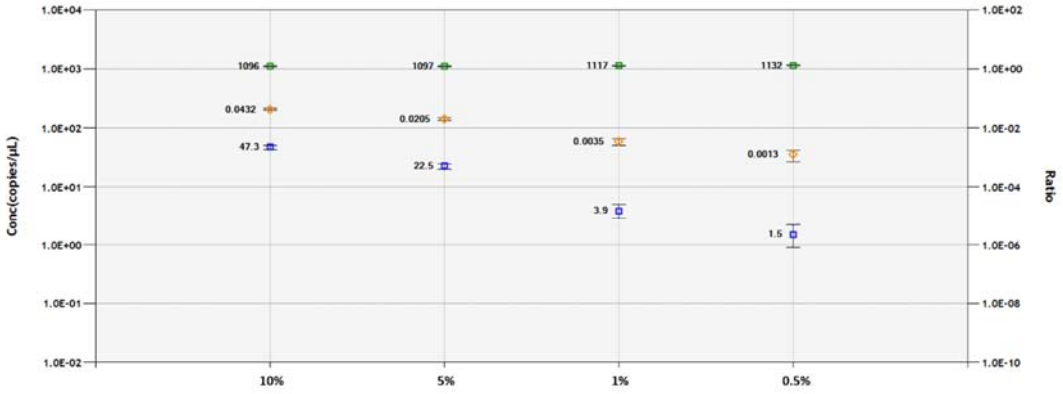
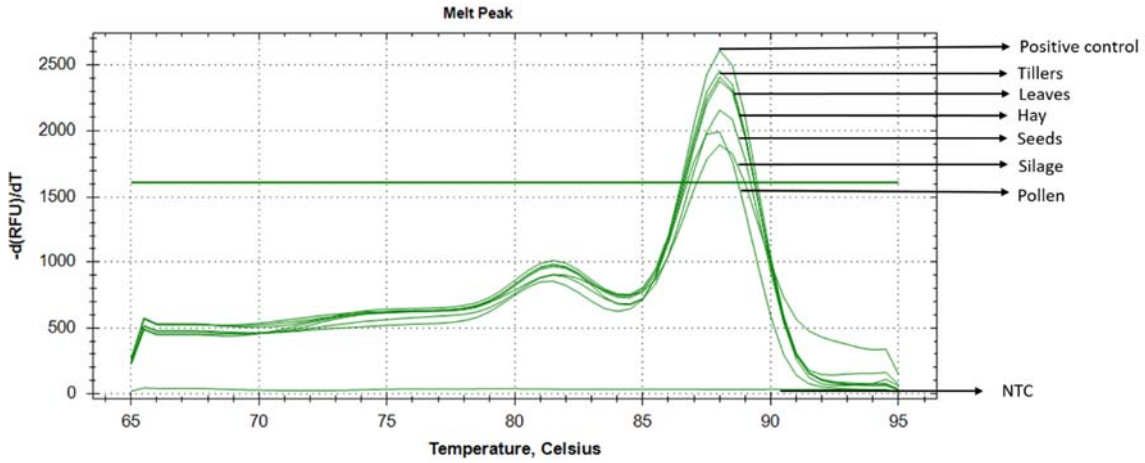


Figure S11: Melting curve assay and TapeStation of qPCR products for 1SST-6G-FFT (a and b respectively), and *hph* (c and d) and using SYBR Green I fluorescence.

a



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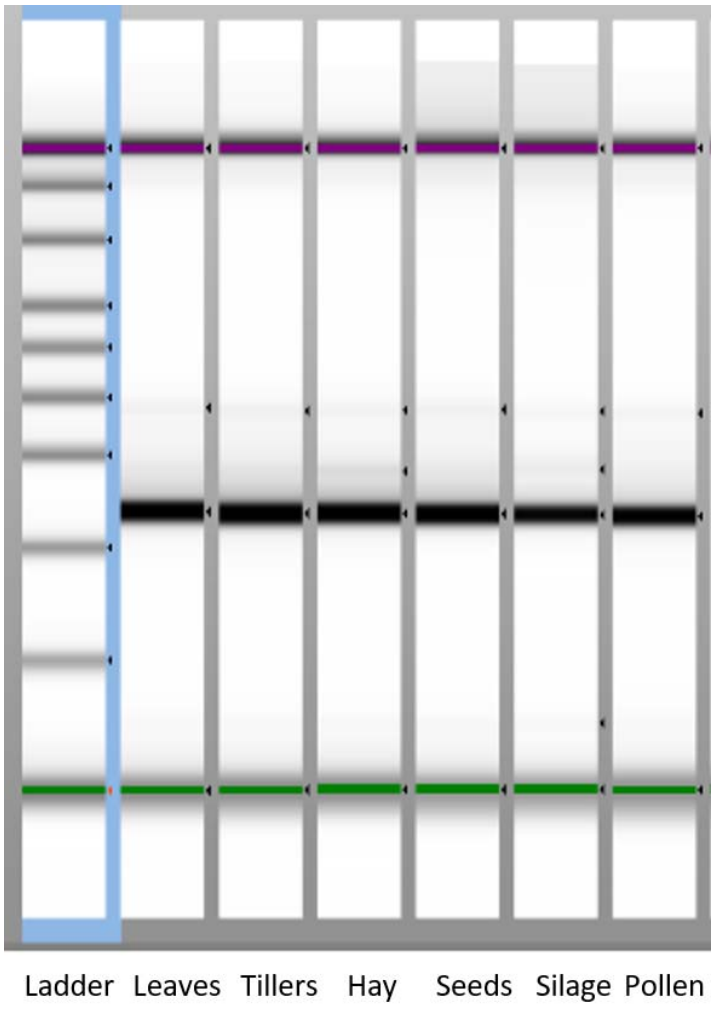

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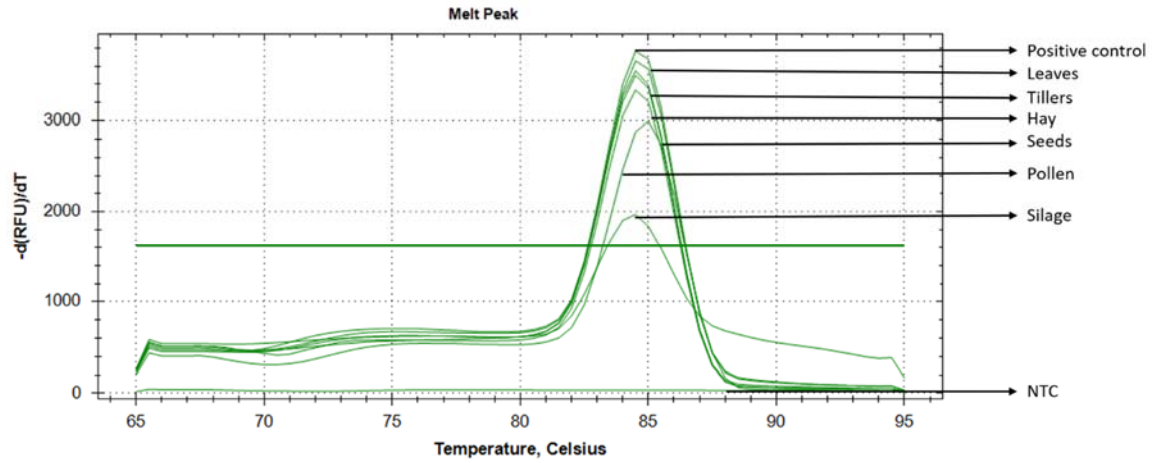
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Temperature, Celsius
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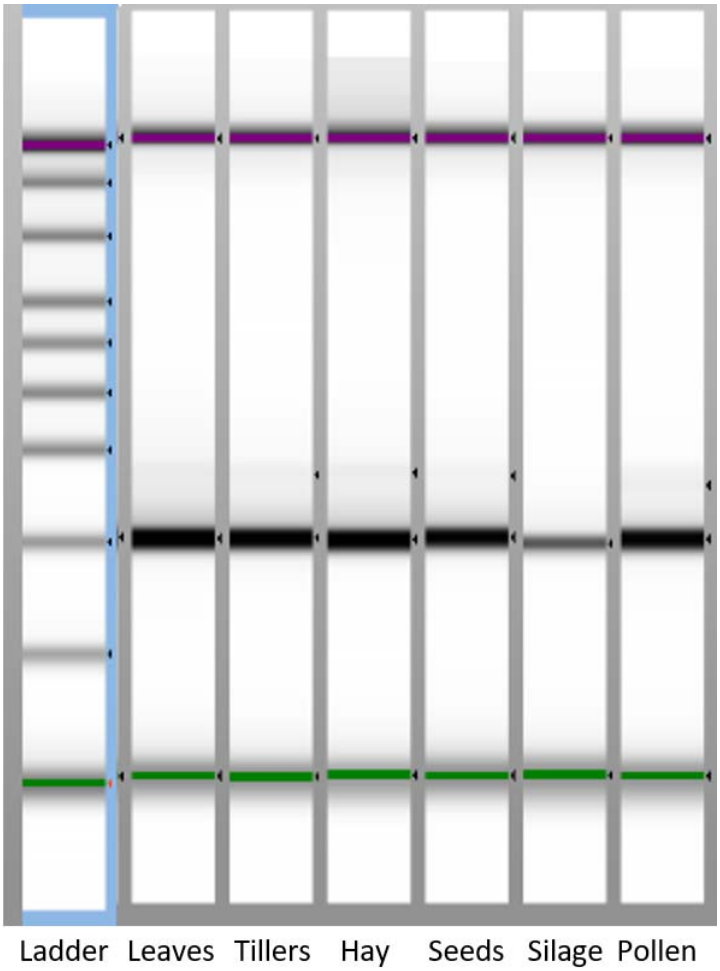
Leaves
Tillers
Hay
Seeds
Pollen
Silage
NTC

d


Ladder
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Silage
Pollen

Chapter 5

Transgenic Res
https://doi.org/10.1007/s11248-018-0087-9

ORIGINAL PAPER

Evaluation of endophyte toxin production and its interaction with transgenic perennial ryegrass (*Lolium perenne* L.) with altered expression of fructosyltransferases

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Received: 19 March 2018 / Accepted: 18 July 2018

Abstract Alkaloid concentration of perennial ryegrass herbage is affected by endophyte strain and host plant genotype. However, previous studies suggest that associations between host and endophyte also depends on environmental conditions, especially those affecting nutrient reserves and that water-soluble carbohydrate (WSC) concentration of perennial ryegrass plants may influence grass-endophyte associations. In this study a single transgenic event, with altered expression of fructosyltransferase genes to produce high WSC and biomass, has been crossed into a range of cultivar backgrounds with varying *Epichloë* endophyte strains. The effect of the association between the transgenic trait and alkaloid production was assessed and compared with transgene free control populations. In the vast-majority of comparisons there was no significant difference between alkaloid concentrations of transgenic and non-transgenic plants within the same cultivar and endophyte backgrounds. There was no significant difference between GOI+ (gene of interest positive) and GOI− (gene of interest negative) populations in Janthritrem response. Peramine concentration was not different between GOI+ and GOI− for 10 of the 12 endophytes-cultivar combinations. Cultivar Trojan infected with NEA6 and Alto with SE (standard endophyte) exhibited higher peramine and lolitrem B (only for Alto SE) concentration, in the control GOI− compared with GOI+. Similarly, cultivar Trojan infected with NEA6 and Alto with NEA3 presented higher ergovaline concentration in GOI−. Differences in alkaloid concentration may be attributable to an indirect effect in the modulation of fungal biomass. These results conclude that the presence of this

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transgenic insertion, does not alter the risk (toxicity) of the endophyte–grass associations. Endophyte–host interactions are complex and further research into associations with high WSC plant should be performed in a case by case basis.

Keywords Genetically modified · *Epichloë* · *Lolium perenne* L. · Higher-energy

Introduction

Perennial ryegrass (*Lolium perenne* L.) is one of the most important pasture grasses in temperate zones worldwide. This pasture plays a key role in providing the grazing feed-base for the dairy and red meat production industries. Ryegrass has remarkable characteristics that makes it popular, of which the most outstanding are high herbage yield and quality, favourable palatability and high grazing persistence (Yamada et al. 2005). Currently, digestibility and energy concentrations are key breeding targets, due to their potential to increase animal production through energy yield and increased intake.

Digestibility has low genetic heritability and has a large number of genes that control the trait. Measuring the trait simply and in large numbers is complex and requires laboratory based screening procedures to be used. As a result of these factors, limited gains have currently been realised by conventional breeding. Different transgenic approaches have been used to increase ryegrass energy, such as accumulation of water-soluble carbohydrates (WSC) in leaf blades (Panter et al. 2017), accumulation of lipids in the leaves (Winichayakul et al. 2008, 2013) and indirectly through improvement in fibre degradability (Faville et al. 2010). Moreover, an elevated concentration of WSC in grasses has been shown to increase milk and animal production (Smith et al. 1998b; Miller et al. 2001).

A perennial ryegrass cultivar with increased energy that was also adapted to Australian conditions could increase animal production in sheep, beef and dairy systems. (Ludemann et al. 2013; Smith et al. 1997). An arbitrary increase of 1 MJ/Kg of dry matter in metabolizable energy concentration of this grass, could lead to an increment of on farm operating profit between 40 and 54% (Ludemann et al. 2015). Therefore, emerging technologies such as genetic-engineering and gene-editing are being used to improve WSC and digestibility in forage grasses.

Genetic variation in the expression of fructosyl transferases has been shown to lead to high WSC concentrations in perennial ryegrass (Rasmussen et al. 2014), that had previously been utilised by plant breeders to develop a range of 'high sugar' cultivars (Humphreys 1989a, b, c). These cultivars have been shown to have the potential to increase animal production, but issues such as regional adaptation, (Smith et al. 1998a, 2007) and the interaction of yield and carbohydrate with seasonal growth patterns and grazing management (Turner et al. 2015; Robins and Lovatt 2016; Chen et al. 2017), has meant that the expression of the trait and subsequent changes in animal performance have not always been consistent.

An alternative approach to selection is the use of genetic modification to improve pasture quality and energy concentration, through the alteration of the expression of fructosyltransferases in conjunction with photosynthetic promoters (Panter et al. 2017; Badenhorst et al. 2018). High energy ryegrass has been produced, through up-regulation of fructan biosynthesis in leaf blades and pseudo-stems (Panter et al. 2017). Glasshouse evaluations of these transgenic plants followed by field trials have suggested an increment between 0.6 and 1.7 MJ/Kg of dry matter in metabolizable energy, compared to their isogenic control (Badenhorst et al. 2018).

Genetically modified (GM) plants are required to undergo rigorous safety assessments, to provide an evaluation of the potential impact on human, animal and the environment prior to commercialisation. Risk assessment of GM cultivars or plants are required to include a detailed molecular characterization, development of tracking and tracing methodologies, assessments of allergenicity, as well as nutritional and toxicological studies. Although, perennial ryegrass is not pathogenic or capable of causing any disease in plants, humans or animals, it can contain a fungal endophyte, which does produce alkaloids that act as deterrents to insect herbivory, but also can affect utilisation as animal feed (Office of the Gene Technology Regulator 2008) and as such any transgene needs to be assessed for its effect on the production of these toxins.

The endophyte of ryegrass is the asexual species of *Epichloë* (previously classified as the form genus *Neotyphodium*: Leuchtmann et al. 2014) that forms mutualistic relationships with their host. The endophyte gains shelter, nutrients and a means of transmission, while the plant gains increased protection from biotic stresses, mammalian and insect herbivores, and nematodes, as well as increased tolerance to abiotic stresses such as drought and lack of nutrients like soluble nitrogen (Hennessy et al. 2016; Bacon 1993). Such resistance against insect herbivorous is conferred through the accumulation of alkaloids, which can have anti-feeding and/or toxic effects.

Alkaloids produced by *Epichloë* endophytes are secondary metabolites that can be classified in four major groups; pyrrolopyrazine compound peramine (Tanaka et al. 2006), indole-diterpenes such as lolitrem B and janthitrem (Guerre 2016), ergot alkaloids (Guerre 2015) and lolines (Blankenship et al. 2001). Peramine and janthitrem I protect its host from invertebrate herbivore attack without detrimental effects to livestock, while lolitrem B is a potent tremorgen that makes it toxic to cattle, and ergovaline has a vasoconstrictor effect in mammals, but also deter feeding by insects so is beneficial at low levels.

Alkaloid profiles vary among different endophyte species and strains. Standard endophyte (SE), is a natural occurring endophyte present in perennial ryegrass (*Lolium perenne*, asexual *Epichloë* endophytes). SE produces lolitrem B which can cause ryegrass staggers and ergovaline which causes heat stress, therefore can be an animal health risk (Kaur et al. 2015). Development of endophyte strains with optimal alkaloid profiles are important in the livestock industry, as over expression of some alkaloids can cause losses in milk production and liveweight gain (Nicol and Klotz 2016). Therefore, it is essential to deliver the specific concentration of alkaloids, to prevent insect attack on plants, but to not affect animal wellbeing.

Some "novel" endophyte-grass host associations, in which alkaloids that act to deter insect herbivory are retained, but those that confer toxicity to mammalian grazers are absent or less abundant include; AR1 (Bultman and Bell 2003) that produces only peramine, NEA2 which produces peramine, ergovaline and low level of lolitrem B (van Zijll de Jong et al. 2008b), NEA3, NEA6 and NEA11 (van Zijll de Jong et al. 2008a), produce peramine and ergovaline, NEA12 (Kaur et al. 2015) and AR37 (AgResearch Ltd, Palmerston North, New Zealand) produce only janthitrem I. These endophyte strains are known to express no lolitrem B and ergovaline, or its production is at levels that do not stress the animal such that welfare and productivity are not compromised. However, further experiments evaluating the effect of these novel endophytes on animal welfare are required, since its effect in mammalian metabolism has not yet been well elucidated. Despite the complexity of grass-endophyte associations the majority or the perennial ryegrass seed in Australia and New Zealand contains endophytes.

The type of endophyte strain and host genotype are the main determinant of alkaloid concentration. However, previous studies have suggested that relationships between host and endophyte depends on environmental conditions, especially those affecting resource availability such as WSC (Ryan et al. 2015). Those studies found a 50% reduction in endophyte and alkaloid concentration when they used a cultivar bred for high WSC, compared with a conventional cultivar (Rasmussen et al. 2007). Factors that change alkaloid concentration may have effects on toxicity, invasion and species diversity (Liu et al. 2011; Ryan et al. 2015). Studying the effects of transgenic perennial ryegrass on endophyte concentration and alkaloid production is important, since it could alter the ecological or environmental context of host-endophyte interactions.

In the present study, a single transgenic event, conferring high-energy and enhanced biomass yield, that has been crossed into a range of cultivar backgrounds with varying endophytes is evaluated for its effect on alkaloid production, in comparison to the transgene free control populations. This study aims to quantify the effects of this single transgenic event on endophyte alkaloid productivity, provide guidance on future approaches of transgenic breeding requirements in relation to endophyte toxin evaluation, and assist in the comprehensive evaluation of a potentially commercialisable transgenic event.

Materials and methods

Generation and selection of the transgenic event

Fructan biosynthesis genes were re-programmed through the transgenic manipulation of two genes

involved in the catalyses of the first step in fructan biosynthesis [sucrose: sucrose 1-fructosyl transferase (1-SST)], and elongation of the growing fructan chain [6-glucose fructosyltransferase (6G-FFT)]. For designing the SST-FFT fusion protein gene, the terminal codon (TAG) of the perennial ryegrass SST gene was removed and a rubisco promoter (enzyme involve in photosynthesis) was added to promote fructan accumulation in leaves. Description of the generation of the transgenic ryegrass plants with altered fructan biosynthesis is reported in Panter et al. (2017). Briefly, perennial ryegrass variety FLp418-20, was selected for use as donor material based on the observed shoot regeneration from embryogenic callus (EC) derived from mature seeds of FLp418 (PGG Wrightson Seeds, Christchurch, New Zealand). Clonal replicates of the genotype FLp418-20, were subjected to transformation with the vector backbone-free expression cassette cGRA000022 (supplement Fig. S1). Biolistic-mediated DNA delivery to embryogenic callus from perennial ryegrass genotype FLp418–20 (called from now on as gene of interest negative GOI−), was used to generate the putative primary T0 transgenic perennial ryegrass event (called from now on as gene of interest positive GOI+). Based on good agronomical performance, increased fructan concentration and altered nutritive characteristics the event 10 containing the cGRA000022 cassette was selected for further analysis. Event 10 field evaluation and its further development using molecular breeding technologies are described in Badenhorst et al. (2018).

Cultivar-endophyte combination selection

Fifteen cultivar-endophyte combinations were assessed (supplementary material Table S2). Seeds of perennial ryegrass cv. Alto, Bronsyn and Trojan containing the common standard endophyte (SE) strain (previously designated standard toxic, ST) (van Zijll de Jong et al. 2008a), two commercial endophytes; AR1 (AgResearch Ltd, Palmerston North, New Zealand), NEA2, (van Zijll de Jong et al. 2008b), and four pre-commercial strains; NEA3 NEA6 (van Zijll de Jong et al. 2008a), NEA11, and NEA12 (Kaur et al. 2015), were obtained from New Zealand Agriseeds Ltd, Christchurch, New Zealand. A total of 20 seeds from each accession were germinated and maintained under glasshouse conditions (with natural day-lengths and mean temperature of 22 °C; DEDJTR Hamilton) at the Victorian Department of Economic Development, Jobs, Transport and Resources (DEDJTR), Hamilton Centre, Victoria, Australia.

Crossing strategy

Description of the full molecular plant breeding strategy implemented for ryegrass with altered fructan biosynthesis is reported in Badenhorst et al. (2016, 2018). Briefly, T0 event plants were crossed with ryegrass cv. Alto, Bronsyn and Trojan containing the selected endophyte strains. The T0 event plants were used as the pollen donor in the pair cross and seed was only harvested from the non- transgenic plant that received pollen, to allow for the maternal transfer of endophyte to the progeny. Seeds from the non-transgenic recipient genotypes (♀-non-transgenic genotype/♂-transgenic event) were germinated in glasshouse.

Gene of interest (GOI) detection

Fresh leaves (∼ 1 g) were harvested into a 50-mL centrifuge tube and snap-frozen in liquid nitrogen. Samples were freeze-dried for 24 h (Genesis 25XL Freeze- Dryer, VirTis, Canton, MA, USA). Genomic DNA was extracted from 300 mg of plant material using the DNeasy Plant Mini Kit (QIAGEN) per manufacturer's protocol.

The presence of the endogenous histone H3 gene (LpHisH3) and the gene of interest (GOI) was detected by real-time PCR, using specific oligonucleotide primer pairs (supplementary data, Table S3) and SYBR Green chemistry (Roche Diagnostics, Basel, Switzerland). PCR cycling conditions were 95 °C for 10 min, 40 cycles of 95 °C for 30 secs and 63.7 °C for 1 min. Results were scored in comparison to positive (plasmid DNA) and negative (non-transgenic plant DNA, no-template) control templates with the endogenous histone H3 gene (LpHisH3) included as a control (Panter et al. 2017).

Endophyte detection

Three random tillers from each plant were harvested by cutting 1 cm from the base, then pooled together, placed into collection microtube racks (Qiagen GmbH, Hilden, Germany) and freeze-dried (Genesis

25XL Freeze- Dryer, VirTis, Canton, MA, USA) for 48 h. DNA extraction was performed using the DNeasy Plant 96-kit (Qiagen, Hilden, Germany) per the manufacturer's protocol.

Multiplex PCR reactions were set up with 0.2 mM dNTPs, 250 nM of each of the six oligonucleotides used for endophyte detection, 0.5 units of Immolase DNA polymerase (Bioline, London, UK) and 1 × Immolase buffer (Bioline) and 25 ng of plant DNA, 10 ng of positive control endophyte DNA or water as the template in a 20 µL reaction volume. Cycling conditions were: 95 °C for 10 min, 10 cycles of 94 °C for 30 s, 65–1 °C per cycle and 72 °C for 1 min, 20 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min followed by a 4 °C hold. Reactions containing plant DNA and endophyte DNA were diluted 1:10 and 1:100 respectively with nuclease-free water. Aliquots of diluted PCR reactions (2 mL) were combined with 7.95 mL of Hi DiTM Formamide (Life Technologies, Carlsbad, CA) and 0.05 mL of the GenescanTM 500LIZTM molecular weight standard (Life Technologies).

PCR products were analysed using an Applied Biosystems 3730 DNA AnalyzerTM (Life Technologies), and raw results were scored against predicted product sizes to identify endophytes with GeneMapperTM v 3.7 software (supplementary data, Tables S4 and S5). Endophyte-specific SSR (Single Sequence Repeat) genotyping and cluster analysis was performed as described by Kaur et al. (2015).

Trial design and quantitative endophyte alkaloid analysis

Once all progeny was sampled and screened for the presence of GOI and endophyte strain, the associations were arranged in a randomised block design created in GENSTAT (Windows 13th Edition; VSN International Ltd, Hemel Hempstead, UK) (Badenhorst 2014). After 6 weeks in the randomised design and under controlled conditions, each plant was harvested approximately 5 cm from the base and freeze-dried (VirTis Genesis 25XL:SP Scientific, Stone Ridge, NY, USA) for 48 h, followed by grinding to 1 mm with a Tecator Cyclotec 1093 sample mill (Foss, Hillerød, Denmark).

For the quantitative alkaloid analysis, samples were extracted twice using 1 mL of methanol:water (80:20, v:v) as the solvent in 20 mg of freeze-dried, ground material. Both supernatants were mixed, dried down and reconstituted in 200 µL methanol:water (80:20, v:v). For quality control purposes, ryegrass samples cultivar Trojan with standard endophyte were used. Quantification and identification of alkaloids were determined by liquid chromatography mass spectrometry (LC–MS).

Series of commercial (peramine nitrate and ergotamine) and in-house isolated (lolitrem B) standard solutions at concentrations of 10, 25, 50, 100, 250, 1000 and 2000 ng/mL were prepared and used to construct concentration curves. An Agilent 1290 UHPLC with a 150 mm × 2.1 mm Themo Hypersil Gold 1.9 µm HPLC column, was used for the analysis of 3 µL aliquots of each sample extract. A gradient mobile phase with a mixture of two solvents: 0.1% formic acid in water (solvent A, Thermo Scientific) and 0.1% formic acid in acetonitrile (solvent B, Thermo Scientific) was used with a flow rate of 0.3 mL/min. Initial conditions were 98% solvent A before initiating a linear gradient to 0% solvent A over 11 min, and this was maintained for 4 min before returning to the initial gradient conditions. Compounds were identified using a Thermo Fisher Orbitrap Velos mass spectrometer (Waltham, MA, USA; Thermo, Bremen, Germany), operating in positive ESI for data acquisition. The masses were acquired in the range of 150–2000 amu and the limit of quantification for the analysis was 1.2 ng/mL and limit of detection 0.36 ng/mL. Relative quantification (expressed as peak area) is presented for janthitrem I in the absent of standards for this compound, as well as relative quantification of ergovaline relative to ergotamine concentration. For peramine and lolitrem B quantitative results with matched standards were calculated.

Statistical analysis

Statistical analysis of derived data was performed using the residual maximum likelihood (REML) model within GENSTAT. Variables were natural log transformed prior to analysis to stabilize the variance. The model used for analysis was a mixed model with endophyte strain, cultivar and host genotype fitted as fixed effects and experimental design factors such as row and column were fitted as random effects. Each alkaloid analysed was measured only in the strain known to produce that alkaloid. The least significant

difference (l.s.d.) at $P = 0.05$ was generated to test for effects arising from differences between endophytes and between host genotypes within each association. Comparisons were made between gene of interest positive (GOI+) and negative (GOI−) treatments within the same cultivar-endophyte combination.

Results

The previously generated event 10 with expression of cGRA000022 cassette was used as input for the detailed evaluation of transgene × host × endophyte evaluation. Seeds of perennial ryegrass (*Lolium perenne L.*) cv. Alto, Bronsyn and Trojan containing the common standard endophyte (SE) and six commercial endophytes were germinated to use as the selected recipient genotypes. The endophyte incidence in the potential recipients was 98%. Selected plants that contained endophyte were used in pair-crosses with selected T0 event plants.

Genotypic analysis was performed on T1/F1 progenies (GOI+) produced from crosses and non-transgenic control samples (GOI−), to identify transgene positive and negative plants. A total of 302 plants were also screened for endophyte presence and identity. Only 3 plants were excluded from further analysis due to failure to identify endophyte presence, based on a collection of diagnostic SSR markers. Therefore, 299 plants were confirmed as endophyte positive (E +) and incidence for the T1/F1 progenies was 99%.

The most prevalent endophyte was AR1, occurring in 36 plants of cultivar Alto (21 GOI− and 15 GOI+), 26 in cultivar Trojan (14 GOI− and 12 GOI+) and 20 in cultivar Bronsyn (12 GOI− and 6 GOI+) (supplementary material Table S2). The strain NEA3 presented the lowest incidence in symbiosis with cultivar Trojan (4 plants; 2 GOI− and 2 GOI+), these were excluded from the experiment due to insufficient experimental replicates. However, the association between NEA3 and cultivar Alto occurred in 27 plants (17 GOI− and 10 GOI+).

All GOI+ and GOI− plants carrying candidate endophytes were subjected to a qualitative analysis for production of peramine, lolitrem B, and ergovaline. Janthritrem response was analysed separately and was semi-quantitative. Each alkaloid was measured only in the strain known to produce it, as described in supplementary material (Table S6). Data were logarithmic transformed ($\log_e$) in the absence of a normal distribution. Different constant values were added to each alkaloid per its residual distribution (Table 1).

Results of transformed means and the least significant differences (LDS) are presented in Table 1. All significant differences reflected lower alkaloid concentration for GOI+ compare with GOI−. Peramine concentrations (reported in parts per million, ppm) for GOI− samples was higher than GOI+ in most combinations, although these differences were not significant (Fig. 1). The Trojan-NEA6 combination in the control (GOI−) was significantly higher than the GOI+ combination, producing 21.3 and 8.0 ppm of peramine respectively. Similarly, in Alto-SE peramine concentration was 64.2 and 25.2 ppm for GOI− and GOI+ respectively.

Even though the aim of this study was evaluating the effect of GOI on alkaloid production, differential responses within cultivars and endophyte strains were apparent. For instance, peramine concentration in a single GOI− cultivar, Trojan with AR1 was 47.3 ppm higher than with NEA2 (56.5 and 9.2 ppm) and SE with cultivar Bronsyn was 25.4 ppm lower than Alto (38.8 and 64.2 ppm).

Lolitrem B (Fig. 2.) with the standard endophyte strain (SE) synthesized considerably higher amounts of this alkaloid compared to NEA2, which is consistent with known expression levels. The combination of Bronsyn-NEA2 and Bronsyn-SE, produced similar concentration of lolitrem B between GOI+ and GOI−. In contrast, lolitrem B concentration for Alto-SE was significantly higher for GOI− than for GOI+ (19.9 and 2.1 ppm). Trojan-NEA2 also presented higher concentration for GOI−, but the difference was not significant.

For most endophyte-cultivar combinations, ergovaline concentrations were the same or higher for GOI− than GOI+ (Fig. 3.). Conversely, for Alto-NEA11, Trojan-NEA11, Trojan-NEA2 and Bronsyn-SE combinations, GOI+ produced lower levels of ergovaline than GOI−, however these differences were not statistically significant when comparing GOI+ and GOI− samples. Trojan-NEA6 showed the highest significant difference in ergovaline concentration for GOI− (14.2 ppm), compare with GOI+ (3.7 ppm).

Janthritrem is a less common alkaloid and there are not commercially available standards for this

Table 1 Transformed means

Trait	Transformation	Cultivar-E	GOI+	GOI−	LSD
Peramine	ln (y + 10)	Alto-AR1	3.86	4.15	0.39
		BronSyn-AR1	3.94	3.82	0.59
		Trojan-AR1	4.30	4.20	0.46
		Alto-NEA11	3.76	3.58	0.61
		BronSyn-NEA11	3.36	3.44	0.48
		Trojan-NEA11	3.25	3.32	0.64
		BronSyn-NEA2	3.39	3.75	0.62
		Trojan-NEA2	2.84	2.95	0.50
		Alto-NEA3	3.54	3.62	0.48
		Trojan-NEA6	2.89b	3.44a	0.51
		Alto-SE	3.56b	4.31a	0.71
		BronSyn SE	3.98	3.89	0.54
Lolitrem B	ln (y + 0.01)	BronSyn-NEA2	− 0.26	− 0.91	1.66
		Trojan-NEA2	0.75	1.23	1.35
		Alto-SE	0.73b	2.99a	1.89
		BronSyn SE	2.45	2.39	1.46
Ergovaline	ln (y + 0.08)	Alto-NEA11	1.04	0.60	0.98
		BronSyn-NEA11	0.41b	1.23a	0.79
		Trojan-NEA11	0.70	0.14	1.04
		BronSyn-NEA2	0.16	0.82	1.00
		Trojan-NEA2	− 1.25	− 1.37	0.81
		Alto-NEA3	1.42b	2.21a	0.79
		Trojan-NEA6	1.32b	2.66a	0.83
		Alto-SE	− 0.22b	1.44a	1.15
		BronSyn SE	0.70	0.83	0.88
Janthritrem	ln (y)	Alto-NEA12	14.56	13.90	1.04
		BronSyn-NEA12	13.52	13.64	0.85
		Trojan-NEA12	13.64	13.96	0.78

Denotes significant difference between GOI+ and GOI− plants within a cultivar/endophyte combination

E endophyte; GOI+ gene of interest positive; GOI− gene of interest negative; LSD least significant difference

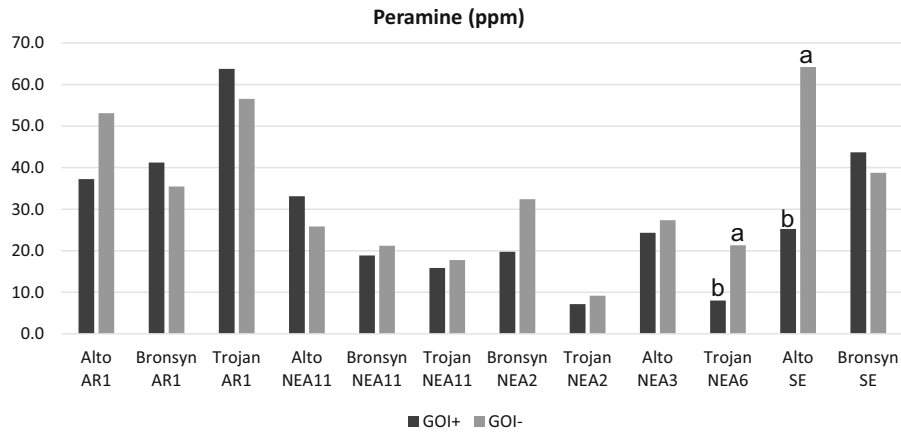

Fig. 1 Peramine concentration of each cultivar-endophyte combination for transgenic lines in dark grey and non-transgenic lines in clear grey. Bars are back-transformed means. a b different letters indicate significant differences at $P < 0.05$. GOI+ gene of interest positive; GOI− gene of interest negative

compound, therefore results are expressed as response (relative peak areas). The NEA12 strain was present in cultivar Alto, Bronsyn, and Trojan (Fig. 4.). Cultivar Trojan and Bronsyn inoculated with NEA12 presented similar responses between GOI+ and GOI−. However, in the combination Alto-NEA12, GOI+ showed

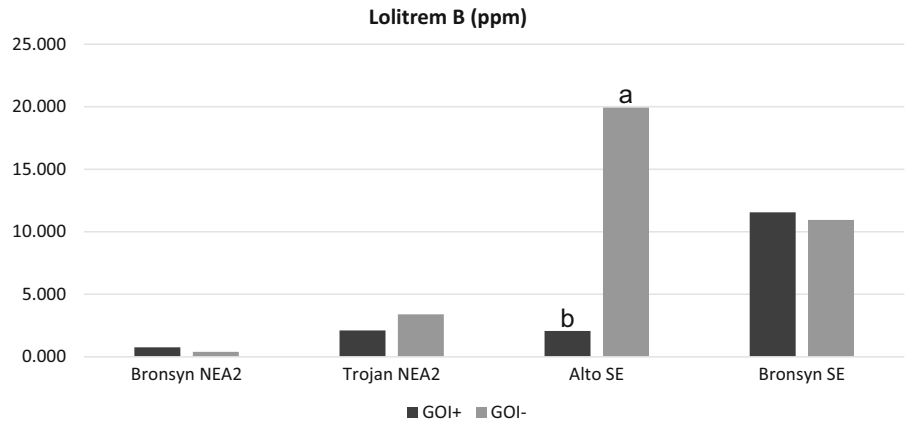

Fig. 2 Lolitrem B concentration of each cultivar-endophyte combination for transgenic lines in dark grey and non-transgenic lines in clear grey. Bars are back-transformed means. GOI+ gene of interest positive; GOI− gene of interest negative

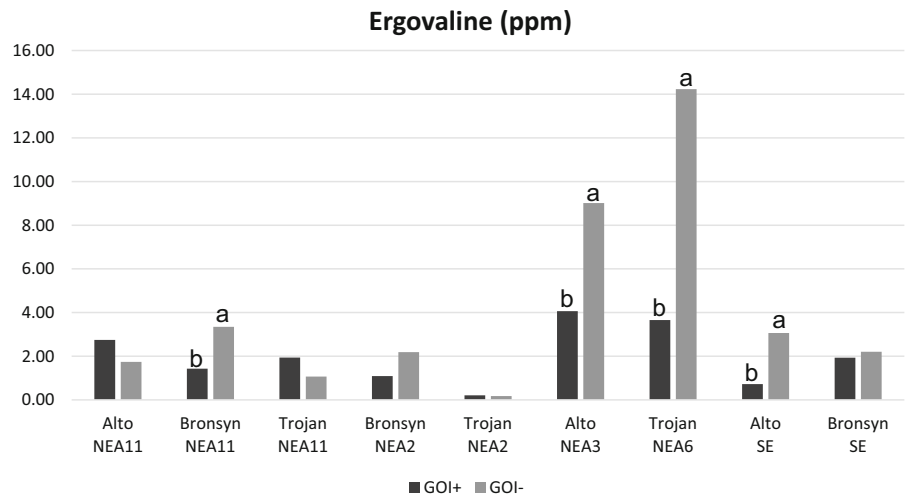

Fig. 3 Ergovaline concentration of each cultivar-endophyte combination for transgenic lines in dark grey and non-transgenic lines in clear grey. Bars are back-transformed means. a b different letters indicate significant differences at $P < 0.05$. GOI+ gene of interest positive; GOI− gene of interest negative

higher janthritrem response than GOI−, but there were no statistically significant differences identified from any combination.

Discussion

High-energy ryegrass has been created by altering fructan accumulation patterns, using fructosyltransferases genes (*1-SST* and *6G-FFT*) that are expressed under the control of a light-regulated promoter fragment (*LpRbcS*) (Panter et al. 2017). The self-incompatible outcrossing nature of perennial ryegrass, makes transgenic breeding a complex process. To fix the transgene within the outcrossing population, an introgression step had to be employed in the breeding system. The detailed transgenic breeding strategy used

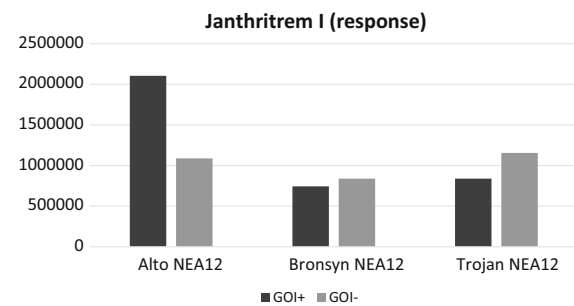

Fig. 4 Janthritrem response of each cultivar-endophyte combination for transgenic lines in dark grey and non-transgenic lines in clear grey. Bars are back-transformed means. GOI+ gene of interest positive; GOI− gene of interest negative

for high-energy ryegrass is described in Badenhorst et al. (2018).

To allow endophyte and transgene transfer to the progeny, pair crosses where the pollen donor was the

T0 event plants and seed was only harvested from the non- transgenic potential recipients were performed, as endophyte is maternally inherited. T1/F1 progenies within different backgrounds were confirmed to have the transgene loci, following a Mendelian inheritance of a single dominant allele with an expected 1:1 segregation ratio for the transgene.

The incidence level of fungal endophyte in the T1/F1 progenies was close to absolute, possibly as the seed was supplied from a commercial seed supplier and they guarantee an incidence higher than 80%. These results highlight the importance of specific selection for host–endophyte associations, based on the interaction between host and endophyte genotype (Wiewióra et al. 2015).

Genetic factors affecting endophyte-host associations have been researched in the last decade. For instance, genetic studies have suggested that qualitative variations in alkaloid profiles are mostly caused by endophyte genotype, while quantitative variations are related with host genotype (Ekanayake et al. 2017; Kaur et al. 2015). However, some studies have suggested that the degree of mutual benefit for endophytic fungi and their hosts genotype can be also conditional on environmental factors such as nutrient availability, especially those affecting WSC concentration.

Rasmussen et al. (2007, 2008) proposed that other factors such as plant leaf carbohydrate content, nitrogen supply and competition with other fungal symbionts can modify the quantitative alkaloid profile in *Lolium perenne* L. Rasmussen et al. (2007) compared a marketed high-WSC cultivar ('AberDove') with a "normal WSC" cultivar (Fennema), inoculated them with AR1 and AR37 strains and found both endophyte concentration and alkaloid production was reduced by 50% in AberDove. However, following detailed WSC measurements of the inoculated material no significant differences were found. They hypothesized that such decrease was due to differences between genotypes within and between both cultivars.

Subsequent studies of the same group investigated whether differences were due to the effects of altered carbohydrates, or host genetic background, by growing multiple cultivars in both high- WSC trait expression and non-expression conditions (using light/temperature treatments). Concluding that low molecular weight (LMW) carbohydrates had a small effect, explaining only $< 6\%$ of the variation in endophyte concentration and the endophyte reduction was caused by differences in compatibility (i.e. differences in the genetic background of cultivars resulting in variable 'affinities' for endophytic infection) (Ryan et al. 2015).

As mentioned earlier, endophyte and host genetic background has an important role in endophyte-host associations and there is a lot of genetic diversity among ryegrass cultivars and endophyte strains. It is the reason why in this study the effect of transgenic high WSC cultivar on alkaloid profile was compared within the same cultivar and endophyte strain, to eliminate those genetic factors.

The quantitative alkaloid analysis of different cultivar-endophyte associations in this study, indicated no significant effect of GOI+ and GOI− plants in most combinations. Though, peramine concentration in GOI+ decreased by 62% (21.3–8.0 ppm) and 61% (64.2–25.2 ppm) in Trojan-NEA6 and Alto-SE respectively. Ergovaline also decreased in GOI+ with Trojan-NEA6 by 74%, (14.2–3.7 ppm), Alto-NEA3 by 54% (9.0–4.1 ppm), Bronsyn-NEA11 by 58% (3.3–1.4 ppm), and Alto-SE by 77% (3.1–0.7 ppm). For lolitrem B only Alto-SE in GOI+ decrease significantly by 89% (19.9–2.1) and janthritrem response did not present differences between GOI+ and GOI−.

Differences in peramine, ergovaline and lolitrem B for Alto-SE and Trojan-NEA6 might not be related with the capacity of alkaloid biosynthesis in GOI+ plants, but potentially due to an indirect effect in the modulation of fungal biomass. The phenomenon called "dilution effect", occurs when an external factor stimulates the growth of the grass plant more than it stimulates the growth of the fungus and has previously been reported by Lane et al. (1997), so that the plant and fungal growth rate are out of synchrony. Transgenic ryegrass plants with altered expression of fructosyltransferases have consistently shown increase in metabolizable energy, up to 1.7 MJ ME kg DM^{-1} in selected T0 events compared with the control genotype, and higher biomass yields, compared to the untransformed control and a commercial high WSC ryegrass (Badenhorst et al. 2018).

The reduction in peramine concentration for Trojan-NEA6 with GOI+ may cause lower pasture protection against insects, since maintenance of a high peramine concentration, minimum of 15–20 ppm, is considered crucial for effective insect

resistance (Popay et al. 2003). However, Alto-SE with GOI + may still deter insect as its concentration was 25.2 ppm. There is no a consistent evidence that re-programming of the expression of fructan biosynthesis genes in ryegrass can alter the association between the host plants and the resident endophyte strain in a positive or negative way. As evaluation of the interaction and alkaloid levels are performed in every new cultivar, agronomic potential of transgenic perennial ryegrass with different novel endophytes should be assessed on a case by case basis, and these data suggest that no new testing regimes are required over and above what is already performed and considered standard practice for ryegrass breeding.

Conclusion

Increased accumulation of fructan in leaf blades of perennial ryegrass would have a potential economic benefit in livestock industry. The presence of a transgenic cassette to enhance water-soluble carbohydrates had little effects, compare with the impact of host and endophyte strain genotype on endophyte persistence and quantitative alkaloid analysis. The few observed changes in peramine and ergovaline concentration does not alter the balance of risk (toxicity) of endophyte–grass associations under the present experimental conditions and are possibly associated with the highest growth rate in genetically enhanced ryegrass. Endophyte–host interactions are complex and further research into associations with high WSC plant is needed.

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Supplement legends

Figure S1: Plasmid map of transgenic cassette in the event 10 ryegrass genome. The transcriptional direction is indicated with arrow, and the perennial ryegrass rubisco promoter, 1SST-6G-FFT fusion protein gene, and FT4 terminator sequences are shown with dark green, blue, and red arrows, respectively. For designing the SST-FFT fusion protein gene, the terminal codon (TAG) of the perennial ryegrass SST gene was removed.

Table S2: Endophyte incidence in T1/F1 progenies. GOI refers to gene of interest positive (+) or negative (-). Plant No. refers to the number of cultivar-endophyte-gene of interest combination plants used in the study.

Table S3: Oligonucleotide primer sequences to determine transgene presence in perennial ryegrass.

Table S4: Oligonucleotide primer sequences to differentiate between genotypes of endophyte within perennial ryegrass plants.

Table S5: Table of SSR marker details and expected product sizes amplified from different genotypes of endophyte within perennial ryegrass plants. ST: standard toxic.

Table S6: List of endophytes used in this study and their expected alkaloid profiles

Table S7: Back-transformed means. E = endophyte; GOI+ = gene of interest positive; and GOI- = gene of interest negative.

Figure S1. Plasmid map of transgenic cassette pGRA000022 in the event 10 ryegrass genome. The transcriptional direction is indicated with arrow, and the perennial ryegrass rubisco promoter, 1SST-6G-FFT fusion protein gene, and FT4 terminator sequences are shown with dark green, blue, and red arrows, respectively. For designing the SST-FFT fusion protein gene, the terminal codon (TAG) of the perennial ryegrass SST gene was removed.

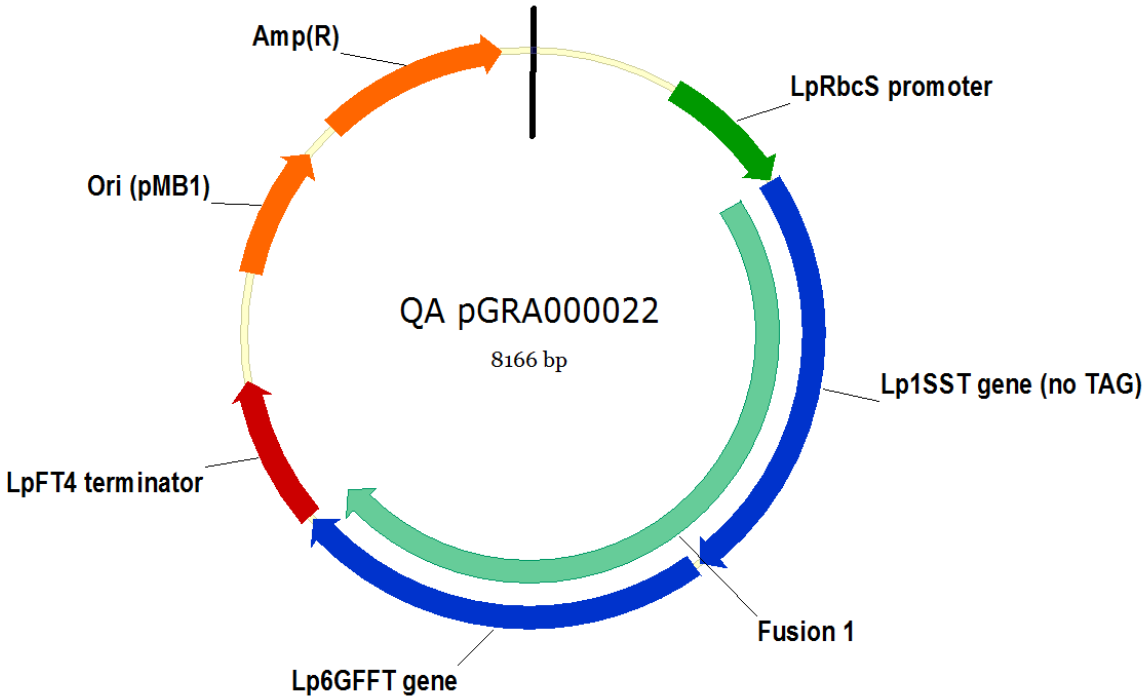


Table S2. Endophyte incidence in T1/F1 progenies. GOI refers to gene of interest positive (+) or negative (-). Plant No. refers to the number of cultivar-endophyte-gene of interest combination plants used in the study.

Cultivar	Endophyte	GOI	Plant No.
Alto	AR1	+	15
		-	21
	NEA11	+	6
		-	10
	NEA12	+	4
		-	6
	NEA3	+	10
		-	17
	SE	+	4
		-	9
Bronsyn	AR1	+	6
		-	12
	NEA11	+	10
		-	14
	NEA12	+	9
		-	6
	NEA2	+	6
		-	9
	SE	+	9
		-	10
Trojan	AR1	+	12
		-	14
	NEA11	+	8
		-	6
	NEA12	+	7
		-	11
	NEA2	+	10
		-	12
	NEA6	+	9

Table S3. Oligonucleotide primer sequences to determine transgene presence in perennial ryegrass.

Target	Assay	Forward Primer (5`-3`)	Reverse Primer (5`-3`)
LpHisH3	qPCR	TGCTTGCCCTTCAGGAGGCT	CTGAATGTCCTTGGGCATGAT
GOI	qPCR	CCCGCGGTGAATTCATGGAG	CGACGACCACCGACAACGC

Table S4. Oligonucleotide primer sequences to differentiate between genotypes of endophyte within perennial ryegrass plants.

SSR Locus	Forward Primer (5`-3`)	Reverse Primer (5`-3`)
NLESTA1QA09	FAM-TGGATATTTTGAAGAAGTTCCAGG	CTAACGATGTATGCGTTTGTTTGG
NLESTA1NGO3	HEX-CGGGCGCACTTGCTTCTCGG	GCCCCGCAGCCTTGTCGTTG
NLESTA1CC05	NED-CGCATACACGTTATGAAGCAGAGG	TTGGGACTTTCCAGAGTTGAGCAG

Table S5. Table of SSR marker details and expected product sizes amplified from different genotypes of endophyte within perennial ryegrass plants. ST: standard toxic.

SSR Locus	Sensitivity	Repeat Motif	Expected Size AR1	Expected Size ST
NLESTA1QA09	Low	(GA)20(G)1(GA)3	189	149
NLESTA1NGO3	High	(GTC)6	226	226
NLESTA1CC05	Intermediate	(TGT)17	217	164

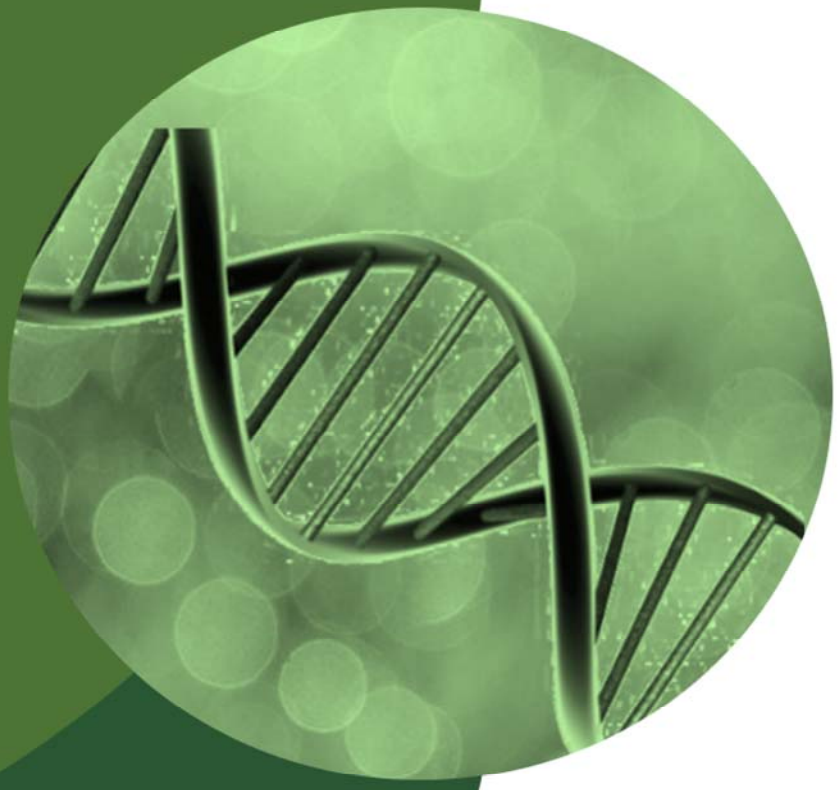
Table S6. List of endophytes used in this study and their expected alkaloid profiles

Endophyte strain	Alkaloid profile	Reference
SE	Peramine	
	Ergovaline	Christensen, *et al.* (1993)
	Lolitrem B	Siegel, *et al.* (1990)
AR1	Peramine	Bultman, *et al.* (2003)
NEA2	Peramine	
	Ergovaline	
	Lolitrem B	van Zijll de Jong, *et al.* (2008b)
NEA3	Peramine	
	Ergovaline	van Zijll de Jong, *et al.* (2008a)
NEA6	Peramine	
	Ergovaline	van Zijll de Jong, *et al.* (2008a)
NEA11	Peramine	Kaur, *et al*. (2015)
	Ergovaline	
NEA12	Janthitrem I	Kaur, *et al.* (2015)

Table S7: Back-transformed means. E = endophyte; GOI+ = gene of interest positive; and GOI- = gene of interest negative.

Trait	Cultivar-E	GOI-	GOI+
Peramine	Alto-AR1	37.229	53.118
	BronSyn-AR1	41.213	35.468
	Trojan-AR1	63.774	56.553
	Alto-NEA11	33.121	25.838
	BronSyn-NEA11	18.847	21.218
	Trojan-NEA11	15.868	17.771
	BronSyn-NEA2	19.755	32.394
	Trojan-NEA2	7.167	9.163
	Alto-NEA3	24.329	27.338
	Trojan-NEA6	8.029	21.312
	Alto-SE	25.234	64.218
	BronSyn SE	43.678	38.764
Lolitrem B	BronSyn-NEA2	0.762	0.395
	Trojan-NEA2	2.105	3.394
	Alto-SE	2.069	3.3943
	BronSyn SE	11.555	10.947
Ergovaline	Alto-NEA11	2.749	1.742
	BronSyn-NEA11	1.430	3.345
	Trojan-NEA11	1.938	1.070
	BronSyn-NEA2	1.092	2.186
	Trojan-NEA2	0.208	0.175
	Alto-NEA3	4.061	9.018
	Trojan-NEA6	3.660	14.231
	Alto-SE	0.725	3.062
	BronSyn SE	1.936	2.202
Janthritrem	Alto-NEA12	2105366.249	1088161.355
	BronSyn-NEA12	744151.558	839028.539
	Trojan-NEA12	839028.539	1155449.496

Chapter 6



General Discussion and Conclusion

In this thesis I describe the development of contemporary tools and methods to characterize transgenic feed. The biosafety assessment of the majority of GM crops available in the market has been based on the premise that they will be used as food that will be consumed by humans, while most GM plants and their bio-products are globally used to feed animals (Bruetschy, 2019). Several articles reviewing the safety assessment procedure of GM food have been published, but little is known about the evaluation of GM feed and whether the same principles used for GM food crops can be applied. To address this gap and answer the first research questions of this thesis, as to whether there was, or should be, any difference in the safety assessment of GM feedstuffs as opposed to human food, a detailed review of the GM regulation process was performed.

The literature review aimed to search the available information on biosafety assessments of GM food and compare them with those of GM animal feedstuffs. The review was published (Giraldo *et al.* 2019) and it is presented as Chapter 2. The first hypothesis, stating that there were differences in the safety evaluation of transgenic feedstuffs as opposed to GM human food, was tested and accepted based upon the results. The review underlines that even when the majority of methods used for the risk evaluation of GM food can also be used for GM feed, several plant parts used to feed animals are human-inedible. So that, the amount of recombinant proteins in some parts of the plant and the level of risk associated with the intake of GM feedstuff in target species should be considered (Giraldo, et al. 2019b).

From Chapter 2 the need of different strategies for the evaluation of GM plants intended for animal consumption was identified, since so far it has focused on the safety for human and the environment, using animal tests mainly as models to extrapolate results to humans. It is estimated that around 70 to 90% of all transgenic plants and their y-

products are destinated to feed animals (Flachowsky et al., 2012). In the EU, where all GM food and feed must be labelled, over 90% of feed material used for livestock contains GM grains and/or GM-derived forage (Kleter, et al. 2017). Additionally, the commercial release of GM crops for exclusive use by livestock, such as Roundup Ready alfalfa (*Medicago sativa* L.) and ongoing research on other GM forage crops including perennial ryegrass and white clover (Smith, et al. 2007), strengthens the requirement of specific strategies to evaluate the safety of transgenic feedstuff (Giraldo, et al. 2019b).

GM crops are fed to a range of livestock species including poultry, sheep, swine and cattle (Nadal, et al. 2017). However, the standard species for GM feed, toxicological and allergenicity studies are mice and rats, whose evaluation is call rat 90-day test, which is compulsory for all transgenic plants in the EU (Hong, et al. 2017). The digestive system and immune responses of rodents are distinct from most livestock species. For instance, ruminant animals including cattle, sheep and goats, use a specialised four-chambered stomach with a unique microbial flora to digest cellulosic compounds found in the plants. Swine have an enzymatic stomach as part of their monogastric digestive system like that of humans, and poultry have two-chambered stomach (France, et al. 2006). Additionally, livestock have a high exposure to GM feedstuffs, which may compromise the majority of their diet over a long period of time (Aumaitre, et al. 2002), while rodents are not usually fed with the entire transgenic crop (leaves, stem, seed, etc) or plant by-products (silage and hay). Some novel traits can lead to an increase the expression of recombinant proteins in specific parts of the plant, which will not necessarily be consumed by rodents under test conditions but would form part of the diet of livestock, altering the level of exposure and therefore the potential risk.

Considering the safe use of GM crops and technological advances, animal studies could be more specific to the anatomical and physiological differences of target feeding species but also minimizing the use of animals to take the ethical considerations of animal testing in to account. More specific assessments, that consider the modification under test and likely impact (or lack thereof), can also streamline the process. The appropriate use of other approaches such as *in silico* and *in vitro* methods, can reduce timelines and costs in the assessment, while avoiding animal testing trials.

In silico mathematical and statistical algorithms and biological models allow improved understanding of biological systems, using computational tools. Amino-acid sequence homology is a bioinformatic approach that is currently being used to predict the potential allergenicity of a novel food/feed protein, by comparing it with known allergen sequences in a database (EFSA, 2008). For instance, van der Voet, et al. (2017), proposed statistical modelling approaches to test equivalence of GM plants using data obtained in historical studies. This has the advantage of using existing information to identify changes in commercial crops that are known to be safe. Although, further development of these models is required, it is a promising area for consideration and all possible *in silico* studies should be considered as the first basic test in the risk assessment of novel crops.

Dynamic *in vitro* models to simulate the digestive system of different animal species, have been used for decades to assess the nutritional value of specific feeds (Boisen and Eggum, 1991). There are multiple systems on the market, varying in size, which have been tested and are highly correlated with *in vivo* systems. In the case of monogastric species, such as pigs and poultry, the digestion is mostly enzymatic, so *in vitro* systems are relatively simple (Bedford and Classen, 1993). Whereas, in polygastric species such as cattle, sheep and goats, the same enzymatic digestion takes place, but the gastric phase is more complex, since they have a special four-chambered stomach (Blümmel, et al. 1997, Calsamiglia and Stern, 1995). These *in vitro* systems can provide an evaluation of the nutritional characteristics/tolerance of the target animals to the transgenic plant under evaluation and can be consider after the *in-silico* studies.

Another important issue that was highlighted by the review was the cost and amount of time the GM deregulation process takes. In general, the regulatory framework for GM plants is based on a safety assessment of new products, and aims to evaluate the potential impact on humans, animals and the environment. Although, practical implementation varies from jurisdiction to jurisdiction, in general it is a slow and expensive process that can take up to 30 years (Napier, et al. 2019) and around $35 million US dollars (McDougall, 2011). Recent advances in genome editing allowing site-specific mutations, avoiding the insertion of exogenous DNA, leading to products that may be partially or completely excluded from GMO regulations in some jurisdictions depending the extent or nature of the genome editing process (Seyran and Craig, 2018). Whereas, other

jurisdictions propose to regulate genome edited plants using the same legislation as used for GM plants.

Despite the rapid growth of transformation methods for novel crops observed in the last few decades, such significant advances in plant biotechnology are not always reflected in the regulatory processes associated with the commercialisation of new crops. This is partially due to risk-disproportionate regulations but is also due to the use of historic technologies that make the assessment profile inefficient and increase the cost. Implementation of emerging technologies can reduce the price and time for the safety evaluation, as well as increasing its accuracy. The first step in the risk assessment of new cultivars is the molecular characterisation of the transgene. Therefore, Chapter 3 addressed the research question on how could a full molecular characterisation of a new transgenic plant be conducted in a more accurate and effective way across a range of crop species?

Traditional methods such as PCR and Sanger sequencing are still being commonly used for molecular characterisation in the safety evaluation of GM food and feed (Guo, et al. 2016, Castan, et al. 2016). These methods are still suitable for some techniques and applications but are time-consuming and laborious. There are more accurate, sensitive and cost-effective methods available, such as long-read sequencing technologies and other *in silico* analytical tools. In Chapter 3, a simple, accurate and rapid workflow for the characterization of transgenic plants was developed.

The use of Nanopore sequencing technologies allowed full molecular characterisation of three different transgenic events. Therefore, the second hypothesis was accepted, since Nanopore sequencing technology simplifies, increases accuracy and reduces labor time in the molecular characterisation of GM plants. In contrast to methods used at present, the nanopore sequencing analysis enables the detection of transgene copy number, insertion site and flanking regions, as well as the presence of unintended insertions and presence of backbone sequences, using a single nanopore flow-cell and following a simple and fast workflow. This method could be used for whole genome sequencing, which would increase the time and cost, but avoid the need for references.

The findings presented in Chapter 3 become even more relevant with the emergence of new plant breeding techniques where the nature of the modification is more complex. For instance, standard PCR-based methods fail to comprehensively reveal the complete characterisation of single trait GM plants (James, 2016). The insertion of endogenous genes in cisgenesis and intragenesis and changes to only a few select nucleotides through genome editing, challenges our current standard methods to characterise GM plants. Compared with random insertions, deletions, and rearrangements of long foreign DNA fragments in the first generations of GMOs, new plant breeding techniques introduce small changes that are more difficult and sometimes even impossible to distinguish, since they look alike changes made by conventional or natural mutation (Holst-Jensen, et al. 2016).

The traceability of GM crops would also benefit from the implementation of new technologies, and although improved methods have been recently developed, optimization is still required. As described in the literature review, the method selected to comply with traceability and labelling requirements must be sensitive and be able to detect the genetic modification(s) from raw agricultural commodities (Giraldo, et al. 2019b). Detection methods have been typically developed for matrices or substrates for human consumption, such as grain or flour, so little was known about traceability of GM feedstuffs products. To address the next research question "How can we use emerging technologies to detect transgenes in different agricultural products?", digital droplet PCR was proposed as an alternative for the traditional PCR. Digital droplet PCR is a potential alternative for detection and identification of transgenic events, due to its capacity to provide absolute quantification, less susceptibility to inhibitors and it does not need standard curves (Racki et al., 2014; Corbisier et al., 2015). In addition to its comparative advantages, it has a similar price per reaction, about 3.66 Euro for ddPCR compared with about 3.33 Euro for qPCR (overall cost for triplicate reactions) (Campomenosi, et al. 2016).

In Chapter 4, Digital droplet PCR was evaluated and compared with classic and standard real-time PCR, to track and trace different GM agricultural products such as pollen, tillers, seeds and fresh leaves and by-products like silage and hay (Giraldo, et al. 2019a). Both, methods were able to identify the transgenes in all plant parts and by-products derived

from high energy ryegrass, a transgenic plant used as case study. However, droplet digital PCR exhibited a higher repeatability and sensitivity. Therefore, the established aim of developing a sensitive and reliable method for traceability of GM forages was accomplished, and the stated hypothesis on the higher sensitivity and reliability of digital PCR was accepted. It is recommended that real time PCR can still be used for transgenic screening processes since it is highly cost-efficient, and digital PCR can be suited for quantitative analysis since it provides absolute quantification of the transgenic event. An integrated GM detection system that includes the development of rapid *on-site* detection methods, NGS for high throughput laboratory screening and digital PCR for accurate and specific confirmations, could improve this area. Results from chapter 4 were published in Frontiers in Plant Sciences (Giraldo, et al. 2019a). This manuscript is a guide for plant breeders and biotechnologists to assess GM forages and their biomass.

These findings are particularly important in jurisdictions, such as the European Union, where new plant breeding techniques are within the remit of rules for regulating GMOs. New genome editing techniques such as site-direct Nuclease 1 (SDN-1), allow small mutations without the integration of recombinant DNA, making their detection more difficult (Hartung and Schiemann, 2014). Implementation of new technologies including digital PCR in the safety assessment process, could decrease the timeframe from years to months, as well as reduce the safety assessment cost, whilst not decreasing the safety assurance.

After a comprehensive molecular characterisation and the development of traceability methodologies for GM crops, environmental safety studies would be the next issue to be addressed in the safety assessment process. Environmental risk evaluations and coexistence strategies for outcrossing forage species and cross-pollinated plants, including canola and maize, are comprehensively documented. Although, environmental safety principles were covered it in the literature review, no further study was performed in this project. Hence, the following step in the safety assessment process are nutritional, toxicological and allergenicity studies. These studies are usually performed following a case by case approach, but they usually involve the use of animals. However, as it was recommended earlier in this document, the use of animals in scientific research should

be avoided when possible. Hence, new technologies to assess *in-planta* metabolism pathway, including "-omics" methods were considered.

In Chapter 5, GM high-energy ryegrass was used as case study, and although it is not toxic or capable of generating any toxicity as a direct result of the transgene incorporation, it can have fungal endophytes. Ryegrass endophytes generate alkaloids, which affects different insects, but also could disrupt ryegrass utilization to feed animals (Office of the Gene Technology Regulator, 2008). The increased energy of the assessed transgenic ryegrass was obtained through the insertion of a fructosyl transferase cassette, which led to a higher water-soluble carbohydrate in leaves (Panter, et al. 2017). Previous studies suggested that plant leaf carbohydrate content, could potentially alter the alkaloid profile of perennial ryegrass (Rasmussen, et al. 2007, 2008). Therefore, the last hypothesis of this project stated that the presence of the transgene modification in perennial ryegrass affects endophyte persistence and quantitative alkaloid concentration.

High-throughput metabolomics was used as a profiling technique to detect unintended effects of high-energy ryegrass crossed into different cultivars and endophytes associations. The aim was to assess the concentrations of different alkaloids when high-energy perennial ryegrass is colonised by different fungal endophyte strains. The results were published in Transgenic Research Journal in late 2018 and concluded that the presence of this genetic modification, did not change the toxicity hazard of such grass-fungal interaction. Therefore, the hypothesis was rejected since the transgene insertion to increase water-soluble carbohydrates had little effects. Endophyte–grass associations are complex and more studies researching such interactions with high water-soluble carbohydrates plants should be considered following a case by case basis. In practice, this study represents an example addressing the toxicological assessment of a GM crop, using a case by case approach and avoiding the need of animal tests.

In this context, the finding developed within this thesis provide useful tools for the safety assessment of transgenic plants. Progress in plant genetic engineering demonstrates that the constant evolution of science is promptly translated into novel biotechnological products. However, this potential will not be released unless the production and evaluation of new plant breeding technologies seem in a social and political framework.

Although, it is impossible to elucidate the complex social, political and scientific interactions of transgenic plants in a single PhD and area of expertise, some outstanding challenges in the regulatory framework were explored, underlining the need of responsible innovation to reconfigure the safety assessment process. Possible strategies to face upcoming challenges are discussed along with some modest recommendations for modification of the regulatory process.

The first challenge to be faced is public acceptance, which has been controversial across all jurisdictions since the first GM crop was released into the market 24 years ago (Briefs, I. S. A. A. A., 2018). Amongst the general population the appreciation of risk relating to GMOs is generally considered to reflect the level of media coverage that the topic receives (Bardin et al. 2017). Socio-psychological studies evidence that people search for GMO information that is consistent with their opinion (favourable or unfavourable), avoiding information that can challenge their ideas (Costa-Font et al. 2008). GMO public acceptance has social, cultural and emotional contexts and although there is a negative perception of GM food in some communities, there is a generalised positive opinion on science, relying on scientists to contribute impartially to public debate (Mallinson, et al. 2018).

The purpose of a regulatory safety assessment of new plant breeding techniques is to protect public safety and encourage public acceptance, by demonstrating that the product has been rigorously evaluated and its consumption does not represent a risk to the consumer (FAO/WHO, 2000). Nevertheless, some studies suggested that the over-regulation of GMOs has contributed to public distrust and reinforced public perceptions that GMOs are hazardous (Herman, et al. 2019). The current pre-market safety assessment has been criticised of not being risk-based, considering that inadvertent changes can happened independently of the breeding technique used for crop development, whether transgenesis or conventional breeding (Fernandez and Paoletti, 2018, Herman, et al. 2018).

An engagement strategy that involves two-way consultation with consumers is essential to achieve public acceptance (Pei and Schmidt, 2019). The power of effective communication was evident in the comparison of two GM crops nutritionally enhanced,

Golden Rice and Omega-3 fish enhanced canola oil. The former was developed by the public sector for humanitarian purposes and not-for-profit (Stein, et al. 2006), while the latter was created by private companies with a market-driven ethos and an important economic component (Betancor, et al. 2015). Despite the great potential health benefits of Golden Rice, it has suffered from significant public opposition affecting regulatory approval, which has taken more than 35 years (Napier, et al. 2019). However, the Omega-3 enhanced canola oil developers invested in an effective communication strategy to different audiences from experimental stages, allowing a commercial application progress without public disruption to be achieved within a 21-year timeline (Napier, et al. 2019).

Strategies to address public acceptance of new potential technologies should involve the investment of more resources in public education. Clear and understandable explanations of the science underpinning NPBT, risk associated with them, and potential benefits, could engage open communication and public discussion. It could also be of value to inform the community about differences among first generation genetic engineering techniques, random mutagenesis, and new genome-editing (Ishii, 2018). Even though, regulators and scientists are highly interested in the techniques themselves, while consumers tend to be more attentive to the final product (Pei and Schmidt, 2019), such information from trusted sources could prevent mis-information usually spread through social media. Creative science communication channels are necessary in order to achieve public acceptance, which could help to avoid over-regulation of these beneficial technologies.

Another area to be consider in this context is the development of regulations relating to new plant breeding technologies. Although, policymakers and scientists consider these technologies as a key solution to the challenges that face agricultural sustainability considering the current growing population, such technologies have met resistance by a variety of organisations (Macnaghten, 2019). Authorisation and risk management processes are highly influenced by politics and new technologies including genome editing, do not match with the current regulatory structure of some countries. For instance, in the European Union the same plant product could be categorized as GMO or non-GMO according to the biotechnological tool used in its production (Jones, 2019).

Other countries such Australia and Argentina are amending their current GMO regulations to fit NPBT (OGTR, April 2019 and Whelan and Lema, 2015).

An integrated system to regulate and assess NPBT, should be implemented with the aim to support the increment of the global food production. Regulation of the product itself, regardless of the process to obtain it, as the US does, is a more logical and reasonable beginning, considering the broad safety history of GM crops. However, when the socio-political regulations are intransigent in the evaluation process, the process itself should be assessed. For example, the safety assessment rigor and timeframe of intragenic and cisgenic transformation events should be lower than first- and second-generation transgenic events, since the former are rearrangements of genes into the same host genome, and it does not involve insertion of foreign genes so inherently has lower safety risk. Therefore, in legislations such us the EU that certifies the process to obtain the crop, a categorization of the processes should be performed. Even categories as simple, moderate and complex transformations, could make the regulatory assessment more efficient, to guarantee a faster use of the technological benefits, without neglecting the safety of the product.

Another factor affecting NPBT commercialization is the mandatory labeling laws implemented in countries such as Australia, New Zealand and the EU. In those counties, the cost of mandatory labeling is incurred by the GM crop producer, while the non-GM producer has a simpler path to market with lower overheads in the production. Additionally, consumer can see the GM label as a warning, akin to a mandatory health label on cigarettes, which can affect their purchasing decision. On the contrary, voluntary labeling applied in countries like Canada and the US, promote non-GM firms to label their product only if it makes their product more valuable, which is reflected in a higher price incurred by the consumer (McCluskey and Winfree 2017). To allow a more fare process for producers and consumers, GM food and/or feed should be voluntarily labelled.

Amendments in the regulatory framework of NPBT, with the objective of responsibly assessing these new tools that can help us to prepare for the upcoming global food challenges, are necessary. The objective is to evaluate NPBT products more effectively, avoiding unnecessary tests. Over-regulation leads to delays affecting the commercial

release of novel products that can positively impact the community at large. Golden rice, a novel variety that could improve the lives of millions with vitamin A deficiencies, is a clear example of an extensive regulatory processes that has taken more than 35 years and it is still ongoing (Napier, et al. 2019).

Conclusion

This research aimed to develop effective methods using emerging techniques for the safety assessment of genetically modified feedstuff. Based on the application of new technologies, it can be concluded that there is space to improve such slow and labored process. The results indicate that there are differences in the assessment of transgenic feedstuff as compare to human food, mainly due to the level of exposure of livestock species to various parts of the plant and differences in their digestive system. It was also demonstrated that using third generation sequencing for molecular characterisation, digital PCR for tracking and tracing and metabolomics for the toxicological assessment of new genetically engineered products, is a way to streamline the regulatory process while not decreasing the safety assurance. However, in order to release the potential of new plant breeding techniques, social and political challenges should also be addressed. A constructive dialogue to create an informed public that can collectively and intelligently agree on a satisfactory minimal path for deregulation in a wide range of scenarios, can be a way to face social challenges, particularly for new events that focused on animal feed. Additionally, a re-structuration of the regulatory framework, that so far has been adequate but could be further improve on a scientific basis, could also help us to be prepared for the upcoming global food challenges.

I genuinely hope the knowledge generated in this thesis will be useful for plant biotechnologists, as they contemplate upcoming challenges in this new plant breeding era.

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Appendix


SAFETY ASSESSMENT OF TRANSGENIC HIGH-ENERGY RYEGRASS
TO IMPROVE PASTURE INDUSTRIES

Appendix I. Giraldo, P.A., Shinozuka, H., Badenhorst, P., Cogan, N.O.I., Spangenberg, G., and Smith, K.F. Safety Assessment of Transgenic High Energy Ryegrass to Improve Pasture Industries. Conference Proceedings of the International Plant & Animal Genome Conference. San Diego, USA, January 13-17 2018.

Safety Assessment of Transgenic High Energy Ryegrass to Improve Pasture Industries

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Perennial ryegrass is one of the most important pasture grasses in temperate zones worldwide. Genetic modification (GM) has been used to increase the metabolizable energy content of perennial ryegrass plants, through up-regulation of fructan biosynthesis in leaf blades. Commercialization of new transgenic cultivars requires data to support the deregulation of the transgenic event and this data considers the impact of the novel germplasm on humans, animals and the environment. One aspect of this data is the development of tools to detect the transgenic event in relevant agricultural products. A novel approach has been developed to evaluate high-energy transgenic ryegrass using emerging technologies, such as droplet digital PCR (ddPCR) and long read DNA sequencing. ddPCR enables absolute quantification of DNA copies to be determined, which allows detection of the transgene in agricultural commodities such as seed, herbage and silage at low levels, with accurate detection at 0.5%. Long read sequencing technology has also been applied to characterize the structure and flanking sequences of the transgenic insertion, through sequencing the transformed plant at moderate genome coverage. Further assessments have also been performed including toxicological and allergenicity studies indicating substantial equivalence to untransformed ryegrass. Nutritional trials using grazing animals are planned, which will validate the on farm production benefits. These novel technologies to assess and trace GM forages are being used to meet regulatory requirements to enable commercialization of this new, potentially large and transformative industry.

Appendix II. Giraldo, P.A., Shinozuka, H., Badenhorst, P., Cogan, N.O.I., Spangenberg, G., and Smith, K.F. Assessment of Transgenic High Energy Ryegrass to Improve the Dairy Industry. Conference Proceedings of the Australian Dairy Conference. Melbourne, Australia, February 13-15 2018.

Assessment of Transgenic High Energy Ryegrass to Improve the Dairy Industry

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Increasing digestibility is a primary goal for pasture grass development, due to its potential to improve animal production through increased intake and energy yield. Economic studies in ryegrass have predicted that an increase in metabolizable energy of 1 megajoule per kilogram of dry matter, could realize on farm profit between 40 and 54%. Genetic modification (GM) has been used to increase the metabolizable energy content of perennial ryegrass plants, through up-regulation of fructan biosynthesis in leaf blades and pseudo-stems. Commercialization of new transgenic cultivars requires data to support the deregulation of the transgenic event and this data considers the impact of the novel cultivar on humans, animals and the environment. We have developed a novel approach to evaluate high-energy ryegrass using emerging technologies. These new methods allow detection of the transgene in agricultural commodities such as seed, herbage and silage at low levels, with accurate detection at 0.5%. Toxicological studies with endophytes, demonstrated the safety of high-energy ryegrass, as there were no statistical differences in alkaloid concentration compared with conventional cultivars. Therefore, high-energy ryegrass not only has the potential to boost production and increase farm profitability but has also been demonstrated to be as safe as its traditional counterpart.

Appendix III. Giraldo, P.A., Shinozuka, H., Spangenberg, G., Cogan, N.O.I., and Smith, K.F. Use of New Genomic Tools to Assess and Trace Genetically Modified Forages. Conference Proceedings of the International Forage and Turf Breeding Conference. Florida, USA, March 24-27 2019.

Use of New Genomic Tools to Assess and Trace Genetically Modified Forages

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Implementation of molecular biotechnology in forage species, such as transgenic technologies, can improve agricultural profitability through achievement of higher productivity, more efficient use of natural resources and decreases in environmental impacts. As part of the regulatory process, all new genetically modified (GM) cultivars are required to go under rigorous safety assesses prior to commercial release. Novel approaches to assess and trace a high-energy transgenic ryegrass have been developed, using emerging technologies such as Oxford Nanopore sequencing and digital PCR. A portable MinION devise was used to detect a transgene with 3.300 bases length. Detailed description of the genetic modification including the insertion site and flaking regions was achieved in a single read of 32.000 bases. Moreover, droplet digital PCR (ddPCR) allowed the absolute quantification of recombinant DNA copies in different agricultural commodities such as seed, herbage and silage at low levels, with accurate detection at 0.5%. The use of these technologies for GM characterization and identification, are not only important to ensure legality and traceability, but also to comply with GM labelling regulations. Additional plant molecular diagnostics platforms are being developed, to enable rapid detection of GM forages on farm, which will allow an on-site detection at the DNA level, rather than at the gene product level. These technologies have shown to be more sensitive and accurate than conventional methods, which could reduce regulatory delays of these potentially large and transformative industry.