Impact of feed processing and mixed ruminal culture on the fate of recombinant EPSP synthase and endogenous canola plant DNA

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Abstract

The impact of feed processing and in vitro ruminal cultures on the persistence of recombinant and canola-specific endogenous DNA was studied using various canola substrates (whole seed, cracked seed, meal and diet). For both, parental and genetically modified substrates, ribulose-1,5-bisphosphate carboxylase/oxygenase gene was amplifiable up to varying time points. Persistence of recombinant DNA, encoding 5-enolpyruvylshikimate-3-phosphate synthase (1363 bp) was detected up to 8 h for meal and 4 h for mixed diet. Upon processing of canola, DNA large enough to contain intact plant genes remains. In an in vitro environment, plant DNA was rapidly degraded upon its release into rumen fluid. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

The increasing use of genetically modified (GM) crops has raised numerous questions regarding the fate of recombinant plant DNA in animals, humans as well as microbial populations inhabiting these systems. In particular, there are major concerns regarding the uptake and persistence of foreign DNA in animal and bacterial cells [1]. Few studies to date have investigated the persistence and survival of DNA throughout the animal digestive tract. Studies involving mice have shown that ingested foreign DNA can persist long enough to cross the intestinal epithelium, and reach leukocytes, spleen and liver cells of the host as well as various fetal organs [2,3]. Upon feeding soya bean leaves to mice, plant-specific ribulose-1,5-bisphosphate carboxylase/oxygenase (Rbc) gene was detected in the liver and spleen [4]. Similar findings have been reported in a study involving chickens and cattle fed GM-based diets [5], where small fragments of endogenous plant DNA was identified in the muscle, liver, spleen and kidney of chickens. In ruminants, detection of the same plant gene fragments was limited to blood lymphocytes and to a lesser extent, the milk in cattle. These differences in gene detection from cattle and chickens may have been due to a greater extent of plant digestion prior to plant material reaching the intestinal tract in the former. Recombinant DNA was not detected in any of the tissues of either animal.

Ruminants consume vast amounts of plant DNA each day and host a diverse and concentrated microbial ecosystem throughout their digestive tract. Despite this, little is known about the fate of plant DNA upon ingestion by these animals. The acquisition of plant DNA by microbes would most likely occur by transformation. There are bacterial species known to be capable of natural transformation [6], including those present in rumen [7]. Fungal species too have been shown to be capable of transformation [8]. Most studies on DNA stability and transformation however, have been conducted under ideal in vitro conditions and thus have limited practical applications. In order
for natural transformation to occur, free DNA has to be available for uptake. It has been observed in ruminants that naked DNA is degraded very rapidly, thus imposing an initial barrier to transformation with plant DNA [9,10]. Though DNA has been reported to lose the capacity to transform bacteria within 1 min of incubation in rumen fluid [9], transfer of genes across species within the rumen does seem possible [11].

Roundup Ready® canola is glyphosate tolerant due to expression of recombinant DNA encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS or EPSP synthase). The purpose of this study was two-fold: firstly, to monitor plant DNA throughout feed processing of parental and Roundup Ready® canola in order to determine the size of plant DNA consumed by ruminants when fed processed diets. The second part of our study addressed the persistence of transgenic (EPSP synthase) and endogenous canola plant DNA (Rbc) during feed digestion by mixed ruminal microbial populations.

2. Materials and methods

2.1. Canola seeds, meals and diets

Parental and Roundup Ready® canola whole seeds were supplied by Monsanto Company (St. Louis, MO, USA). The respective canola meals were prepared at Texas Engineering Experiment Station, Texas A&M University (TX, USA). Briefly, the canola seeds were conditioned (average discharge temperature 74.4°C) and flaked to a thickness of 0.254–0.381 mm. The flakes were then cooked (discharge temperature 96.1–101.7°C), dried, and extracted in hexane heated to 54.4°C (1 part solvent:1 part solid). The meal was recovered at 93.9°C.

The diets from the respective meals were prepared at the Lethbridge Research Centre (Lethbridge, AB, Canada) and contained 6.5% of each of the canola meals (parental or Roundup Ready®) on a whole percentage basis. For preparation of the diets, canola meal was mixed with the other constituents of the diet and pelleted at 100°C.

2.2. Canola leaf

Leaf samples were obtained from canola plants grown in a phytotron facility at the Lethbridge Research Centre under standard conditions and were used as positive or negative controls (as indicated).

2.3. Batch culture substrates

For batch culture experiments, the substrates tested were parental (P) and Roundup Ready® (R) of each of the following: whole canola seeds (PWS and RWS), cracked canola seeds (PCS and RCS), meals (PM and RM), and diets (PD and RD). Whole canola seeds were cracked using a rolling pin. Meals and diets were ground to pass through a 1-mm screen.

2.4. Batch culture fermentation

To prevent possible background and contamination from Roundup Ready® canola or soybean containing diets, a rumen fistulated Jersey steer maintained on alfalfa hay was used in this study. The rumen contents were collected, via a cannula, 2 h after the animal was let out to graze. Approximately 1.5 l rumen fluid was collected by straining digesta through four layers of cheesecloth into a flask flushed previously with CO₂. Solid digesta was sealed in a bag and both fractions were taken back to the laboratory for processing, under anaerobic conditions.

Inoculum was prepared by blending 1.5 l rumen fluid with 375 g of solid digesta for 45 s × 3 pulses. The homogenate thus obtained was passed through four layers of cheesecloth and the strained fluid was added to two volumes of buffer pre-warmed to 39°C as previously described [12] to obtain the rumen fluid inoculum. The rumen fluid inoculum (20 ml) was dispensed into triplicate 35-ml serum vials containing 250 mg of each of the substrates (PWS, RWS, PCS, RCS, PM, RM, PD, or RD). The vials were sealed and incubated at 39°C on a rotary shaker for 0, 2, 4, 8, 12, 24, or 48 h. The 0-h vials were processed immediately after the addition of inoculum. For the remaining incubations, triplicate vials were removed from the shaker at the indicated time points and placed on ice prior to processing. The batch culture fermentations were processed step-wise to obtain feed-particle-associated bacteria, fluid-associated bacteria and supernatants as previously standardized in our lab [13]. Briefly, the complete contents of each vial were transferred into a 50-ml falcon tube, and centrifuged at 300 × g (10 min; 4°C). The supernatant (supernatant A) was aliquoted for volatile fatty acid (VFA) measurements and the remaining pellet (pellet A), containing plant debris and feed-particle-associated bacteria, was used for DNA isolation. A sub-sample (2 ml) of the supernatant A was centrifuged at 10 000 × g (4 min, room temperature) and the resulting supernatant (supernatant B) which contained free DNA was transferred to a fresh 2-ml tube and used for DNA isolation. The remaining pellet (pellet B), containing fluid-associated bacteria [13], was also used for DNA isolation. VFA concentrations in the supernatant from the first centrifugation step (500 × g) were determined by gas chromatography as described previously [14].

2.5. DNA isolation

DNasey Plant Mini kit (Qiagen Inc., Mississauga, ON, Canada) was used for isolating DNA from canola plant leaves (parental and Roundup Ready®) which were used as controls.

DNA isolation from canola seeds, meals and diets was
accomplished by using a modified CTAB extraction from a previously published procedure [15]. Briefly, 1 g of seed, meal or diet was ground in liquid nitrogen using a pestle and mortar. The ground material was added to 4 ml of 2-mercapto-ethanol/CTAB solution pre-warmed to 65°C in a 50-ml centrifuge tube, capped, and incubated for 1 h. An equal volume (4 ml) of chloroform/isooamyl alcohol (24:1) was added and the mixture centrifuged at 7500 x g (10 min, 4°C). The aqueous phase was transferred to a fresh tube and the organic extraction was repeated twice. DNA was precipitated with 0.6 volume isopropyl alcohol (4°C for 2 h). The DNA pellet was washed twice with 70% ethanol and re-suspended in 3 ml TE buffer at 65°C for 1 h, followed by addition of DNase-free Ribonuclease A (Sigma, St. Louis, MO, USA) to a final concentration of 20 μg ml⁻¹ and incubated according to standard procedures. Following the RNase treatment, organic extraction was carried out twice on DNA as described above. The aqueous phase was transferred to a clean tube and DNA was precipitated by adding 0.1 volume of 3 M sodium acetate solution and 2 volumes of ethanol. The precipitated DNA was washed twice with 70% ethanol, air dried for 20 min, and finally resuspended in 500 μl TE.

DNA isolation from batch culture pellets containing plant debris and feed-particle-associated bacteria (pellet A) was also performed using the CTAB extraction procedure described above. DNA extraction from fluid-associated bacteria (pellet B) was done using the Wizard® Genomic DNA Purification kit (Promega Ltd, Madison, WI, USA) and from supernatant B was used comparing the QiAamp DNA Mini kit (Qiagen Inc., Mississauga, ON, Canada), respectively.

2.6. DNA quantification and PCR

Extracted DNA was quantified spectrophotometrically and used for PCR. PCR amplification of a 540-bp fragment of *Brassica napus* Rbc was used as control to detect endogenous canola DNA (GenBank Accession No. X75334). It was detected using forward primer, Rbc F (5'-GGC TGA CGT CGT CAC GTA G-3') and reverse primer, Rbc R (5'-CGT TGC CTG CCA CAG GAT TAA GG-3'). PCR conditions used were: 95°C for 5 min, 30 cycles of 94°C for 20 s, 50°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 10 min. The EPSPS whole gene was detected using forward primer, EF1 (5'-TCA CGG TGC AAG CAG CAG CCG TCC AGG-3') and reverse primer, ER1 (5'-TCA AGC AGC CTT AGT GTC GGA GAG TTC G-3') to amplify a 1363-bp region. PCR conditions used were: 94°C for 5 min, 22 cycles of 94°C for 15 s, 68.5°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min.

All PCR reactions (50 μl) contained the following (final concentrations): 1× PCR buffer, 0.2 mM dNTP mix, 0.5 μM each of forward and reverse primer, 1.5 mM MgCl₂, and 2.5 U *Taq* Polymerase (Invitrogen, Burlington, ON, Canada). 100 ng DNA template was used for PCR. A negative control without template DNA, as well as appropriate controls using non-transgenic leaf (P) and/or transgenic leaf (R) was included in each run. PCR was performed on a PTC 100 thermocycler (MJ. Research Inc., Watertown, MA, USA).

2.7. Gel electrophoresis

5 μl of genomic DNA isolated from canola substrates was applied to a 0.7% (w/v) agarose gel [17]. For all PCR reactions, 20 μl of PCR products was analyzed on 1.5% (w/v) agarose gels.

2.8. Statistical analysis

VFA production data for each substrate was analyzed by ANOVA [18]. The treatment effects were compared against each other using LS MEANS with PDIFF procedure.

3. Results and discussion

3.1. DNA isolation and PCR

DNA from canola leaf tissues was used as controls for all PCR analyses. DNA extraction from the seeds, meals and diets was found to be of the best quality when the described CTAB extraction method was used and therefore this procedure was used to isolate DNA for batch culture samples. Initial attempts at PCR amplifications of DNA from the batch culture showed that 100 ng template DNA produced good amplification and was therefore used for PCR set-up.

3.2. Canola processing

Genomic DNA isolated from seeds (PWS and RWS) was high molecular mass (approximately 23 kb) (Fig. 1) and that from the canola meals (PM and RM) was highly fragmented but still had significant amounts of 23-kb DNA, while those from diets (PD and RD) contained
comparatively lower amount of high molecular mass DNA. As expected, these results affirm that feed processing degrades plant DNA, most likely due to high temperatures during the processing. It has been reported previously that the treatment of oilseed rape meal (canola) resulted in complete degradation of DNA and that heating maize grains to 95°C for 5 min resulted in an inability to amplify a 577-bp gene sequence by PCR [19]. In the present study, during canola meal preparation, temperatures of 95°C were used and during diet processing temperatures of 100°C were achieved. Despite this, we were able to detect high molecular mass DNA and could successfully amplify full-length plant genes (EPSP synthase) from mixed diets containing canola meal.

The 540-bp Rbc fragment (endogenous plant DNA) was detected in all parental and Roundup Ready® substrates (Fig. 2A), while the EPSPS gene (1363 bp) was only detected in Roundup Ready® leaf and derived substrates viz;

RWS, RM, RD (Fig. 2B). Despite using the same amount of DNA (100 ng) for PCR reactions, the 1363-bp band from RM was less intense than that isolated from Roundup Ready® seed and leaf, and amplification from RD DNA was even lower. These results are expected and suggest that fragmentation of DNA (transgene or otherwise) occurs during feed processing. In this case, processing decreased the amount of 1363-bp DNA fragments in meals and diets. The diets contained 6.5% of the respective canola meals and thus the transgenic DNA to total plant DNA ratio in RD would be expectedly lower, explaining the faint EPSPS band observed in the RD (Fig. 2B, lane 9) as compared to meal (RM, Fig. 2B, lane 8). This reduction could have also arisen as a result of fragmentation of EPSP synthase during diet preparation. Presence of the EPSPS fragment in the diets was tested at least three times from three different samples. The results indicate that endogenous (Rbc) and recombinant plant genes (EPSP synthase) are present and can be detected from processed canola diets.

3.3. Batch culture incubations

Total VFA concentrations were found to increase throughout fermentation for all the substrates tested, indicating continuous digestion of plant material (Fig. 3). Within each treatment of GM or parental canola (PWS, PCS, PM and PD), VFA concentrations did not differ significantly (P > 0.05). Acceptance of GM crops is dependent on substantial equivalence between the parental and transgenic lines [20]. Bioavailability of nutrients is a key component when comparing the equivalence of two feeds. We found that, in vitro digestion of parental and Roundup Ready® substrates appeared equivalent, in terms of digestive end-products, which account for a major proportion of absorbable energy for ruminants.

Mean values of whole seed VFA were significantly lower than cracked seeds (P < 0.05). This is accounted by the
fact that the seed coat acts as a barrier to digestion of plant material which explains the observation that cracking the seeds resulted in a greater accumulation of fermentation end-products [21]. VFAs from both whole and cracked seeds were significantly lower (P < 0.05) than meals and diets, while differences between meal and diet values were not significant (P > 0.05). Thus processing of meals and diets may have improved digestibility by increasing the plant material susceptible to microbial degradation.

Neither the endogenous 540-bp Rbc nor the recombinant 1363-bp EPSPS fragments were detected in supernatants (supernatant B) of batch culture incubations. However, bacterial DNA, was present in these samples (data not shown). Because nuclease activity is present in rumen fluid [22,23], we initially speculated that free plant DNA in the supernatant may have been degraded between the time taken for sampling and isolation of DNA. Thus batch culture incubations were repeated and DNA from the various fractions was isolated immediately (without a time lag). Plant DNA, however could still not be detected suggesting that plant DNA does not accumulate in the fluid fraction of rumen contents. The extremely high concentration of bacterial cells in rumen fluid compared to plant cells is perhaps the reason for detection of bacterial DNA in supernatant samples. Despite degradation of DNA by nucleases, the starting amount of bacterial DNA upon cell lysis must be high enough such that it can be amplified. On the other hand, plant DNA released into the aqueous environment from rumen fluid seems to be a small proportion of total DNA present. These results are supported by the observation from another study which showed that the 1914-bp cry1A(b) maize gene persisted for less than 1 min in rumen fluid and a 350-bp bla fragment survived for only 1 min [9].

Persistence of endogenous plant DNA (Rbc) isolated from pellets containing plant debris (pellet A) was consistently the same within each treatment group (Fig. 4), as was substrate digestion, indicated by VFA concentrations (discussed earlier). The 540-bp sequence was amplifiable for all time points from PWS, RWS, PCS, RCS, for up to 8 h in PM and RM, and for up to 4 h in PD and RD (Fig. 4A–H). These results indicate that plant DNA persistence is inversely related to plant cell digestion. Plant DNA

Fig. 4. PCR analysis for persistence of 540-bp canola-specific Rbc fragment throughout a ruminal batch culture incubation with parental (P) and Roundup Ready® (R) canola whole seed (A and E), cracked seed (B and F), meal (C and G) and diet (D and H). Lane 1: 100-bp DNA ladder Plus (M); lane 2: negative control contained all components of the PCR reaction except DNA template (–); lane 3: P leaf positive control (P+); lane 4: R leaf positive control (R+); lanes 6–12: represent time points 0, 2, 4, 8, 12, 24 and 48 h.

Fig. 5. Detection of 1363-bp EPSPS transgene from ruminal batch culture incubation with Roundup Ready® (R) canola whole seed, cracked seed, meal and diet. Lane 1: 1-kb DNA ladder (M); lane 2: negative control contained all components of the PCR reaction except DNA template (–); lane 3: parental leaf negative control (P–); lane 4: R leaf positive control (R+); lanes 6–12: represent time points 0, 2, 4, 8, 12, 24 and 48 h.
DNA from whole and cracked seeds could be detected for the longest incubation time, while total VFA production was expectedly the lowest for these substrates. Although, mean VFA values did not significantly differ between meals and diets, the Rbc gene was detected for a longer incubation time in the meals (8 h) than in diets (4 h for PD and RD) which is most likely due to lower concentration of canola DNA in diets as well as degradation of canola DNA upon processing. Similar trends were seen for 1363 bp EPSPS whole gene in Roundup Ready\textsuperscript{0} canola DNA from whole and cracked seeds, which was expectedly the lowest for these substrates. Although, the complete gene was detected at all time points in the whole and cracked seed samples, for up to 8 h in the RM, and for up to 4 h in the RD (Fig. 5A–D). Both Rbc and EPSPS are low-copy genes and their equal lengths of detection in RM and RD indicates similar rates of degradation.

The 466-bp bacterial-specific sequence could be amplified from fluid-associated bacteria (pellet B) throughout the 48 h incubation for all substrates whereas Rbc was not detected. The 466-bp fragment was also detected at all time points in the DNA isolated from pellet A, which contained plant material and particle-associated bacteria (data shown for PM, PD, Fig. 6A; and RM, RD, Fig. 6B). The detection of bacterial DNA for the 48-h incubation in meals and diets suggests that DNA isolated throughout the incubation was of PCR-quality and corroborates the fact that plant DNA was not present in detectable amounts past 8 h in meal and 4 h in diet preparations. These results also indicate that transformation of bacteria by the fragments of endogenous (Rbc) and recombinant (EPSPS) plant DNA studied here did not occur, despite transformation by fragments of DNA this length being plausible [24].

Transformation by DNA fragments can take place only if certain barriers are overcome as discussed previously [25]. First, DNA must be accessible to competent microorganisms. This would seem to be an initial limitation in the ruminant system since we found upon release, DNA is rapidly degraded. A second barrier is successful incorporation of foreign DNA into the competent recipient cells. It is well documented that recombinant events are highly dependent on sequence similarity [26] and the newly acquired DNA must be of use to the recipient cells. Stable transformation of ruminant microbes by recombinant DNA found in Roundup Ready\textsuperscript{0} canola was not detected in the present investigations and seems unlikely at least up to 48 h. However, transfer of genes across kingdoms within the rumen ecosystem has previously been demonstrated [11]. What appears to be the determining factor then, is the balance between the high number of microbes in ruminant fluid, to the number constantly in direct contact with plant material. This critical balance could reduce the effect of rapid DNA degradation acting as a barrier against transformation.

Despite this study being an analysis of plant DNA throughout in vitro incubations, the use of intact plant substrates instead of naked DNA, provides important information on the fate of endogenous and recombinant canola DNA in a ruminal environment. That plant DNA was not detected in the supernatant, but only in pellets containing plant debris, suggests that presence of plant DNA in ruminant systems is directly related to intact plant cells. Essentially, disappearance of plant DNA fragments is analogous to the digestion of plant cells in the rumen. This seemingly is the limiting step for transformation of rumen bacteria because once DNA is released into the aqueous ruminal environment, it is degraded almost immediately. Plant DNA fragments reaching the intestinal tract of ruminants and being available for absorption there [5] are most likely the result of undigested plant material passing through the rumen to the intestines, where plant cells can then be lysed, releasing DNA. The presence of intact plant genes in diets fed to ruminants warrants studies of DNA fragments in vivo and we are currently addressing this issue in trials with sheep fitted with ruminal and duodenal cannulae.

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References