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# DNA stability in plant tissues: implications for the possible transfer of genes from genetically modified food

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**Abstract The potential for transfer of antibiotic resistance genes from genetically modified (GM) plant material to microbes through genetic recombination in the human or animal gut is a consideration that has engendered caution in the use of GM foods. This study was aimed at defining the optimal physical and chemical conditions necessary to ensure sufficient fragmentation of DNA in plant tissues to a size where it would be unlikely to be stably transferred to bacterial gut microflora. The ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit (Rubisco SS) genes are of similar size (approximately 1.4 kb) to transgenes present in GM plants. DNA analysis and PCR amplification of Rubisco SS genes showed that fresh maize and maize silage contained high molecular weight DNA and intact Rubisco SS genes. Relatively high temperatures and pressurised steam were necessary to degrade fully genomic DNA and Rubisco SS genes in maize and wheat grains, the source of most animal feedstuffs. Furthermore, chemical expulsion and extrusion of oilseeds resulted in residues with completely degraded genomic DNA. These results imply that stringent conditions are needed in the processing of GM plant tissues for feedstuffs to eliminate the possibility of transmission of transgenes.** 

*Key words:* Rubisco; Genetically modified organism; DNA; β-Lactamase gene; Maize ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit

## 1. Introduction

Genetically modified (GM) crops are being developed for a variety of reasons, including resistance to herbicides and pests, length of shelf-life in the case of supermarket products, efficiency of processing and improved nutritional value. In some cases, the gene of interest is linked to a selectable marker gene which confers antibiotic resistance [1]. Such resistance, if transferred to pathogenic micro-organisms, might exacerbate the problem of resistant strains of bacteria. For example, an American cottonseed construct contains an *aad* gene, which confers resistance to streptomycin and spectinomycin. This has not been granted approval for marketing in the European Union [2]. Spectinomycin is a useful drug used in the therapy of infections caused by *Neisseria gonorrhoeae*, particularly in pregnant women, where the use of other antibiotics may be contra-indicated. Therapeutic options for this group are limited. Already a clinically significant resistance to third generation cephalosporins used in the treatment of gonorrhoea and other sexually transmitted diseases has been reported worldwide [3–5]. This is of particular relevance when considering GM crops that carry the $bla_{TEM}$ gene from which cephalosporin resistance has evolved.

Horizontal gene transfer across related species and even unrelated organisms is a well-documented phenomenon. Gene exchange mechanisms include transduction, transformation and conjugation, with the latter being the mechanism with the broadest host range of transfer [6]. The implications of this in the context of biosafety are far reaching. One particularly important example of this is the case of animal feedstuffs, where material from transgenic plants encounters bacteria in the ruminant gut. It is not clear whether the transfer of such genes from transgenic plant to microbe can be completely excluded; for example, it has been shown that oral bacteria can be transformed by naked DNA in human saliva [7,8]. Moreover, DNA from an M13 mp18 phage ingested by mice was detected in their gastrointestinal tract as fragments of up to 1 kb in length [9]. It was able to penetrate the intestinal wall into the nuclei of spleen and liver, where it was found to be covalently linked to the host DNA [10]. There is evidence to suggest that foreign DNA originating from daily intake of food may be covalently linked to the host DNA [11]. The likely consequences of such genetic recombinations for mutagenesis have not been investigated. However, it was observed in a cell-free system that specific patterns of de novo methylation of foreign DNA occur as a result of insertion of foreign DNA into the mammalian genome, thus triggering extensive changes in cellular DNA methylation patterns at sites far away from the locus of insertional recombination [12]. These alterations may contribute to the potential of foreign DNA to induce oncogenesis. Therefore one aspect of the current debate on the safety of GM food is to know what conditions are necessary in installations preparing animal feeds to prevent gene transmission, i.e. what conditions of food processing would ensure sufficient disruption of DNA.

Food ingredients are likely, in the future, to originate from a variety of GM sources containing a number of different transgenes. GM maize incorporating a β-lactamase gene which confers resistance to penicillin [13] has, for example, generated great interest in view of the small but finite possibility of the genetic material being transferred to potentially pathogenic microflora, thus rendering them antibiotic-resistant. DNA is susceptible to degradation by heat, pressure and other physical and chemical processes, which might be used in food processing. We were therefore interested in defin-

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ing physical or chemical treatments of plant tissues, the major component of animal feedstuffs, that would fragment plant DNA to a size where it would be unlikely to be stably transferred to the bacterial microflora.

Since we had no access to GM plant material at the start of this investigation, we targeted a family of genes which is abundant in the chloroplasts of higher plants, namely the maize ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit (Rubisco SS) (*RbcS1* and *RbcS2*) genes, which are approximately 1.6 kb and 1.3 kb in length, respectively, compared to the β-lactamase gene which is approximately 1.2 kb in length. There is no reason to believe that the conditions necessary to fragment the DNA of GM plant material would be any different from those of unmodified material.

## 2. Materials and methods

### 2.1. Plant tissues

Samples of animal feed ingredients were obtained from commercial sources. They included oilseed rape, linseed and soybean, before and after oil extraction by expulsion and extraction (involving 60 min in a mixture of hydrocarbon solvents and drying at more than 100°C); wheat grain of the variety Riband that was subjected to heat treatment, both dry and moist, at a range of temperatures and pressures and for a range of durations; smaller samples of 20 varieties of wheat with the highest and lowest crude protein and fibre contents were subjected to dry heat treatment at 93°C; fresh maize leaves and maize silage, from the University of Leeds Farm; fresh maize cobs and maize grains from a local supermarket; maize grains were subjected to dry heating at three different temperatures and durations; maize gluten (solvent extracted and dried at 100°C for at least 60 min) and flaked maize (rolled at more than 100°C); fresh wet sugarbeet and dried sugarbeet pulp (at least 200°C); fresh ryegrass and ryegrass silage.

### 2.2. DNA isolation

This procedure utilised the Nucleon PhytoPure® extraction kit (Amersham). Samples were ground in liquid nitrogen and the genomic DNA isolated according to the manufacturer's guidelines. Following ethanol precipitation, genomic DNA was further purified by centrifugation at 13 000 rpm and filtration through a 0.45 μm membrane then resuspended in TE buffer. This procedure generated DNA that was a good template in polymerase chain reaction (PCR) assay reactions. Purified DNA was incubated with calf thymus RNase A in order to remove any contaminating RNA from the DNA, which obscured low molecular weight DNA fragments on agarose gels. The concentration of DNA was measured by UV spectrophotometry [14].

### 2.3. Agarose gel electrophoresis

Fragmentation of DNA was assessed by agarose gel electrophoresis followed by ethidium bromide staining. DNA samples containing approximately 1 μg of DNA with 0.2 volumes of gel loading buffer (1% xylene-cyanol, 1% bromophenol blue, 50% glycerol in 1×TAE buffer) were applied to 1% agarose gels in TAE buffer containing 1 μg/ml of ethidium bromide [14]. Samples were separated by electrophoresis at 50 V/cm for 30 min to 1 h or until the xylene-cyanol dye migrated 3/4 of the way down the gel. UV-fluorescent DNA fragments were photographed using a UVP imagestore system and their sizes determined by comparison with marker DNA fragments (an *Eco*RI/*Hin*dIII digest of lambda DNA and/or a kilobase DNA marker and/or a 100 bp DNA ladder).

### 2.4. PCR analysis

The structure of the small subunit gene family (*RbcS1* and *RbcS2*) of the maize chloroplast enzyme Rubisco was analysed in DNA samples. A series of oligodeoxy-nucleotide primers were synthesised, spaced at intervals of up to approximately 600 bp apart in the maize *RbcS* genes, a cut off size which is considered to be the minimum length of DNA for which recombinations are likely to occur [15,16]. The use of a set of primers that amplify fragments of increasing lengths was expected to determine the frequency of DNA strand breaks in selected regions of the *RbcS* genes. Where there is a DNA strand break between two primer binding sites, PCR amplification should not take place efficiently and this would be revealed by a lowered level, or absence, of PCR products as detected by agarose gel electrophoresis. In this way, the precision of analysis of DNA fragment sizes could be extended to the level of single genes.

The PCR reaction mixture (50 μl) contained 5 μl of 10×PCR buffer (supplied by the manufacturer), 0.2 mM of each dNTP (Pharmacia), 50 pmol of each of forward and reverse primers (Genosys), 2 U of Taq DNA polymerase (HT Biotechnology) and 1 μl of DNA template. PCR was performed in a Perkin Elmer thermocycler with an initial denaturation step of 5 min at 95°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C and extension for 1 min at 72°C with a final extension at 72°C for 10 min. The PCR assay was standardised using four maize *RbcS* genomic clones, pTN20 and pTN21 which span segments of the *RbcS1* gene and pBSS23 and pBSS21 which span segments of the *RbcS2* gene.

## 3. Results

The effects of grinding and milling on the yield and quality of DNA from wheat samples was investigated. The effect of grinding frozen plant tissues was tested since this was a step in the DNA isolation procedure and it was important to test that DNA was not fragmented during its isolation. Since milling of grains is used in a variety of food processing protocols, this variable was also tested for possible effects on DNA fragmentation. Wheat grains were subjected to mechanical grinding and milling at different grist sizes. Grinding and milling did not appear to have any significant effect on the average molecular weight of DNA isolated, which was high molecular weight, of at least 20 kb (results not shown).

### 3.1. Analysis of DNA from commercial feedstuffs

Chemical extraction of feedstuffs, followed by mechanical treatment as in the case of oilseed rape meal and other oilseed cakes, appeared to leave a residue containing only highly fragmented DNA (results not shown). Expelled linseed and rapeseed, dried sugar beet pulp, extracted supersoy and soybean all yielded completely degraded DNA, while intact high molecular weight DNA was readily extracted from linseed leaves and dry grains, wheat leaves and grains, fresh wet sugar beet pulp, fresh ryegrass and ryegrass silage, soybean leaves and grains, three varieties of maize grains, maize leaves and maize silage, as well as from rapeseed leaves and grains (Fig. 1).

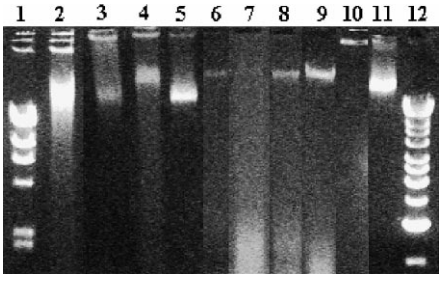


Fig. 1. Analysis of genomic DNA isolated from a variety of plant tissues by agarose gel electrophoresis. Lane 1, *Hin*dIII/*Eco*RI lambda DNA marker; lane 2, DNA isolated from dry maize grains steam-treated at 60°C for 30 min; lane 3, DNA isolated from dry soybean grains; lane 4, ryegrass silage; lane 5, dry wheat grains; lane 6, rapeseed; lane 7, extracted rapeseed cake; lane 8, unprocessed linseed grains; lane 9, fresh sugarbeet; lane 10, flaked maize; lane 11, fresh maize grains; lane 12, kilobase DNA marker ladder.

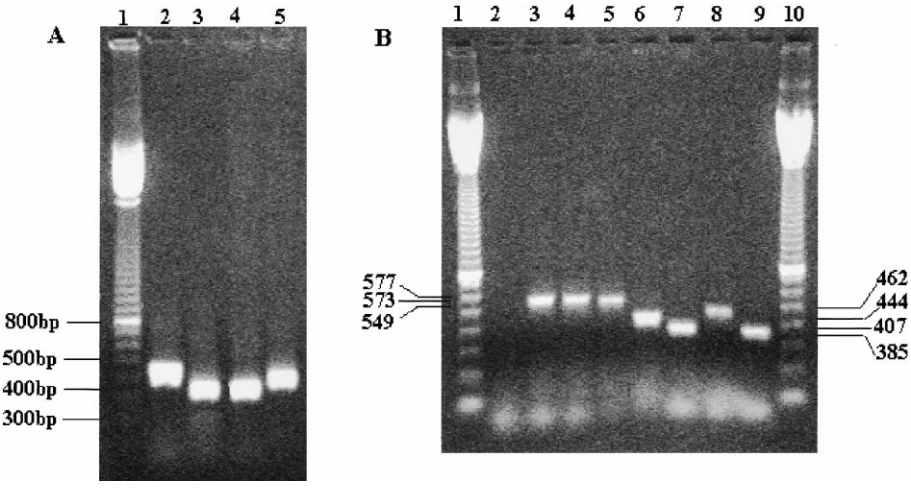


Fig. 2. A: Agarose gel electrophoresis of PCR products amplified from maize *RbcS* plasmid templates. Lane 1, 100 bp DNA ladder; lane 2, a 462 nucleotide PCR product of plasmid pTN20 from maize *RbcS1*; lane 3, a 407 nucleotide PCR product of plasmid pBSS21 from maize *RbcS2*; lane 4, a 385 nucleotide PCR product of plasmid pBSS23 from maize *RbcS2*; lane 5, a 444 nucleotide PCR product of plasmid pTN21 from maize *RbcS1*. B: Agarose gel electrophoretic analysis of PCR products amplified from maize genomic DNA templates. The primer pairs F2-B2, F2-B3, F2-B4, F3-B5, F4-B6, F5-B7 and F6-B8 (see Table 1 for sequences) were used to amplify specific sequences of maize *RbcS1* and *RbcS2* genes using spun and filtered genomic DNA isolated from fresh maize leaves as a template. Lanes 1 and 10, 100 bp DNA ladder; lane 2, control PCR without DNA template; lane 3, a 577 nucleotide PCR product obtained with the F2-B4 primer pair; lane 4, a 573 nucleotide PCR product obtained with the F2-B3 primer pair; lane 5, a 549 nucleotide PCR product obtained with the F2-B2 primer pair; lane 6, a 444 nucleotide PCR product obtained with the F4-B6 primer pair; lane 7, a 407 nucleotide PCR product obtained with the F6-B8 primer pair; lane 8, a 462 nucleotide PCR product obtained with the F3-B5 primer pair; lane 9, a 385 PCR product obtained with the F5-B7 primer pair.

### *3.2. PCR amplification of specific maize RbcS gene sequences*

The specificity of the PCR primers was established using four bacterial plasmid templates containing maize *RbcS1* and *RbcS2* genomic clones generating PCR products of up to 462 bp (Fig. 2A). The positive PCR results obtained thus indicated that primers were correctly designed and that the conditions for PCR were optimal. The primers designed to amplify PCR products from the above plasmid templates together with four primer pairs designed to amplify *RbcS* gene sequences from maize genomic DNA (see Table 1 for primer sequences) were used to amplify PCR products from maize genomic DNA isolated from fresh maize leaves. These primers produced PCR products ranging in size from 385 bp to 577 bp (Fig. 2B).

Intact high molecular weight genomic DNA ($>21$ kb) was readily extracted from fresh maize leaves, dry maize grains and maize silage as assessed by agarose gel electrophoresis (Fig. 3A, lanes 2–4, respectively), while DNA was substantially degraded in maize gluten and flaked maize (Fig. 1, lane 12). Using purified genomic DNA from fresh maize leaves, heat-treated and untreated dry maize grains and maize silage as templates in PCR reactions, specific sequences within the maize *RbcS1* and *RbcS2* genes were amplified with all the primer pairs used, generating PCR products of up to approximately 600 bp (Fig. 3A,B), but no products were obtained with DNA from maize gluten meal or flaked maize (results not shown).

Dry maize grains were subjected to dry heat at either 90°C, 92°C or 94°C for 5, 10 and 15 min. Gel electrophoresis of DNA showed that there was a substantial amount of intact high molecular weight DNA ($>21$ kb) in maize grain samples treated at 90°C for 5, 10 or 15 min (Fig. 3A, lanes 5–7, respectively), and using the primer pair designed to amplify specific sequences of the maize *RbcS* gene family, we were able to obtain a PCR product of 577 bp (Fig. 3B, lanes 6–8, respectively). Heating maize grains to 92°C for 5, 10 or 15 min, however, greatly reduced the amount of DNA obtained (Fig. 3A, lanes 8–10, respectively). The bulk of high molecular

Table 1
PCR primers used in this study

| Primer pair | Primer sequences | Product size |
|---|---|---|
| F1-B1 | [F1] 813–835 5′-TACGGCAACAAGAAGTTCGAGAC-3′<br>[B1] 1012–991 5′-AACATGGGCAGCTTCCACATGG-3′ | 200 nt product for F1-B1 pair (813–1012) |
| F2-B2 | [F2] 724–745 5′-TGCATGGACTGATGTGTGTTGC-3′<br>[B2] 1272–1253 5′-ACCCGAAGCAAAACCGAACG-3′ | 549 nt product for F2-B2 pair (724–1272) |
| F2-B3 | [B3] 1296–1276 5′-ACCAAGCAAGCAAAGGGTACG-3′ | 573 nt product for F2-B3 pair (724–1296) |
| F2-B4 | [B4] 1300–1280 5′-AGAAACCAAGCAAGCAAAGGG-3′ | 577 nt product for F2-B4 pair (724–1300) |
| F3-B5 | [F3] 8–31 5′-GCCCAATTCTGTAGATCCAAACAG-3′<br>[B5] 469–447 5′-GTATGTACTCGCTGCTTGCTTGC-3′ | 462 nt product for F3-B5 pair (8–469) in the *RbcS1* gene |
| F4-B6 | [F4] 994–10135′- TGTGGAAGCTGCCCATGTTC-3′<br>[B6]1437–1413 5′-TAGCAGGTATAGAGGTAGCCAATGC-3′ | 444 nt product for F4-B6 pair (994–1437) in the *RbcS1* gene |
| F5-B7 | [F5] 123–145 5′-CCAAGCAAGCAAGCTCGATCTAC-3′<br>[B7] 507–484 5′-AGGTACGACAGCGTCTCGAACTTC-3′ | 385 nt product for F5-B7 pair (123–507) in the *RbcS2* gene |
| F6-B8 | [F6] 656–677 5′-ATGTGGAAGCTGCCCATGTTCG-3′<br>[B8] 1062–1042 5′-TGTTCCTCACCCAACGAATCG-3′ | 407 nt product for F6-B8 pair (656–1062) in the *RbcS2* gene |

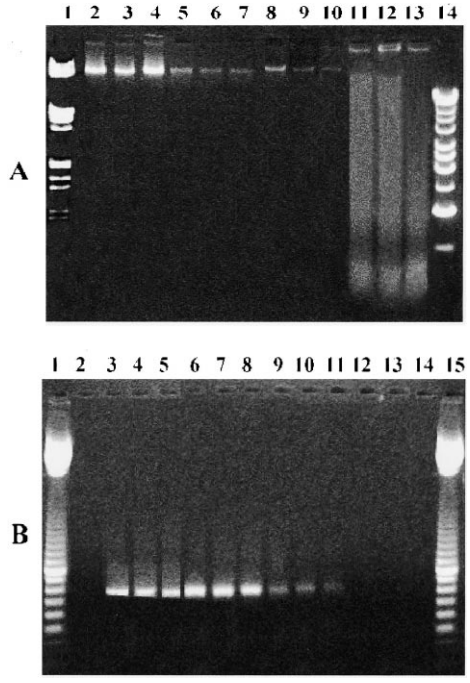


Fig. 3. A: Analysis of maize genomic DNA by agarose gel electrophoresis. Lane 1, *Hin*dIII/*Eco*RI lambda DNA marker; DNA isolated from: lane 2, fresh maize leaves; lane 3, dry maize grains; lane 4, maize silage; lanes 5–7, DNA isolated from dry maize grains subjected to dry heating at 90°C for 5, 10 and 15 min, respectively; lanes 8–10, DNA isolated from dry maize grains subjected to dry heating at 92°C for 5, 10 and 15 min, respectively; lanes 11–13, DNA isolated from dry maize grains subjected to dry heating at 94°C for 5, 10 and 15 min, respectively; lane 14, kilobase DNA marker ladder. B: Agarose gel electrophoretic analysis of PCR products amplified from maize genomic DNA templates. The primer pair F2-B4 (refer to Table 1 for sequences) was used to amplify a 577 nucleotide bp product from the maize *RbcS1* gene using genomic DNA isolated from the samples described in A above. Lanes 1 and 15, 100 bp DNA ladder; lane 2, negative control without DNA template; PCR products amplified using: lane 3, fresh maize leaves; lane 4, dry maize grains; lane 5, maize silage; lanes 6–8, dry maize grains subjected to dry heat at 90°C for 5, 10 and 15 min, respectively; lanes 9–11, dry maize grains subjected to dry heat at 92°C for 5, 10 and 15 min, respectively; lanes 12–14, dry maize grains subjected to dry heat at 94°C for 5, 10 and 15 min, respectively.

weight DNA obtained was presumed to be partially fragmented and this was clearly shown by the reduction in the amount of PCR product obtained (Fig. 3B, lanes 9–11, respectively). At 94°C for at least 5 min, the DNA was degraded (Fig. 3A, lanes 11–13) and this was confirmed by the complete absence of PCR products (Fig. 3B, lanes 12–14, respectively).

## 4. Discussion

The results of this study show that treatment of plant tissues at temperatures of 95°C or above for more than a few minutes is sufficient for degradation of DNA to take place to the extent that it should be incapable of transmitting genetic information. These conclusions are based on both the size of DNA fragments and the survival of specific gene sequences, with similar results being obtained in both cases.

Two aspects of plant DNA stability have been addressed in this work: on the one hand the extraction of oil, starch and sugar from oilseeds, maize grain and sugar beet, respectively; on the other the heat treatment of maize grains. In the former case, the treatments included chemical and/or physical methods, and where high temperatures were involved, the DNA was broken down into small fragments to the point that specific gene sequences detected by PCR were no longer present. In the case of wet sugar beet pulp, where high temperatures were not incurred, DNA was still intact but when the pulp is dried, DNA was severely fragmented. In the case of wheat grains, a temperature of at least 95°C was required for air-dried grains, while steam treatment significantly reduced the temperature at which fragmentation occurs. From the point of view of the risk of transmission of unwanted genetic determinants from animal or human food, it seems that there is little likelihood of transmission from the extracted oilseed and maize products such as soybean meal and maize gluten, which are the major forms that are traded internationally. For locally produced feedstuffs that are not subjected to high temperatures, such as wet sugar beet pulp, silage and cereal grains, intact DNA is still present and potentially capable of being taken up by microbes in the digestive tract.

Our results clearly show that DNA remained stable in silage and so it would seem sensible not to use ensiled GM crops as animal feed in the event of a significant risk of transmission of a transgene from GM source to the gut of farm animals being identified. Although some of the DNA extracted from silage is undoubtedly of microbial origin, the fact that specific *Rubisco* SS sequences were detected by PCR in silage makes it clear that intact maize DNA was still present.

Our results show that if a temperature of 85°C is maintained by steam for 10 min, then DNA is partially fragmented, but some fragments could remain at above the size of a transgene. It will be necessary to carry out further research with material from commercial processing before firm conclusions can be drawn about the viability of DNA in commercial animal feeds.

In conclusion, we have shown that processing of plant material involving temperatures of above 95°C for more than a few minutes fragments DNA to the extent that genetic information is unlikely to be retained. Materials which have not been subjected to such treatments not only have non-fragmented DNA but also retain specific PCR-detectable sequences suggesting that DNA is intact. This study therefore has implications for the current debate on the safety of processed GM food consumed by animals and humans.

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