Genetic determinants of mycotoxin synthesis in genus *Fusarium*

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Abstract

Havránková H., Pazlarová J., Ovesná J. (2011): **Genetic determinants of mycotoxin synthesis in genus** *Fusarium*. Czech J. Food Sci., **29** (Special Issue): S86–S92.

The fungi of the genus *Fusarium* occur worldwide mainly on cereal grains. They are considered to be not only plant-pathogenic, but also the producers of secondary metabolites – mycotoxins. In our study, we focused on the analysis by Q-PCR of two genes expression – *Tri4*, *Tri10* – involved in the production of *Fusarium* mycotoxin deoxynivalenol. Reference genes *β-tub* and *UBC* were found to be suitable for the expression level normalisation using the program geNorm. The level of expression of the target genes was analysed on four isolates of *Fusarium graminearum* grown *in vitro*. In all four isolates, *Tri10* gene expression level was lower than that of *Tri4* gene expression.

Keywords: *Fusarium*; trichothecene biosynthesis; *Tri4*; *Tri10*; real-time PCR; geNorm

Genus *Fusarium* occurs worldwide on a variety of different plant hosts, especially on the crop and small grain cereals. The members of this genus cause serious plant diseases, most often root, leaf and head blight (scab). These infections lead to the reduction of the yield and grain quality and therefore to global losses. Serious consequences for animal and human health are caused not only by the fungi themselves but also by their metabolic derivatives – mycotoxins. Mycotoxins are associated with plant diseases, as well as with cancer and other growth effects in animals and humans (Desjardins 2006; Leslie & Summerell 2006).

Chemically varied group of *Fusarium* mycotoxins are sesquiterpeniod compounds – trichothecens. Four types of trichothecens have been identified (A–D) differing by the functional group on cyclohexane/tetrohydropyran core. The most important toxin of type B trichothecens is deoxynivalenol (DON). Type B-trichothecens belong to the more phytotoxic mycotoxins, and their major producers being *F. graminearum* and *F. culmorum* (Foroud & Eudes 2009). Trichothecene-producing *Fusarium* species and thus trichothecens themselves are virtually ubiquitous in natural and agricultural ecosystems around the world, so their total removal is unrealistic. It is therefore important to develop methods of reducing trichothecene contamination of food and feed to a level not affecting the animal and human health (Desjardins 2006).

In past decades, an outline of the trichothecene biosynthetic pathway has been established based on molecular genetic analysis (Kimura *et al*. 2007). Trichothecene biosynthesis starts with cyclisation of farnesyl pyrophosphate to trichodien followed by a series of oxidation, isomerisation, cyclisation, and esterification (Foroud & Eudes 2009). Genes that are involved in trichothecene biosynthesis

Supported by the Ministry of Agriculture of the Czech Republic, Projects No. QI111B044, No. MZE 0002700604, and by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. OC 09031.

(*Tri* genes) are located within the 25 kb *Tri5* cluster (12 genes), *Tri1–Tri16* cluster, or in separated loci (*Tri15*, *Tri101*) (Seong *et al.* 2009). The *Tri4* gene encodes a cytochrome P450 monooxygenase involved in the second step of trichothecene biosynthesis – trichodiene transformation to isotrichotriol (Kimura *et al*. 2007). As a gene at the beginning of the biosynthetic pathway, *Tri4* was one of the most studied *Tri* genes – for example also for PCR-based identification of toxinogenic *Fusarium* species, as described by Lacmannová *et al*. (2009). Two genes from the known *Tri* genes have the regulatory function and encode the transcription factors TRI6 and TRI10. Other *Tri* genes were corregulated by TRI6 and TRI10 perhaps as a TRI6 transcription complex according to several studies (Tag *et al.* 2001; Peplow *et al.* 2003; Seong *et al*. 2009).

The aim of this study is to choose the most stable reference genes using software geNorm and to analyse the expression of two functionally different target genes – *Tri4*, *Tri10* – involved in the trichothecene biosynthesis. The experiments were performed with four isolates of *Fusarium graminearum*.

MATERIAL AND METHODS

Fungal material and growth conditions. *Fusarium graminearum* isolates 81-1, 71M1, 35M1, and 4M1 were used. They were provided by the Department of Mycology of the Crop Research Institute, Prague-Ruzyně (Šíp *et al*. 2008). The isolates were inoculated on cellophane-covered potato dextrose agar (Hi Media Laboratories Pvt. Ltd., Mumbai, India) with the addition of chloramphenicol (Sigma, St. Louis, USA). Seven-day incubation was performed at 25°C. The mycelium was removed with a sterile scalpel, immediately frozen by liquid nitrogen and stored at –80°C.

DNA isolation, RNA isolation and purification. The frozen mycelium was disrupted by grinding in a mortar. DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions, and stored at –20°C. RNA was isolated by phenol-chloroform extraction using the commercial product TRIzol® Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The isolated RNA was purified using RNeasy Mini Kit and RNase-Free DNase Set (Qiagen, Germany) according to the manufacturer's instructions and stored at –80°C. The concentration of the isolated nucleic acid was determined spectrophotometrically by measuring the absorbance at 260 nm (Nano-Photometer™; Implen GmbH+Co.KG, Munich, Germany). The purity of the nucleic acid was verified by measuring the absorbance at 230 nm, 260 nm, and 280 nm. The RNA isolated was subjected to electrophoresis; RNA integrity was verified by evaluating the signal quality of the large and small RNA ribosomal subunits in the electrophoreogram. Two independent RNA isolations of all samples were performed (technical repetitions 1, 2).

Preparation of cDNA. The isolated and purified RNA was reverse-transcribed using TaqMan® Reverse Transcription Reagents in a thermal cycler Verity® (Applied Biosystems Inc., Delaware, USA). The reaction mixture and thermal cycling conditions were compiled according to the manufacturer's instructions. Each 50 µl reaction mixture contained 1 µg of total RNA, 1× RT buffer, 5.5mM $MgCl_2$, 500µM of each dNTP, 1.25µM oligo $d(T)_{16}$, 1.25µM random hexameres, 0.4 U/µl RNase inhibitor, 1.25 U/µl MultiScribe™ Reverse Transcriptase, and Nuclease Free Water (Ambion Inc., St. Austin, USA) up to the total volume. The temperature profile was as follows: incubation at 25°C (10 min), reverse transcription at 48°C (30 min), inactivation of reverse transcriptase at 95°C (5 min), cooling at 4°C (∞). The cDNA samples were stored at –20°C.

Primers. Primers used in this study were as follows: Tri4-F (TAT TGT TGG CTA CCC CAA GG), Tri4-R (TGT CAG ATG CGC CTT ACA AA); product length 95 bp; Tri10-F (TCT GAA CAG GCG ATG GTA TGG A), Tri10-R (CTG CGG CGA GTG AGT TTG ACA), product length 391 bp; βtub-F (GGT AAC CAA ATC GGT GCT GCT TTC), βtub-R (GAT TGA CCG AAA ACG AAG TTG), product length 296 bp, 46 bp intron (Ponts *et al.* 2007); Ef2-F (CTG TGT TCT TAC CAT GAC), Ef2-R (CAA CAG GTT AGC ACC A), product length 387 bp (Boutigny *et al.* 2009); UBC-PS.1 (TCC CCT TAC TCT GGC GGT GTC), UBC-PS.2 (TTG GGG TGG TAG ATG CGT GTA GT), product length 104 bp (Lysøe *et al.* 2006).

Thermal gradient PCR. To determine the preliminary optimal primer annealing temperature for the subsequent real-time PCR, the thermal gradient PCR was performed with all primers.

The reaction mixture contained 1 µl of cDNA corresponding to 20 ng of total RNA, 1× Power

SYBER® Green PCR Master Mix (Applied Biosystems, Inc., Delaware, USA), 200nM of each primer and Nuclease Free Water up to the final volume of 20 µl. The mixture was prepared according to the manufacturer's instructions. The amplification was performed in a thermal cycler PTC-200 (MJ Research, Inc., Watertown, USA). The thermal profile included polymerase activation at 95°C (10 min), 45 cycles with denaturation at 95°C (30 s), annealing (temperature gradient 56; 57; 58.6; 61.1; 62°C – 30 s), extension at 72°C (1 min), and final extension at 72°C (10 min). The preliminary optimal primer annealing temperature for the subsequent real-time PCR was 57°C.

Real-time PCR. The reaction mixture for real-time PCR had the same composition as the mixture for gradient PCR. To detect the possible contamination, β-tubulin primers that are designed in two different exons were used. Nuclease Free Water was used as a negative control, the isolated and diluted (10 ng/µl) gDNA as a positive one. The amplification was carried out in 7900HT Fast Real-Time PCR System (Applied Biosystems Inc., Delaware, USA) under the same cycle conditions as those for gradient PCR; dissociation phase (95°C – 15 s, 60°C – 15 s, 95°C –15 s) was added instead of the final extension. Each PCR amplification was performed in triplicate. A 2-fold serial cDNA dilution for each gene was prepared to determine PCR efficiency as described previously (Ponts *et al.* 2007). The specificity of the resulting PCR products was checked by the dissociation curve and electrophoresis analysis.

Real-time PCR data analysis. Real-time PCR data were collected and processed in program SDS 2.2.2 (Applied Biosystems Inc., Delaware, USA). The mathematical model of relative quantification published by Pfaffl (2001) was used for the data evaluation. Quantification was based on threshold cycle (C_T) (also known as a crossing point (C_p)) – the cycle number at which fluorescence exceeds the minimal detection limit. Relative quantification of the experimental data was performed using programs MS Excel (Microsoft Corporation, USA) and geNorm (http://medgen.ugent.be/~jvdesomp/genorm/#introduction, accessed April 23, 2010) according to the instructions described previously (Vandesompele *et al.* 2002). The data of all target genes were expressed relative to the expression of the least expressed gene. The most stable reference genes, determined by the geNorm, were included into the normalisation factor. The relative expression value of each target gene was divided by the normalisation factor.

RESULTS AND DISCUSSION

The basic prerequisites for gaining reproducible real-time PCR data are the cultivation, isolation, and purification under the same conditions, and verification of the nucleic acid integrity and purity. Working with RNA of poor quality (degraded, contaminated) may strongly devaluate the experimental results obtained (Fleige & Pfaffl 2006). The values of the ratio A_{260}/A_{280} ranged from 1.750 to 1.879 for DNA and from 2.078 to 2.134 for RNA, which corresponds to the criteria of nucleic acid purity (Qiagen 2006a,b). The validity of the criterion $A_{260}/A_{230} > 1.8$ for all RNA samples was also verified, the ratio A_{260}/A_{230} ranged from 2.257 to 2.500.

The analysis of the dissociation curves and electrophoreograms after real-time PCR at the annealing temperature 57°C showed only specific products in PCRs with primers βtub, EF2, UBC, and Tri4 (for example see the dissociation curve for sample 71M and electrophoreogram after real-time PCR at the annealing temperature 57°C with

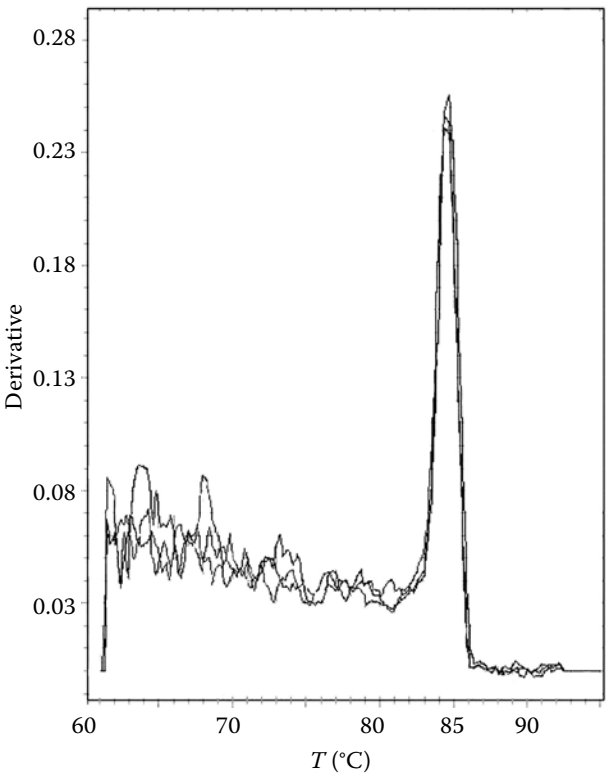

Figure 1. Dissociation curve for sample 71M after real-time PCR at annealing temperature 57°C with primers βtub

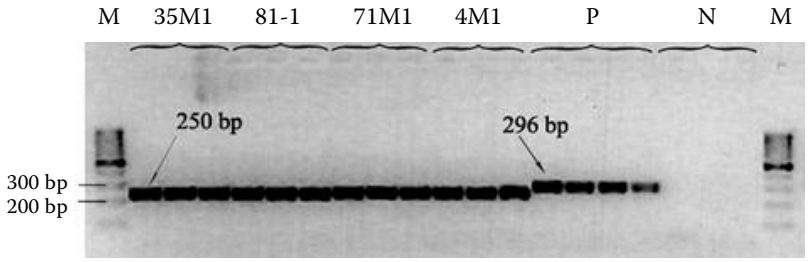

Figure 2. Electrophoreogram after real-time PCR at annealing temperature 57°C with primers βtub. M – DNA size marker (100 bp); samples – 35M1, 81-1, 71M1, 4M1; P – positive control (gDNA); N – negative control (Nuclease Free Water). Triplicates of each sample are shown. Formation of only specific 250 bp long products (cDNA)

primers βtub in Figures 1 and 2, respectively). On the contrary, real-time PCR with primers Tri10 at the annealing temperature 57°C generated apparent non-specific products (Figure 3). Changing the reaction conditions by increasing the annealing temperature to 63°C resulted in the emergence of only specific products (Figure 4).

PCR amplification efficiency is an important indicator of the accuracy and reliability of analysis using real-time PCR. The values of the efficiencies obtained ranged from 87% to 98%. Although some efficiency values did not reach the recommended 90% value (Applied Biosystems 2008), these values were still included in the analysis, because quantification of the gene expression using program geNorm takes in its algorithm the differences in real-time PCR efficiencies into account.

The program geNorm evaluated the obtained C_T values and determined *βtub* and *UBC* in both technical repetitions as the most stable reference genes (Figure 5). To determine the minimum number of reference genes required for the reliable normalisation factor, pairwise variation value V is used. Pairwise variation indicates the degree of variation between the two pairs of the normalisation factors – before and after adding the reference gene. Pairwise variation limit below which the addition of another reference gene is

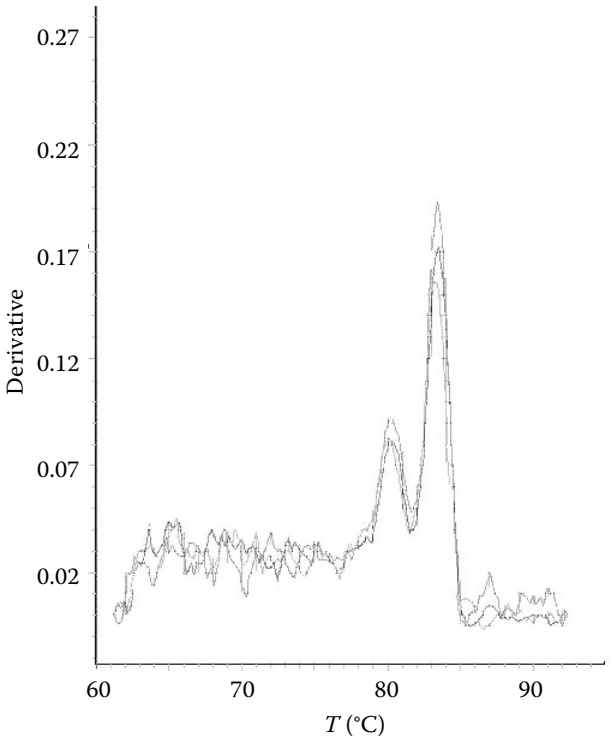

Figure 3. Dissociation curve for sample 71M after real-time PCR at annealing temperature 57°C with primers Tri10

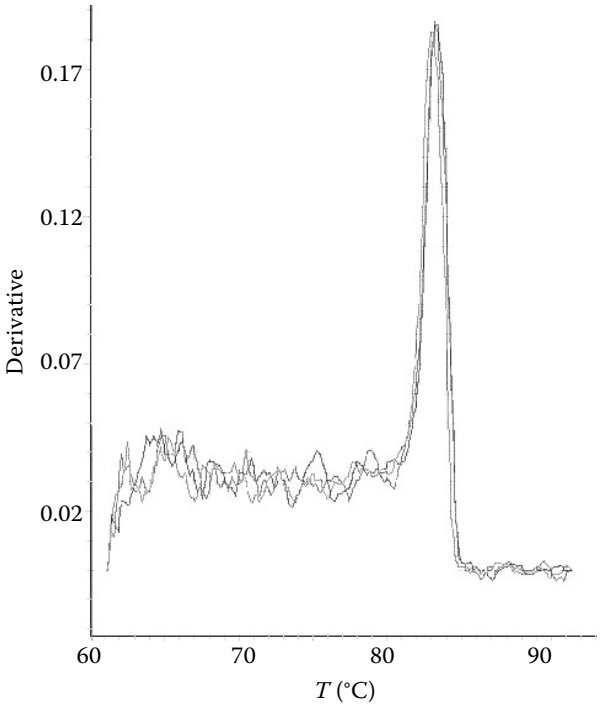

Figure 4. Dissociation curve for sample 71M after real-time PCR at annealing temperature 63°C with primers Tri10. Increasing the annealing temperature to 63°C resulted in the emergence of only specific products

geNorm PrimerDesign

Change Data	Beta	EF2	UBC	Normalisation Factor
1	1.00E+00	1.00E+00	1.00E+00	0.5942
2	2.46E+00	2.50E+00	2.00E+00	1.3720
3	2.21E+00	2.32E+00	1.73E+00	1.2307
4	1.47E+00	2.67E+00	1.20E+00	0.9966
M < 1.5	0.287	0.452	0.323	

Change Data	Beta	EF2	UBC	Normalisation Factor
1	1.00E+00	1.00E+00	1.00E+00	0.5859
2	2.38E+00	2.44E+00	2.37E+00	1.4041
3	1.77E+00	2.21E+00	1.96E+00	1.1549
4	1.38E+00	2.55E+00	1.64E+00	1.0525
M < 1.5	0.265	0.351	0.207	

Figure 5. Output geNorm data of reference genes *βtub*, *Ef2*, *UBC* for technical repetition 1 (above) and 2 (below). The input data were relative gene expression values of reference genes in each sample – 35M1 (1), 81-1 (2), 71M1 (3), 4M1 (4); geNorm determined value of normalisation factor and gene expression stability measure (M); the higher is M value; the lower is stability of gene expression. The gray box indicates the most stable reference gene, the black box the least stable one

not required is 0.15 (Vandesompele *et al.* 2002). Pairwise variation value in both technical repetitions did not exceed the limit value – 0.149, 0.117 – thus just two reference genes were included in the normalisation factor (Figure 6).

The lowest expression level of both target genes was found in the sample 35M1. Therefore the expression level of the target genes in the remaining samples was related to the expression in sample 35M1. Relative and normalised expression values of the target genes in both technical repetitions were plotted in the graph (Figure 7).

Tri10 gene expression was lower in all samples and repetitions than *Tri4* gene expression (Figure 7). We can assume that this was due to the functional differences of these target genes. Based on the experiments with *Fusarium sporotrichioides* in culture (Tag *et al.* 2001; Peplow *et al.* 2003) and *Fusarium graminearum* during plant infection (Seong *et al.* 2009) it was previously concluded that *Tri10* regulates many genes for primary and secondary metabolism. TRI10 with TRI6 as a transcription complex probably positively regulates other *Tri* genes, including *Tri4* which encodes oxygenase involved in trichothecene biosynthesis. In addition, *Tri6* negatively regulates the expression of *Tri10* (Tag *et al.* 2001; Peplow *et al.* 2003; Seong *et al.* 2009). This assumption probably corresponds to our result showing that *Tri10* has a lower expression than *Tri4*.

The four isolates of *Fusarium graminerum* differed in the target genes expression (Figure 7). As mentioned before, the lowest expression level was recorded in isolate 35M1.

The samples with the highest detected level of expression were 81-1 and 4M1. Šíp *et al.* (2008) in their *in vivo* study on *Fusarium* isolates on wheat cultivars found a higher DON production capac-

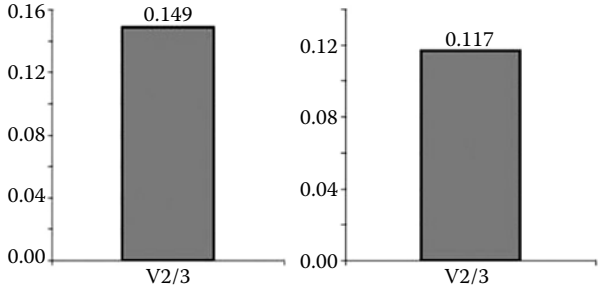

Figure 6. Pairwise variation value for reference genes *βtub*, *Ef2*, *UBC* in technical repetition 1 (left) and 2 (right). Pairwise variation value in both technical repetitions did not exceed the limit value (0.15)

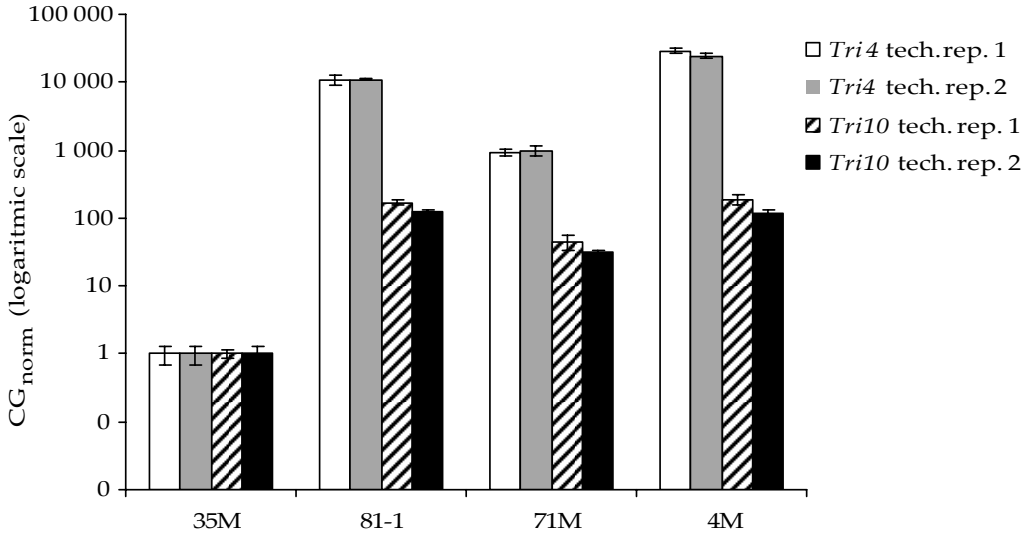

Figure 7. Relative and normalised expression value (CG_{norm}) of target genes – *Tri4*, *Tri10* – in both technical repetitions, in all samples – 35M1, 81-1, 71M1, 4M1. The lowest expression level was recorded in isolate 35M, the highest one in samples 81-1 and 4M1. *Tri10* gene expression was in all samples and repetitions lower than *Tri4* gene expression

ity of isolates 4M1 and 35M1 and a lower one of isolate 81-1. However, DON content in the *in vivo* experiments depends on many factors – such as environmental conditions, resistance of the cultivar, pathogenicity of then isolate, etc. – and therefore these *in vivo* results cannot be compared with our findings obtained in *in vitro* experiments.

CONCLUSION

The main goal of this study was to determine the expression levels of two target genes – *Tri4, Tri10* – related to trichothecene (DON) production in four isolates of *Fusarium graminearum*. The basic necessity for this was to optimise the methods and conditions to gain really reproducible and valid gene expression data. We have optimised the isolation, purification, and also real-time PCR conditions for all primer pairs βtub, UBC, EF2, Tri4 and Tri10. As a stable reference controls for the gene expression normalisation were determined, using program geNorm, genes *βtub*, *UBC*. The results obtained indicate that *Tri10* gene expression is lower than *Tri4* gene expression. But we must emphasise that all experiments were performed on *Fusarium graminearum* isolates grown *in vitro*, so these results give only a necessary basis for subsequent experiments on *Fusarium* spp. grown *in vivo*.

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Received for publication July 29, 2011
Accepted after corrections November 24, 2011

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