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"The application of gene expression profiling in the characterization of physiological effects of genetically modified feed components in rats"

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Dipl.-Ing. agr. Anja Hartmann
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Gutachter

1. Gutachter: Prof. Dr. M. Schwerin
(Forschungsinstitut für die Biologie landwirtschaftlicher Nutztiere, Dummerstorf; Universität Rostock)

2. Gutachter: Prof. Dr. G. Flachowsky
(Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit, Braunschweig)

3. Gutachter: Prof. Dr. Karl-Heinz Südekum
(Universität Bonn)

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ABBREVIATIONS

ADF	acid detergent fibre
bit	binary digit
bp	base pair
Bt	Bacillus thuringiensis
BXN	bromoxynil-resistant
CaMV	cauliflower mosaic virus
cA	crude ash
cDNA	complementary deoxyribonucleic acid, copy deoxyribonucleic acid
cF	crude fibre
cM	centi Morgan
cP	crude protein
CtxB	Cholera toxine subunit B
Cy3-UTP	Cy3 aminoallyl uridintriphosphat
Cy5-UTP	Cy5 aminoallyl uridintriphosphat
cRNA	complementary ribonucleic acid
DD	differential display
DDRT-PCR	differential-display reverse transcription - polymerase chain reaction
DEG	differentially expressed gene
DM	dry matter
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dsDNA	double strand deoxyribonucleic acid
dUTP	desoxyuridintriphosphate
EE	ether extract
EFSA	European Food Safety Authority
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
EST	expressed sequence tag
FAO	Food and Agriculture Organization
FC	fold change
FDR	False Discovery Rate
for	forward
geneID	gene identification
GM	genetically modified
GNA	galanthus nivalis lectin

GO	gene ontology
GSA	Gene Set Analysis
GV	genetisch verändert
IGA	Individual Gene Analysis
ILSI	International Life Science Institute
kDa	kilo Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
Lowess	locally weighted regression scatterplot smoothing
M	molar mass
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
n	number
N	nitrogen
NCBI	National Centre for Biotechnology Information
NDF	neutral detergent fibre
NfE	nitrogen free extract
nptII	neomycine phosphotransferase II
NPU	net protein utilization
ORF	open reading frame
PAGE	parametric analysis of gene set enrichment
PCR	polymerase chain reaction
PER	protein efficiency ratio
PMT	photo multiplier tube
QTL	quantitative trait loci
r	Pearson´s coefficient of correlation
rAlb-nptII	recombinant, nptII expressing potato of the cultivar Albatros
rAlb-VP60	recombinant, VP60 expressing potato of the cultivar Albatros
rBV-VP60	recombinant, VP60 expressing baculovirus
rev	reverse
RHDV	Rabbit Haemorrhagic Disease Virus
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
SAGE	Serial Analysis of Gene Expression
SAM	Significance of Microarray Analysis
SD	standard deviation

SNP	single nucleotide polymorphism
TCID	tissue culture infective dose
TIFF/tif	tagged image file format
TSP	total soluble protein
VP60	virus protein of 60 kilo Dalton
WHO	World Health Organization
wtAlb	Conventional, non-transgenic potato of the cultivar Albatros
wtBV	Wildtype baculovirus
vs.	lat. *versus*

Nucleotides

A	adenine
C	cytosine
G	guanine
T	thymine

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1 INTRODUCTION

Modern biotechnology e.g. recombinant DNA technology is more and more used in plant breeding to enhance crop productivity or to improve health and nutrition benefits for consumers (e.g. increased vitamin contents of crops, lower level of anti-nutrients) by integrating new genes into plants (BOUIS *et al.*, 2003).

However, such new agricultural products have aroused an increased concern about the safety of genetically modified (GM) plants which are introduced into the food chain (ILSI, 2003). The possible appearance of “unintended effects”, e.g. compositional or nutritional changes as a result of the genetic modification process, is one of the key elements of safety assessment procedures (e.g. KUIPER *et al.*, 2003; EFSA, 2008). Novel dietary bioactive substances are an additional aspect of concern (ILSI, 2004).

So far, a main consensus of most safety assessments has been that GM plants without substantial changes in their composition do not significantly differ in their nutritional value from those of their nearest isogenic varieties and do not affect the health of the organisms which ingested the plants. Detailed overviews were given by FLACHOWSKY (2005), FLACHOWSKY *et al.* (2007) and EFSA (2008). Only few findings have proven possible “unintended effects” in genetically modified plants such as an increased protein content in GM rice (MOMMA *et al.*, 1999) or augmented glycoalkaloid content in GM potatoes (HASHIMOTO *et al.*, 1999). In addition, the minority of the publications reported on physiological effects of GM plants when ingested by animals in feeding trials (e.g. PRESCOTT *et al.,* 2005: altered immunogenicity in mice; MALATESTA *et al.*, 2002: influence on zymogen synthesis in mice).

However, the adequacy of conventional methods for the nutritional evaluation of GM foods has been criticized (MILLSTONE *et al.* 1999; CLARK *et al.* 2001; LIU-STRATTON *et al.*, 2004). Conventional approaches for detecting effects of genetically modified food crops have been primarily based on measurements of single compounds or features. LIU-STRATTON *et al.* (2004) in contrast suggested studying expression, proteomic and metabolomic profiles of genetically modified foods. They also proposed expression profiling in tissues from experiments with humans or animals who have ingested such foods and to compare them with the corresponding traditional food as a reference for a complete safety assessment and in order to increase the probability of detecting unexpected effects. Indeed, the examination of large scale gene expression changes could be a sensitive and useful method for characterizing physiological side effects of GM foods, especially in the light of known impacts of macro- and micronutrients as well as dietary bioactive molecules on gene expression (DE CATERINA *et al.*, 2004). First

expression profiling assays within this context have been carried out by NARASAKA *et al.* (2006) and EFSA (2008).

The present work aimed to evaluate the application of comparative expression profiling by using microarray technology in the characterization of “unintended” physiological effects of GM feed components. Within this study the physiological impact of specific GM feed components was characterized in feeding experiments with rats. Firstly, a recombinant VP60 (viral antigen) expressing potato was fed. Secondly, a recombinant potato, which expressed solely a marker gene that is used for plant selection, was given. In an additional experiment inactivated recombinant VP60-baculovirus were administered. Expression profiles were compared to control groups which were fed the near-isogenic non-GM potatoes or wildtype baculovirus additives, respectively.
As a prerequisite for this expression study different approaches for data analysis in microarray experiments were compared, as it is known that they can have a profound influence on the results and on the conclusions (QUACKENBUSH, 2001). Special attention was turned to criteria for defining differentially expressed genes (DEG) since that is the fundament on which most of subsequent analyses and interpretation are usually based. To determine which genes show significant expression changes, it is common to perform a t-test and a “False Discovery Rate” (FDR) correction. The FDR correction adjusts/corrects the p-values for high numbers of expected false positives which can arise in microarray studies because thousands of comparisons are done simultaneously (ALLISON *et al.*, 2006). Though, there is no common agreement for which FDR level significance should then be assumed. According to literature thresholds varying between 5 % – 20 % (FDR) are used in combination with different fold change filters (FC ≥ 1.3 to 2.0) to define genes, that are “differentially expressed” between the two experimental groups (BOOTH *et al.*, 2004; ADARICHEV *et al.*, 2005; RUIZ-BALLESTEROS *et al.*, 2005; ZHU *et al.*, 2005; HAN *et al.*, 2006; PONSUKSILI *et al.*, 2007). It is consequential that using different thresholds and filter criteria can have a profound influence on the number of DEGs (PAN *et al.*, 2005). However, the lists of DEGs are the base for the identification of affected biological processes and thus for the conclusion to be drawn from the results. Therefore, a second aim of this work was to study the effect of different thresholds and filters (FDR, FC) on the number of DEGs and on biological processes identified as being diet-affected. These results built the precondition for the characterization of physiological effects of GM feed components and were based on data of the feeding experiment carried out with rats fed a recombinant baculovirus feed additive.

2 LITERATURE REVIEW

2.1 Structural and functional genome analysis in farm animals

In general genomics aim to gain a comprehensive understanding of the structure and the function of genomes (HOCQUETTE, 2005). The term “genomics” was proposed by Thomas Roderick in 1986 to characterize the scientific discipline of mapping, sequencing and analyzing genomes (MCKUSICK, 1997). In present literature genomics is described as the study of genes and their function in an organism (WHITFIELD, 2007) and may be divided into structural genomics and functional genomics. Structural genomics deal with the characterization of the physical nature. The target of research is DNA which corresponds to the organisms’ genetic background, whereas functional genomics research concentrate on the key molecules which give life to the cells: RNA, proteins and metabolites (HIETER & BOGUSKI, 1997; HOCQUETTE, 2005).

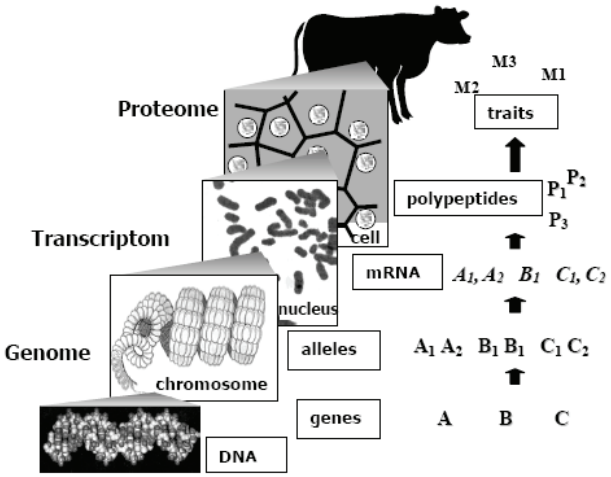

Figure 1: Molecular basis of the phenotype (modified from NEUSCHWANDER, 2001)

DNA sequence serves as “blueprint” of an organism and determines the basic amino acid sequences in proteins and ultimately determines the structural and functional nature of encoded proteins (see Figure 1). The sequence of a gene typically has a regulatory, a coding and a non-coding part. The gene regulatory sequence consists of DNA patterns that control how this gene is to be utilized at a given time point and under specific conditions. The coding sequence is composed of a series of coding regions (exons) whose information is transcribed into messenger RNA (mRNA), interspersed with non-coding regions (introns) whose information cannot be translated into RNA; although they might have other functions. RNA is further modified (introns are spliced, RNA is polyadenylated and capped) and transported into the cytosol. Ribosomes bind to the cap

structure and the RNA is translated into a polypeptide. Finally, posttranslational modifications convert the polypeptide into a functional protein. Regulation of expression takes place at different steps between transcription and synthesis of the final active protein product. All biological processes are regulated by the information flow in this pathway. Although this process is controlled by the basic genetic determinants, external or environmental factors can also have an impact; e.g. disease challenge, exposure to environmental toxins or nutrient supply (NEUNSCHWANDER, 2001; REECE, 2003; DAWSON, 2006; MARTENS *et al.*, 2007).

2.1.1 Structural genomics

Structural genomics was referred to as physical (physical localization of the genes along the chromosomes) and genetic mapping (representation of the genetic distances between genes; this is assessed on the basis of their recombination rate) and sequencing of a genome (MCKUSICK, 1997). HIETER & BOGUSKI (1997) stated that structural genomics represented the initial phase of genome analysis with a clear end-point – the construction of high-resolution genetic, physical and transcript maps of an organism. Other authors did not limit the term to the gene sequence but also include determination of three-dimensional structures of the encoded proteins and derivation of knowledge about their function (THORNTON *et al.*, 2000; BRENNER, 2001). According to SCHWERIN (2000) structural genome analysis comprises also the identification of the genetic variation which determines phenotypic variation.

Nowadays, the individual can be characterized at the level of its genomic DNA or its genotype. The genotype refers to the full hereditary information (DNA). The vast majority of DNA sequence is equal between mammalian organisms. Humans for instance differ by some animal species only 1 % in terms of DNA sequence. Among individuals of the same species differences are even less. In human 99.9 % of DNA is identical from person to person. The reason why individuals look and act differentially is attributed to only 0.1 % DNA which differs amongst them (HOCQUETTE, 2005). These differences or alternative forms of genes that are located at a specific position on a specific chromosome are called alleles. The alleles that are present in an individual constitute its genotype. Different alleles can produce variation in inherited characteristics and result in a variation of phenotypic traits. The phenotype of an organism comprises its actual physical properties, such as height, milk yield etc. Genetic variation, in the form of multiple alleles of many genes, exists in most natural populations (REECE, 2003).

2.1.1.1 Identification of trait-associated genetic variants

For hundreds of years man has improved successfully livestock production (e.g. growth rate, meat quality, egg production) by selection and improving the environmental conditions for the animals (KAPPES, 2003). Nevertheless, in traits with a low heritability, with late development or negative correlated traits, further progress is limited (SCHWERIN, 2003). The use of genomic information can help to overcome these limitations; it did and will increase selection efficiency in livestock (ELSEN, 2003). This has especially become an interesting approach since improvements in the efficiency of DNA sequencing have allowed the sequencing of several genomes. The human and the mouse genome for example are completely sequenced. Assemblies of 33 other mammal genomes, including agriculturally relevant genomes like pig and cattle are available and 40 mammal genome projects are in progress (http://www.ncbi.nlm.nih.gov/genomes).

The availability of such extensive sequence information enables a better understanding of the genome structure and the searching for trait associated genetic variants (HOCQUETTE, 2005). Identified genetic variants are independent of age, gender, environmental factors and generally evenly distributed across the whole genome whereas traditional breeding selection criteria like performance etc. are prone to these factors. Consequently, in modern breeding beneficial genetic variants, which are known to be associated with a certain phenotype (e.g. meat quality), can be used to improve this trait (NEUENSCHWANDER, 2001). To select for those advantageous genetic variants two types of genetic markers are typically used:

- Linked markers are loci that are sufficiently close to the trait-gene on the chromosome such that in general, alleles at the marker and the trait-gene are inherited together.
- Direct markers are loci that code for the functional polymorphism in the gene that controls variation in the trait.

Linked markers can only be used at population level to predict the phenotype after the association between the marker alleles and the trait-gene has been confirmed. Inheritance of the marker and that trait-gene is studied in a family and once confirmed only valid within that family. Moreover, the association may change in subsequent generation through recombination events. Commonly mutations or microsatellite markers are used. Microsatellite markers usually contain five to 20 copies of a short sequence motif that is between 2 bp and 4 bp in length and repeated in tandem. The number of repeats varies between individuals resulting in a large number of alleles for a given locus.

Direct markers or causal mutations in contrast are more useful because once the functional polymorphism is known it is possible to predict the effect of particular alleles in all animals in a population. The mutations are either insertions or deletions of DNA sequences or changes in nucleotide sequence (single nucleotide polymorphism: SNP). These alterations in sequence may either have no effect on the protein coded by the gene or result in a change of amino acid sequence and may affect functionality of the protein. However, causative mutations are hard to find and difficult to verify (DEKKERS & HOSPITAL, 2002; DEKKERS, 2004; WILLIAMS, 2005). Nevertheless, they have been proven to be especially useful to advance monogenetic traits e.g. inherited diseases (NEUENSCHWANDER, 2001). In the case of monogenetic traits the variation is directly controlled by a single gene and in the simplest case this gene will only have two alleles. Although most traits that are important in livestock production are generally more complex, such examples exist (e.g. coat color, muscle hypertrophy, glycogen content in skeletal muscle of pigs and ovulation rate). In general they include genetic disorders and genetic defects (DEKKERS, 2004). Multifactorial traits like e.g. growth rate and milk yield have a very large range of variation in the observed phenotype. The variation in such quantitative traits is controlled by several loci (region that harbors one or more genes affecting the trait) and each of them is responsible for a small amount of the overall variation (quantitative trait loci: QTL; ANDERSSON, 2001; WILLIAMS, 2005). Examples for loci already used as gene test in commercial breeding are given in Table 1. To identify marker or loci that underlie a phenotypic variation, two approaches or a combination of those are generally used:

- association tests using candidate genes
- genome scans based on linkage mapping with anonymous DNA markers

The candidate gene approach has the advantage that even loci with small effects can be detected. Nevertheless, this strategy will only be successful provided that the candidate represents a true causative gene, which is hard to find. Moreover, certain a priori knowledge about the function of genes in order to select a candidate, which is going to be tested, is required. A genome scan will always find the map location of a trait locus with major effects; provided that an appropriate genetic model has been used, a reasonable sample size is available and that the marker set provides full genome coverage. However, genome scans can only identify regions of chromosomes that affect the trait. Usually an area of 10-20 cM is defined but the exact position and number of QTL in the region is unknown (ANDERSSON, 2001; DEKKERS & HOSPITAL, 2002; GELDERMANN, 2005).

Table 1: Examples of gene tests used in commercial breeding for different species (D = dairy cattle, B = beef cattle, C = poultry, P = pigs, S = sheep) by trait category and type of marker (modified from DEKKERS, 2004)

Trait category	Direct marker	Linked marker	
Congenital defects	BLAD(D)		SHUSTER *et al.* (1992)
	Citrulinaenemia (D,B)		DENNIS *et al.* (1989)
	DUMPS (D)		SCHWENGER *et al.* (1993)
	Mannosidosis (D,B)		BERG *et al.* (1997)
	RYR (P^a)	RYR (P^b)	[a]FUJII *et al.* (1991), [b]HANSET *et al.* (1995)
Milk quality	κ-casein (D)		MEDRAN & AQUILAR-COROVA (1990), RINCON & MEDRANO (2003)
	β-lactoglobulin (D)		
	FMO3 (D)		LUNDEN *et al.* (2002)
Meat quality	RYR (P^c)		[c]FUJII *et al.* (1991),
	RN/PRKAG3 (P^e)	RN/PRKAG3 (P^f)	[e]MILAN *et al.* (2000), [f]CIOBANU *et al.* (2001)
		A-FABP/FAB4 (P)	GERBENS *et al.* (1998)
		H-FABP/FAB3 (P)	GERBENS *et al.* (1999)
		CAST (P^g,B^h)	[g]CIOBANU *et al.* (2004), [h]BARENDSE (2001)
		THYR (B)	BARENDSE *et al.* (2001)
		Leptin (B)	BUCHANAN *et al.* (2002)
Feed intake	MC4R (P)		KIM *et al.* (2000)
Disease	Prp (S^i)	B blood group (C^j)	[i]BELT *et al.* (1995), [j]HANSEN *et al.* (1967),
	F18 (P^k)	K88 (P^l)	[k]VOGELI *et al.* (1997), [k]MEIJERINK *et al.* (2000), [l]JØRGENSEN *et al.* (2003)
Reproduction	Booroola (S^m)	Booroola (S^n)	[m]WILSON *et al.* (2001), [n]LORD *et al.* (1998)
	Inverdale (S^o)	ESR (P^p)	[o]GALLOWAY *et al.* (2000), [p]ROTHSCHILD *et al.* (1996)
	Hanna (S^q)	PRLR (P^r)	[q]MCNATTY *et al.* (2001), [r]VINCENT *et al.* (1998)
		RBP4 (P)	ROTHSCHILD *et al.* (2000)
Groth and composition	MC4R (P^s)	CAST (P^u)	[s]KIM *et al.* (2000), [u]CIOBANU *et al.* (2004)
	IGF-2 (P^t)	IGF-2 (P^u)	[t]GEORGES *et al.* (2003), [u]JEON *et al.* (1999), [u]NEZER *et al.* (1999)
	Myostatin (B)		GROBET *et al.* (1998)
	Callipyge (S)		FREKING *et al.* (2002)
Milk yield and composition	DGAT (S^v)	PRL (D^w)	[v]GRISART *et al.* (2002), [w]COWAN *et al.* (1990)
	GRH (D)		BLOTT *et al.* (2003)
	κ-casein (D)		MEDRANO & AQUILAR-COROVA (1990), RINCON & MEDRANO (2003)

2.1.1.2 Limitations of structural genomics

On the one hand, structural genomics provide information about molecular functions, such as what a protein binds to or reacts with (BRENNER, 2001). Moreover, it can help to improve the understanding of the genetic background of trait characteristics and variation. A marker assisted selection can thus contribute to the genetic improvement of the phenotype. On the other hand, this information does not provide information about what all the genes do, how cells work, how organisms form or how they react in disease states (LOCKHART & WINZELER, 2000). Nor do they account for environmental influences when we look at trait characteristics in the organism (SCHWERIN, 2003). In addition, gene-gene interactions are not detected in structural analysis although they play an important role in trait characteristics. Thus, the realization that the genome sequence fails to explain the fundamental nature of many biological processes has led to profound changes in this field of genomic research (WHITFILED, 2007). Not only

mapping and sequencing but also the investigation of genome function became a major objective (HIETER & BOGUSKI, 1997).

2.1.2 Functional genomics

Instead of focusing on DNA and sequence, functional genomics research concentrates on RNA, proteins and metabolites (see Figure 1) in order to extend the knowledge about what these molecules do and how they react in the organism (HIETER & BOGUSKI, 1997; HOCQUETTE, 2005). By analyzing RNA, proteins or metabolites insight into which genes are “in use” in certain tissues or in a concrete situation can be gained. This might help to enlarge the knowledge about disease development or trait performance which is determined by genetic and environmental factors (SCHWERIN, 2003). Moreover, functional genomics enlarge the dimension of biological investigation from studying single genes, transcripts, proteins or metabolites to studying all genes, transcripts (transcriptomics), proteins (proteomics) or metabolites (metabolomics) simultaneously. It aims to integrate knowledge from all levels of scientific areas instead of studying them by independent approaches (HIENDLEDER *et al.*, 2005; see Figure 2).

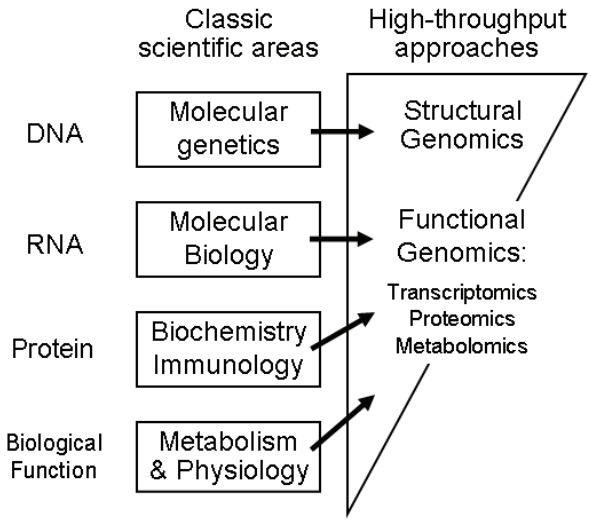

Figure 2: Levels of gene expression and their approaches of genome analysis (modified from HOCQUETTE *et al.* (2005)

With the completion of e.g. the bovine genome sequence and an eased availability of high-throughput technologies, these developments could also be advantageous for agricultural research. A simultaneous measurement of changes in expression of thousands of genes in relation to environmental and physiological challenges can now be done. By correlating genes and pathways with metabolic changes, functional genomics

has the potential to identify new candidate genes to improve genetic selection programs and to improve performance and well-being of the animal (PÉREZ LASPUIR & FERRIS, 2005; HOCQUETTE *et al.*, 2007).

2.1.2.1 Expression profiling – Characterizing the influence of genetic and environmental factors on trait expression

Gene expression can be analyzed at RNA or protein level. In most cases protein production is the ultimate output and RNA is an intermediate on the way to the functional protein. Nevertheless, RNA based approaches are commonly used because measuring RNA levels is generally less difficult, more sensitive and has a higher through-put than protein-based approaches. Moreover, mRNA levels are immensely informative about cell state and activity of genes. For most genes, changes in mRNA abundance are related to changes in protein abundance (LOCKHART & WINZELER, 2000). Several tools for studying gene expression at the level of transcription are available and can be divided into three major groups (KOZIAN & KIRSCHBAUM, 1999):

- Hybridization-based techniques (e.g. northern blotting, DNA arrays),
- PCR-based techniques (e.g. reverse transcription PCR (RT-PCR), differential display (DD, DDRT-PCR)
- Sequence based techniques (e.g. Serial analysis of gene expression (SAGE), DNA sequencing chip)

Measuring the expression of genes (transcription level) can help gaining information about cells´ activity under certain conditions and it is widely used in different fields of human but also of agricultural research. In general, two different questions are asked when investigating expression levels. Either the aim is to measure the expression level of a particular gene in different samples (individuals). Or information about which genes (gene families) are differentially expressed under different conditions (genetic or environmental) such as healthy *versus* diseased states and high productive *versus* low productive tissues is needed (NEUENSCHWANDER, 2001; HOCQUETTE *et al.*, 2005). With the availability of high-throughput technologies like DNA arrays, the second question has become more and more important. Examples of some expression studies in cattle including array technology are given in Table 2. Nowadays for several farm animals commercial DNA arrays are available (e.g. pig, chicken, cattle) though custom made arrays are still used especially in cases when tissue specific or specialized arrays are needed.

Table 2: Examples of expression studies in cattle (modified from HOCQUETTE *et al.*, 2005 and DAWSON, 2006)

Technical approach	Topic	Literature
Microarray	Differential gene expression associated with pathogenic microorganisms, disease and immune function	MOODY *et al.*, 2003; DAVIES *et al.*, 2006; KLENER *et al.*, 2006; MARSH *et al.*, 2006; PATEL *et al.*, 2006
Microarray	Differential gene expression associated with pregnancy	ISHIWATA *et al.*, 2003; HERATH *et al.*, 2004; LOOR *et al.*, 2005, 2006
Microarray	Differential gene expression associated with milk performance, muscle development and meat quality	REVERTER *et al.*, 2003, LEHNERT *et al.*, 2004; LEHNERT *et al.*, 2007
Microarray	Differential gene expression in genes that are important in follicular development and embryo development	USHIWAZA *et al.*, 2004; CORCORAN *et al.*, 2006; KLEIN *et al.*, 2006; ZIALEK *et al.*, 2007
Transcriptomics, Proteomics	Differential expression between different tissues and organs	CHO *et al.*, 2002; SUCHYTA *et al.*, 2003; TALAMO *et al.*, 2003
Differential Display	Differential expression between different genetic types	DORROCH, 2001

The major outcome of these studies is the identification of genes or physiological pathways which may be of importance in biological functions, since their expression differs depending either on genetic or environmental factors (HOCQUETTE, 2005). This can be seen as the main advantage of functional genomics (i.e. gene expression studies in contrast to structural genomics). Results account for environmental effects on the phenotype whereas in investigating only genetic factors (genotype), this aspect is left aside. Especially for quantitative traits it is essential that genetic as well as environmental factors are influencing the phenotype and the agronomical trait, respectively. In short, the individual's phenotype is the sum of effects of the genotypic and the environmental effects (phenotype = genotype + environmental effects; see also Figure 3). Thus, gene expression studies can provide information about both genotypic and environmental influence on a phenotype and they are nowadays more and more used in livestock research.

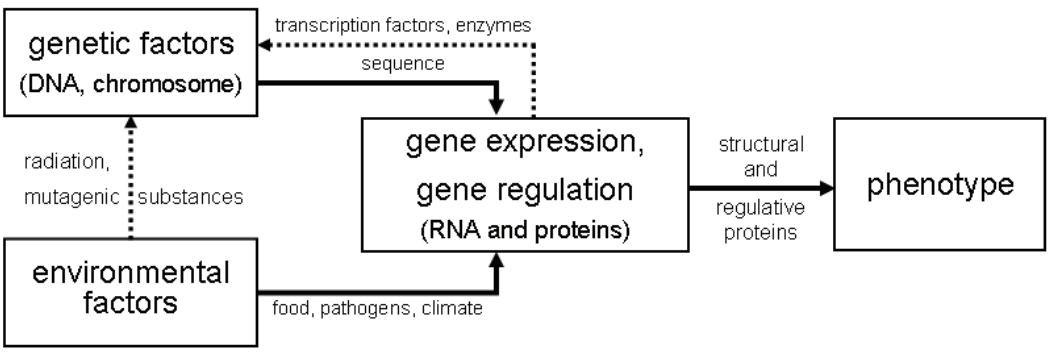

Figure 3: Influence of genetic and environmental factors on gene expression and phenotype (modified from NEUSCHWANDER, 2001)

Genetic factors

Genetic factors are especially interesting for animal breeding. Thus, the knowledge about expression differences of certain genes in samples, which differ in agricultural interesting traits (e.g. breeds with high milk yield and low milk yield), can help to identify associations between the trait and the genetic variants which occur in the population. BASS *et al.* (1999) showed for example the association of myostatin with the number of muscle fibers in cattle by using gene expression analysis. Modern techniques like microarray analysis, which allow the simultaneous measurement of the expression level of thousands of genes and whole genomes, respectively enable the scientist to identify new molecules and resulted in a wide spread application of this approach in livestock but also in other fields of research. BERNARD *et al.* (2007) were able to identify the gene DNAJA1 using microarray technology which explains up to 63 % of the variability in meat tenderness in charolais cattle. PONSUKSILI *et al.* (2005) discovered several possible candidate genes for body composition by expression analyses in pigs. WAYNE and MCINTYRE (2002) proposed to combine global gene expression analysis with QTL mapping to identify candidate genes that are associated with the trait of interest. RON *et al.* (2007) applied this approach to their study and combined a mouse mammary gland gene expression analysis and comparative mapping for identifying candidate genes in QTL of milk production traits in cattle. In addition, large-scale expression profiling enables the identification not only of single candidate genes but of whole trait associated biological pathways which alter among different samples. In that way a better understanding of the molecular mechanisms, underlying the performance or phenotypic trait characteristics, could be gained. PONSUKSILI *et al.* (2007) found an overrepresentation of differentially expressed genes in lipid metabolism, protein synthesis, cell growth and cell proliferation when comparing two commercially used pig breeds (German Landrace *versus* Pietrain).

Environmental factors

Additionally, gene expression studies allow the identification of genes which are affected by different “environmental” conditions (e.g. diets, temperature and infection). Thus, physiological effects on the organism can be estimated by relating differentially expressed genes to their known function or their involvement in physiological pathways. SCHWERIN *et al.* (2003) for example observed significant changes in the transcription level of genes involved in the metabolism of stress response in pigs, chronically fed a protein-restricted diet (based on soy protein isolate) in comparison to pigs fed a casein-

based diet. In addition, the enlightenment of molecular mechanisms underlying the organisms' reactions towards certain environmental challenges, as for instance infection, can be studied. WELLNITZ & KERR (2004) proved the involvement of TNF-α, lactoferrin and serum amyloid A in the inflammatory response towards Staphylococcus aureus infection in bovine mammary gland epithelial cells. It may even be possible to derive practical recommendations for livestock production. BYRNE *et al.* (2005) showed the involvement of genes that are associated with protein turnover, cytoskeletal remodeling and metabolic homeostasis due to nutritional restriction in steers. LOOR *et al.* (2006) proposes for example a controlled energy intake for cows during the periparturient period. Based on findings from a microarray study and real-time RT-PCR results they found this could confer an advantage to the animal by activating hepatic molecular adaptations well ahead parturition. These last two examples emphasize the importance of gene expression studies in relation to nutrition, which have become more and more important also in human research and which have led to the development of the research field of nutrigenomics.

2.1.2.2 Interaction of nutrients and gene expression – nutritional genomics

Nutritional genomics deals with the investigation of the interaction of genomic and dietary factors and how these interactions influence for instance health (BROWN & OUDERAA, 2007). In the strict sense, nutritional genomics includes two main research fields: nutrigenomics and nutrigenetics. The science of "nutrigenomics" implies the study of how molecular expression, metabolism and ultimately health in individuals are altered due to ingested nutrients or other food components (FOGG-JOHNSON & KAPUT, 2003; GERMAN, 2005). The term has to be distinguished from "nutrigenetics" which comprises how genetic variation affects the interaction between dietary components and the health and disease potential of the individual (DEBUSK *et al.*, 2005). The general term nutritional gen-"omics" also implies the integration of high-through-put techniques in nutritional studies. Diet-gene specific interactions have been recognized and investigated for a long time. But modern profiling technologies (e.g. transcriptomics) bring a new perspective to this approach because dietary effects on thousand of genes can be studied simultaneously (ROBERTS *et al.*, 2001). These "omics" technologies applied in the context of nutrition and health have the potential to deliver biomarkers that respond to a certain nutrient, treatment or diet or to discover bioactive food components as well as to assess the safety and functionality of new foods and food ingredients (DANIEL, 2002; KUSSMANN *et al.*, 2006).

This seems especially applicable because it is known that dietary components can affect gene expression by acting as signals which are monitored by the nutrient sensors in the biological system. This activation can either be a direct interaction of the nutrient with transcription factors or can happen indirectly by regulating the secretion or intracellular action of one or more hormones (for example insulin, glucocorticoids, thyroid hormone) which themselves alter gene expression (DE CATERINA & MADONNA, 2004). Nutrients like carbohydrates, lipids, proteins or calcium are directly involved in hormone secretion and action. Glucose can for instance increase lipogenesis and the expression of lipogenic genes by stimulating the release of insulin from pancreas (KERSTEN, 2001). However, in addition to hormone-mediated alterations of the cellular metabolism these nutrients are able to interact with nuclear receptors and response elements. Several molecular studies have demonstrated that dietary chemicals or their metabolites interact in that way and can bind directly to nuclear receptors (Table 3 and FOGG-JOHNSON & KAPUT, 2003).

Table 3: Transcription factor pathway mediating nutrient-gene interactions (MÜLLER & KERSTEN, 2003)

Nutrient	Compound	Transcription factor
Macronutrients		
Fats	Fatty acids Cholesterol	PPARs, SREBPs, LXR, HNF4, ChREBP SREBPs, LXRs, FXR
Carbohydrates	Glucose	USFs, SREBPs, ChREBP
Proteins	Amino acids	C/EBPs
Micronutrients		
Vitamins	Vitamin A Vitamin D Vitamin E	RAR, RXR VDR PXR
Minerals	Calcium Iron Zinc	Calcineurin/NF-ATs IRP1, IRP2 MTF1
Other food components		
	Flavonoids	ER, NFkB, AP1
	Xenobiotics	CAR, PXR

AP1, activating protein1; CAR, constitutively active receptor; C/EBP, CAAT/enhancer binding protein; ChREBP, carbohydrate responsive element binding protein; ER, oestrogen receptor; FXR, farnesoid X receptor; HNF, hepatocyte nuclear factor; IRP, iron regulatory protein; LXR, liver X receptor; MTF1, metal-responsive transcription factors; NFkB, nuclear factor kB; NF-AT, nuclear factor of activated t cells; PPAR, peroxisome proliferators-activated receptor; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; SREBP, sterol-responsive-element binding protein; USF, upstream stimulatory factor; VDR, vitamin D receptor.

In that way gene expression can reflect for example metabolic stress, which describes changes in the plasma and/or cellular concentration of nutrients and metabolites, which might lead to the disturbance of cellular functions (MÜLLER & KERSTEN, 2003). Amino acids for instance are known to control gene expression of key enzymes involved in their metabolism. CHOP (CCAAT/enhancer-binding homologous protein) encodes for a

ubiquitous transcription factor that accounts for cellular response to dietary deprivation of several amino acids (BRUHAT *et al.*, 1999; DE CATERINA & MADONNA, 2004). Another gene that shows transcriptional increase in response to amino acid deficit is the insuline-like growth factor-binding protein-1 (IGFBP-1; DE CATERINA & MADONNA, 2004). Fatty acids regulate the activity of several transcription factors including PPAR, LXR or HNF-4 by binding directly to the specific factor (JUMP, 2004). PPARα is mostly expressed in liver and is up-regulated during food deprivation. It is involved in cellular proliferation, fatty-acid oxidation but also in acute-phase response (MÜLLER & KERSTEN, 2003). Endogenous or exogenous toxic substances are sensed amongst others by the xenobiotic receptors CAR and PXR which then mediate regulation of xenobiotic metabolism mainly through cytochrome P450 enzymes (FRANCIS *et al.*, 2003; TIMSIT & NEGISHI, 2007). Against the background of these examples, gene expression patterns could be also regarded as “dietary signatures” which may be used for elucidating the effects of certain diets or nutrients on physiology and health (DANIEL, 2002). These interactions between nutrients and health of an organism are of common interest. Nutritional deficiencies or disorders such as diabetes, cardiovascular diseases or obesity are known to be influenced by diet. Consequently determining the composition of foods and understanding how food components can modulate health, may contribute to the successful management of diet-related diseases (WHITFIELD, 2007). The awareness about this link between nutrition and health has stimulated increasing interest in nutrition-based health management and especially in functional foods. These foods are developed in order to improve the nutritional characteristics and to affect health positively, beyond the traditional nutritional value (BROWN & VAN DER OUDERAA, 2007). Among those, genetically modified plants with improved nutritional characteristics are also to be found.

2.2 Genetically modified plants in animal feeding

Genetically modified plants have been altered in their genetic composition by means of genetic engineering. The modification involves the insertion of a specific gene, generally with desired characteristics, into the genome of the plant to be modified. The typical insert (gene construct) is composed of a promoter element (on/off switch for gene expression), the gene sequence which codes for the specific selected feature and the terminator element which functions as stop signal (MARZOK, 2004). In addition, a marker gene is generally attached which usually confers resistance to an antibiotic or herbicide and allows only cells, that have integrated and expressed the foreign

sequences, to survive during plant regeneration in culture (ROSELLINI *et al.*, 2007). Such plant transformation methods have opened up new possibilities for agricultural research in terms of optimization of plant production and the development of new crops with improved constituents.

2.2.1 First and second generation crops

The worldwide cultivation of genetically modified crops (e.g. soybean, maize, rapeseed or cotton) increased distinctively from 1.7 (1996) to 90 million ha/year (2005) in the recent years (GÓMEZ-BARBERO & RODRÍGUEZ-CEREZO, 2007; FLACHOWSKY 2007). Most of these crops belong to the so-called **"first generation"** plants which are characterized by the modification of agronomically important input traits such as tolerance against pesticides, herbicides or insects (some examples are given in Table 4). In general, these plants do not have any intended changes in nutrient composition or nutritional quality. A well known example of this group is insect resistant Bt-maize which has been developed by genetic modification to resist European borer infection (TONY *et al.*, 2003).

The **"second generation"** genetically modified plants include the functional/novel foods which are defined as foods and food ingredients that have so far not been used for human consumption to a significant degree within the European Community (European Regulation of Novel Foods and Novel food ingredients, No. 258/97). They are characterized by the modification of certain output traits meaning that they have nutritional or health benefits including improved nutritional quality or nutrient availability and/or reduced amount of toxic, allergenic and antinutritive compounds, respectively (examples are given in Table 5; PERSLEY, 2003; ILSI, 2004; FLACHOWSKY, 2005). The so-called "golden rice" is an example of this group. It produces ß-carotene (provitamin A). With the development of this rice line one intended to alleviate vitamin A deficiency in developing countries (AL-BABILI & BEYER, 2005). The main objects of genetic modification of plants with respect to food and feed production are summarized by MATISSEK (1998) as follows:

A) Direct increase in yield by creating high productive plant varieties

B) Enhancement of productivity by elimination or reduction of disturbing biotic and abiotic influences and limitations (pest and disease control)

C) Improvement of post harvest management and storage features

D) Improvement of food and feed qualities by improving nutritive value or eliminating unfavorable ingredients and increasing health benefit substances

Table 4: Examples of "first generation" GM crops with improved agronomically input traits (tolerances; modified from CLARK & LEHMAN, 2001)

Type of tolerance	Transgene	Comments
Herbicide	Glyphosate	Non-selective, broad spectrum herbicide; inhibits EPSPS enzyme of the shikimic acid pathway that produces phenylalanine, tyrosine and tryptophane; e.g. Roundup
	Bromoxynil	Post-emergence herbicide for dicot weeds; acts as potent inhibitor of electron transport at the photosystem II site; uncouples oxidative and PS phosphorylation; e.g. BXN cotton
Insect tolerance	Bt	Highly selective, naturally occurring insecticide, although selectivity appears to be lost when introduced transgenically; crystals formed during sporulation of *Bacillus thuringiensis*, a soil microbe, contain proteins (endotoxins) that become toxic when ingested by particular classes of insects
	Lectin proteins	Carbohydrate-binding plant proteins, similar to protease and amylase inhibitors; different plant families produce different lectins; e.g. the snowdrop lectin (GNA) of EWEN & PUSZTAI (1999) protects transgenic tobacco, potatoes and lettuce against sap-sucking insects, as peach potato aphid (*Myzus persicae*)
Virus tolerance	Coat proteins, satellite RNAs, replicase, antisense, defective interfering, Cis-acting elements, movement proteins, ribozymes	
Fungal tolerance	Phytoalexins, ribosome inactivation proteins, chitinases and glucanases	
Bacterial tolerance	Lysozymes, lytic peptides, toxines, H_2O_2	
Stress tolerance	- Drought tolerance (e.g. elevated concentrations of praline or fructans to enhance osmolality) in tobacco - Oxidatative stress tolerance, via anti-oxidant enzyme over-expression, conferring drought or chilling tolerance in tobacco, alfalfa and cotton - Cold tolerance in tomato and tobacco - Salinity tolerance in tobacco	

Group D comprises the functional foods discussed above. Promising functional foods belonging to this group are plant-based or edible vaccines. During the past two decades numerous species of plants have been genetically modified to produce vaccines (FLOSS *et al.*, 2007). On the one hand, this could be an interesting approach for the treatment and prevention of diseases (CARTER *et al.*, 2002) and a milestone on the road to creating inexpensive and easily deliverable vaccines (SWAMY KRISHNA TRIPURANI *et al.*, 2003; WARZECHA & MASON, 2003). CASTANON *et al.* (1999) did successfully insert the major structural protein VP60 of rabbit hemorrhagic disease virus (RHDV) in potatoes. Rabbits immunized with leaf extracts from potatoes expressing the VP60 were fully protected against RHDV. Further examples of plant-based vaccines are reviewed by STREATFIELD & HOWARD (2003). On the other hand, the genetic modification ultimately results in the introduction of new proteins into food or feed plants and therefore the safety of the newly introduced protein as well as potential unintended effects caused by the process of transgene insertion has to be assessed (TAYLOR & HEFLE, 2001; CELLINI *et al.*, 2004).

Table 5: Examples of "second generation" GM crops with nutritionally improved traits intended to provide health benefits to consumers and domestic animals (modified from ILSI, 2004; review)

Crop/Species	Trait	Transgene
Alfalfa	+ Phytase	Phytase (Aspergillus)
	+ Resveratrol	Resveratrol glucoside
	Lignin ↓	Down-regulation of caffeic acid 3-O-methytransferase and caffeoyl CoA 3-O methyltransferase
Lupin	Methionine ↑	Seed albumin (sunflower)
Maize	Methionine ↑	mRNA stability by intron switching Dzr1 target
	Protein with favorable amino acid profile	α-Lactoalbumin (porcine)
	Vitamin C ↑	Wheat dehydroascorbate reductase (DHAR)
Potato	Inulin molecules ↑	1-SST (sucrose:sucrose 1-fructosyltransferase) and the 1-FFT (fructan:fructan 1-fructosyltransferase) genes of globe artichoke (Cynara scolymus)
	+ Sulphur-rich protein	Non-allergenic seed albumin gene (Amaranthus hypochondriacus)
	Solanin ↓	Antisense sterol glycol transferase (Sgt) gene
Sorghum	Improved digestibility of livestock feed	Mutated Brown midrib (Bmr) reduced activity of caffeic acid O-methyltransferase (COMT), a lignin-producing enzyme
Soybeans	Improved amino acid composition	Synthetic proteins
	Immunodominant Allergen ↓	Gene silencing of cystein protease P34 (34kDa)
Sweet potato	Protein content ↑	Artificial storage protein (ASP-1) gene

↓ = reduced content of the specific substance

↑ = elevated content of the specific substance

+ = additionally producing the specific substance

2.2.2 Effect of genetic modification and conventional breeding on nutrient composition in plants

Although traditional plant breeding results in changes of the heritable traits of organisms, the great variety of new crops does not differ significantly from older varieties regarding composition. Nevertheless, in few cases unintended effects did occur in traditional breeding (ILSI, 2004, see Table 6). Modern biotechnology in turn offers more accurate and efficient methods of improving certain traits in the plant (ILSI, 2004; TAYLOR, 1997). Thus, the occurrence of unintended effects should be considered particularly.

Unintended effects are defined as "consistent" differences between the genetically modified plant and its nearest isogenic counterpart (control line) which exceed normal expected variance between lines and the primary expected effects of the introduced target gene (EFSA, 2008). These differences include the formation of new metabolites or altered levels of existing metabolites which can include nutritional, anti-nutritional as well as toxic or allergenic factors (KUIPER *et al.*, 2001; PERSLEY, 2003; CELLINI *et al.*, 2004; EFSA, 2008). Such effects may occur due to genetic rearrangements, gene disruption or sequence changes as a consequence of the recombination event because DNA integration is random (RISCHER & OKSMAN-CALDENTEY, 2006; ILSI, 2007). In

addition, somaclonal variation – a reprogramming during plant regeneration – or pleiotropic effects of the newly introduced gene might cause such interactions (MOHAN JAIN 2001; SHEPARD, 2006).

Table 6: Unintended effects in traditional breeding (modified from ILSI, 2004)

Host plant/trait	Unintended effect	Reference
Barley/Powdery mildew resistance	Low yield	THOMAS *et al.* (1998)
Celery/Pest resistance	High furanocoumarins content	BEIER (1990)
Maize/High lysine content	Low yield	MERTZ (1992)
Potato/Pest resistance	Low yield, high glycoalkaloid content	HARVEY *et al.* (1985)
Squash, Zucchini/Pest resistance	High cucurbitacin content	COULSTON & KOLBYE (1990)

A well-known case of unintended effects is the newly introduced allergenic potential of methionine-rich soybeans (Glycine max) in which the 2S albumin gene from the Brazil-nut was inserted and in that way identified as the major allergen of Brazil-nuts (NORDLEE *et al.*, 1996). In that case the allergenic properties of the Brazil-nut were transferred unintentionally to the soybean. Nevertheless, the Brazil-nut was a known allergenic food but in other cases the allergenic potential of plants or certain genes might not be known before (due to gene silencing) and could appear due to genetic modification.

Moreover, the use of marker genes for selection of cells with successful construct-insertion, which often code for resistance to antibiotics, are a common concern. However, according to FAO/WHO (1996) the transfer of antibiotic resistance genes from plants to micro-organisms in the human gastro-intestinal tract is unlikely to occur. In addition, FUCHS *et al.* (1993) proved that the ingestion of genetically engineered plants which are producing the NPTII protein (coding for resistance against several antibiotics) poses no safety concerns.

“Substantial equivalence” is a commonly used concept to identify unintended effects in genetically modified foods or feeds. The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) of the United Nations recommend this concept as the most practical approach to address the safety evaluation of foods, feeds or food components derived from modern biotechnology (FAO/WHO, 1991, 1996, 2000). It is based on the idea that existing foods with a long history of safe use can serve as basis for comparing the properties of a GM food with an appropriate counterpart (KUIPER *et al.*, 2001). The application of this concept is not a safety assessment per se but it helps to identify differences between conventional and GM crops which could then be used to

decide about further safety assessment. The key element in the concept of substantial equivalence is a compositional analysis of main nutritional components (ILSI, 2004).

Table 7: Unintended effects in genetic engineering breeding (modified from CELLINI *et al.*, 2004)

Host plant	Trait	Unintended effect	Reference
Canola	Over-expression of phytoene-synthase	Multiple metabolic changes (tocopherol, chlorophyll, fatty acids, phytoene)	SHEWMAKER *et al.* (1999)
Potato	Expression of yeast invertase	Reduced glycoalkaloid content (-37 to -48 %)	ENGEL *et al.* (1998)
Potato	Expression of soybean glycinin	Increased glycoalkaloid content (+16 to 88 %)	HASHIMOTO *et al.* (1999)
Rice	Expression of soybean glycinin	Impaired carbohydrate transport in the phloem	DUECK *et al.* (1998)
		Increased vitamin B_6 content (+50 %)	MOMMA *et al.* (1999)
Rice	Expression of provitamin A biosynthetic pathway	Formation of unexpected carotenoid derivatives (ß-carotene, lutein, zeaxanthin)	YE *et al.* (2000)
Wheat	Expression of glucose oxidase	Phytotoxicity	MURRAY *et al.* (1999)
Wheat	Expression of phosphatidyl serine synthase	Necrotic lesions	DELHAIZE *et al.* (1999)

Although many surveys have proven substantial equivalence between GM foods and their conventional parental or near isogenic lines (reviewed by FLACHOWSKY, 2005) in some studies unintended side effects due to genetic engineering breeding (see Table 7) were observed. By expressing glucose oxidase in wheat MURRAY *et al.* (1999) found reduced fungal infection as expected but the plant exhibited also phytotoxicity. HASHIMOTO *et al.* (1999) observed increased glycoalkaloid contents in transgenic potatoes due to insertion of soybean glycinin. In general, the concept of substantial equivalence works well for evaluating “first generation” crops. However, the application for “second generation” crops with an intended modification of one or more nutritional characteristics is difficult. For these plants investigating nutritional equivalence in animal feeding trials may be a useful alternative or additional part of safety considerations (AUMAITRE *et al.*, 2002; FLACHOWSKY, 2005).

2.2.3 Dietary and physiological effects of genetically modified plants investigated by measuring conventional traits in feeding trials

According to FAO/WHO (2000), animal testing may be considered necessary if the characterization of the GM food indicates that available data are insufficient for a thorough safety assessment. As mentioned above, this might particularly be the case for “second generation” crops. In addition, compositional analyses provide the basis for the safety assessment of new crops but they should be considered only as part of the overall

nutritional assessment as they give limited information on the bioavailability of nutrients (ILSI, 2004).

Nevertheless, the key element of investigating nutritional equivalence in feeding studies is consistent with the concept of substantial equivalence. The condition of animals fed with GM crops needs to be compared to animals fed with a food/feed derived from a suitable comparator. This in turn might be difficult to obtain regarding foods/feeds with intentionally changed nutritional composition (ILSI, 2004).

Within the recent years many feeding trials with GM ingredients have been carried out using conventionally measured traits (e.g. digestibility of nutrients, weight gain, general health and performance characteristics and blood chemistry). These studies were conducted with several species like mice, rats, rabbits, cattle, broilers, pigs etc. Most of them did not exhibit health risks for the ingesting organism. Detailed overviews are given by FLACHOWSKY (2005), FLACHOWSKY *et al.* (2007) and EFSA (2008). FLACHOWSKY *et al.* (2007) conclude that in general, genetically modified plants without substantial changes in their composition do not significantly differ in their nutritional value from those of their nearest isogenic varieties and do not affect health of the organism that ingested the plant. Including very recent studies most surveys prove that the tested GM crops are as safe and nutritious as their non-GM counterparts (some examples are given in Table 8) though it has to be taken into account that the parameters which were investigated vary between the different studies.

However, few findings report on physiological effects of GM plants when ingested by animals in feeding trials or in cell culture experiments. FENTON *et al.* (1999) argued for greater care before incorporating plant lectins into GM foods because they found that snowdrop lectin binds to human white blood cells. A very controversial study of EWEN & PUSZTAI (1999) revealed trophic effects of GM potatoes expressing a snowdrop lectin on rats' intestines. PRESCOTT *et al.* (2005) observed an altered immunogenicity of GM peas in mice. MALATESTA *et al.* (2002) found an influence of GM soybean on zymogen synthesis in pancreatic acinar cells of mice. SÉRALINI *et al.* (2007) detected signs of hepatorenal toxicity when re-analyzing a data from a feeding study with GM maize in rats. They concluded that longer experiments were essential in order to indicate the real nature and extent of the possible pathology and that from the specific study the GM corn could not be regarded as a safe product. Results from SAGSTADT *et al.* (2007) suggested that a specific GM maize line might induce significant changes in white blood cell populations in fish which are associated with an immune response.

Table 8: Examples of feeding studies with GM plants investigating conventionally measured parameters

GM plant	Investigated parameters	Animal	Result	Authors
Glyphosate tolerant soybeans	**Plant**: Crude nutrient & antinutrient composition **Animal**: Growth, feed conversion (R, C, B), fillet composition (C), milk production & composition (D)	Rat (R), Broiler (B), Catfish (C), Dairy cows (D)	≈	HAMMOND *et al.* (1996)
Glyphosate tolerant soybeans	**Animal**: Growth, food intake, organ weights of liver and spleen, histopathology of several organs, IgE & EgG antibodies in sera	Rat	≈	TESHIMA *et al.* (2000)
Glyphosate tolerant maize	**Plant**: Crude nutrients, amino acid & fatty acid profile, sugar, starch **Animal**: N-digestibility, PER, NPU	Rat	≈	CHRENKOVA *et al.* (2002)
Glyphosate tolerant soybeans	**Plant**: Crude nutrients, amino acids, minerals **Animal**: Growth performance, carcass and organ yields	Broiler	≈	MCNAUGHTON *et al.* (2007)
Myristic acid-rich rapeseed	**Plant**: Crude nutrients, amino acid, minerals Fatty acid profiles, Glucosinolate contents **Animal**: Digestibility, feeding value, feed intake, weight gain Back & intramuscular fat composition	Pig	≈ fatty acid profiles changed ↑ of glucosinolate contents ↓ of feed intake & weight gain	BÖHME *et al.* (2007)
Glufosinate tolerant sugar beets & maize	**Plant**: Crude nutrients, amino acid & fatty acid profile, NDF-fraction **Animal**: digestibility, feeding value	Pig, ruminant	≈	BÖHME *et al.* (2001)
Glufosinatet olerant maize	**Plant**: Nutritional profile of diets, **Animal**: weight gain, feed intake/efficiency, hematology, clinical chemistry, coagulation urinalysis, organ weight and gross and microscopic pathology	Rat	≈	MALLEY *et al.* (2007)
Bt maize	**Plant**: Crude nutrients, sugar, starch, non-starch polysaccharides, minerals, amino acid & fatty acid profile **Animal**: feeding value	Pig	≈	REUTER *et al.* (2002)
Bt maize	**Plant**: Crude nutrients, amino acids, anti-nutrients, secondary metabolites **Animal**: nutritional performance variables, hematology, clinical chemistry, coagulation & urinalysis, organ weights, gross & microscopic appearance of tissues	Rat	≈	MACKENZIE *et al.* (2007)
Bt maize (grain & silage)	**Plant**: Crude nutrients, ADF, NDF, minerals, lignin, starch **Animal**: body weight, health parameters, milk production and composition, hematology, clinical chemistry	Dairy cows	≈	FAUST *et al.* (2007)
Bt maize	**Animal**: Health performance, body weight, feed consumption, hematology, blood chemistry, urinalysis, organ weights, gross & microscopic appearance of tissues	Rat	≈	HAMMOND *et al.* (2006)
Bt maize	**Plant**: Crude nutrients, starch, sugar, minerals, glycoalkaloids, protease inhibitor activity **Animal**: feed intake, body weight, feed efficiency, organ weights, blood chemistry	Rat	≈	EL SANHOTY *et al.* (2004)

PER = protein efficiency ratio, NPU=net protein utilization; ADF = acid detergent fibre; NDF = neutral detergent fibre;
↓ = reduction of specific content or parameter; ↑ = increase of specific content or parameter; ≈ = no changes observed

Although the observed changes might not be meaningful in terms of health concerns these results demonstrate the need for the use of sensitive methods or tools to ensure the safety. Moreover such outcomes have given rise to discussions about the adequacy of the currently used methods (KÖNIG *et al.*, 2004; LIU-STRATTON *et al.*, 2004) which usually belong to the target-based approaches.

2.2.4 The use of target and non-target approaches in the physiological characterization of genetically modified plants and animals ingesting these feeds

When characterizing GM foods or physiological effects caused by their ingestion, target- and non-target-based approaches can be followed. **Targeted approaches** are hypothesis driven and focus on gaining information about specific known macro- and micronutrients, toxic allergenic or bioactive compounds present in the GM crop. The spectrum of compounds is determined by information gained from analyses of conventionally bred crops (ILSI, 2004). However, these approaches have severe limitations with respect to unknown anti-nutrients and natural toxins especially in less well known crops (KUIPER *et al.*, 2001). To increase the chances of detecting unintended effects due to the genetic modification of plants, profiling technologies (**non-target approaches**) such as transcriptomics, proteomics and metabolomics have the potential to provide useful additional information and to broaden the extensiveness of comparative analyses. Profiling methods provide an open-ended broad view of the complex cellular metabolism of the organism. These non-targeted approaches may be of particular relevance for second-generation GM food crops e.g. which lead to enhanced nutritional profiles. They may not replace conventional analyses but could be useful to confirm and supplement other results (EFSA, 2006). LIU-STRATTON *et al.* (2004) suggested studying expression, proteome and metabolome profiles of genetically modified foods as well as of tissues from experiments with humans or animals that have ingested such foods and to compare them to the corresponding traditional food as a reference. A first application of expression profiling using microarray technology in analyzing physiological effects of GM crops was carried out successfully by NARASAKA *et al.* (2006). In their study they investigated the effect of GM wheat flour on gene expression in liver and intestine of rats and concluded that microarray technology could be applicable to the field of food safety analysis. KÖNIG *et al.* (2004) proposed the use of sets of genes as biomarkers for cells´ responses to toxins and allergens and points out an increasing sensitivity of test systems by using profiling methods.

2.3 Expression profiling methods - transcriptomics

Several methods are widely used for transcript quantification: e.g. differential display, northern blotting, in situ hybridization and reverse transcription polymerase chain reaction (RT-PCR). Apart from RT-PCR the use of these methods is limited by their low sensitivity (BUSTIN, 2000). Moreover, they focus mostly on the analysis of few molecules whereas in functional genomics transcript levels are studied in a more global way, meaning that transcription of thousands of molecules can be monitored at the same time. This has become possible since the development of new high-throughput methods (HOCQUETTE *et al.*, 2007). Commonly used and very powerful tools are the high-density oligonucleotide and cDNA arrays (microarray; LOCKHART & WINZELER, 2000).

2.3.1 Microarray analysis – a holistic profiling method

The cornerstone for this technology was led by Stephen P.A. Fodor in the late 1980s who showed that peptides may be synthesized on small silicon chips by means of photolithographic synthesis. It became rapidly clear that this method might also be used to synthesize and fix short DNA fragments on silicon or glass chips (KUNZ *et al.*, 2004). The term array refers to the regular arrangement of oligonucleotide (oligo array) or cDNA (cDNA array) representations (probes) of genes on a solid support (YE & DAY, 2003). cDNA probes are generally products of the polymerase chain reaction (PCR) generated from cDNA libraries or clone collections which are then spotted onto the glass slide or nylon membrane. For oligo arrays a different fabrication system is usually applied. These probes are synthesized in situ either by photolithography or by ink-yet technology. The advantage is an increased probe density on the array with the drawback that probe sequence length is restricted. Alternatively, pre-synthesized oligonucleotides can also be deposited onto glass slides (WATSON *et al.*, 1998; SCHULZE & DOWNWARD, 2001).
One of the first surveys using this microarray technology to study global gene expression was carried out by DERISI *et al.* (1996). Nowadays microarrays are becoming rapidly a fundamental tool in discovery-based genomic and biomedical research (WANG *et al.*, 2006).

Aspects of experimental design and workflow of microarray experiments

Before conducting a microarray experiment certain aspects have to be taken into consideration. Choosing an appropriate experimental design for the issue which is to be investigated can be challenging because it may have an impact on the results obtained.

The first decision to be made is which microarray system (**cDNA** or **Oligo** array) should be used. However, this question is mostly answered by the accessibility and cost of the array platform (scanner and other technical requirements) in the certain working field. Nevertheless, it can be mentioned that oligo arrays (35 – 80 bp) (CAUSTON *et al.*, 2003), as design for instance by the company Affymetrix, are potentially vulnerable to single base changes due to polymorphisms or sequencing errors in the original sequence used for oligonucleotide design. False mismatches may occur and result in an underestimation of gene expression (MAH *et al.*, 2004). cDNA arrays, which are usually spotted chips with probe sequences ranging from 200 – 1500 bp, are less sensitive to single base pair exchanges because of probe sequence is longer. Nevertheless, they are more prone to cross-hybridizations (i.e. gene families) and may include latent non-specific sequences (i.e. repetitive elements). Moreover, the printing process of cDNA arrays may cause variations in probe concentrations (MAH *et al.*, 2004).

A second point to be taken into consideration concerning the experimental design is the decision for **one or two color arrays**. On the one hand, two color approaches allow the direct comparison of sample pairs hybridized on the same array. In that way variability due to processing multiple microarrays per assay can be minimized and results theoretically in an increased sensitivity (BENDIXEN *et al.*, 2005; PATTERSON *et al.*, 2006). On the other hand, it has been reported that fold change values obtained from two color experiments often have a systematic dependence on intensity, the so called intensity-dependant dye bias, which is mostly observed in low intensity signals (CAUSTON *et al.*, 2003). Though certain normalization methods (e.g. Lowess: QUACKENBUSH, 2002) compensate for this (YANG *et al.*, 2002b). One color designs avoid this problem and in general are advantageous because of a greater design simplicity and flexibility. Regarding reproducibility, sensitivity and accuracy PATTERSON *et al.* (2006) reported good consistency between the data from one and two color experiments. They noted that both approaches provide similar levels of biological insight. DE REYNIÈS *et al.* (2006) in contrast found that one color based experiments have a higher level of reproducibility. Taken altogether it can be resumed that both systems have advantages and disadvantages and the decision to use either one color or two color arrays will often be determined by cost, experimental design considerations and personal preference (PATTERSON *et al.*, 2006).

The **basic steps of a two color microarray experiment** are shown in Figure 4. First RNA is extracted and purified from tissues of interest and labeled with a fluorescent dye.

One of the most popular applications is to compare the gene expression levels of two different groups e.g. different conditions. Thus, the two samples are labeled either with two different dyes (two color array) and hybridized on the same array, or the samples are hybridized on two different arrays and labeled with the same dye (one color array). After hybridization of the labeled RNA to the arrays they are excited by laser and scanned at the appropriate wavelengths (channel) of fluorescent dye. Each signal in every cDNA element on the slide provides a measurement of the specific expression level of the corresponding gene (CAUSTON *et al.*, 2003; BENDIXEN *et al.*, 2005). Signals from the two samples being investigated can then be compared.

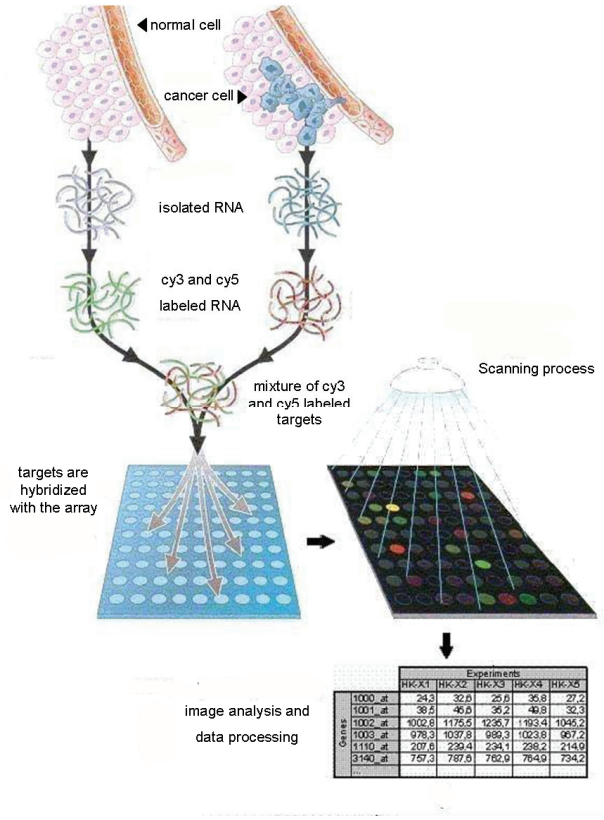

Figure 4: Workflow of a gene expression experiment using a two color platform (modified from LANGE, 2004)

Image processing and quality filtering of two color microarrays

Regardless of the product or design used in the experiment, the arrays are scanned following hybridization and independent grey scale images are generated for each experimental variable which is investigated (CAUSTON *et al.*, 2003). This is typically done by a 16-bit scanner which creates TIFF (Tagged Image File Format) images. These images contain various pixel measurements, which are averaged, for each spot (gene) on the array and have an output range of 2^{16}-1 = 65535 units per pixel (YANG *et al.*, 2002c). Thus a 16-bit tif image is obtained from each channel and each array,

respectively. These images are then transformed into data matrixes using image analysis software. Typically these matrixes include both spot and background intensities for every probe/spot on the array (BENDIXEN *et al.*, 2005) in order to correct for background specific noise. Once expression is extracted, a quality filtering is carried out. As a first quality criterion, visual inspection of the generated image is done (CAUSTON *et al.*, 2003) and spots that look suspicious are manually flagged. Moreover, some image analysis software tools offer the choice to automatically flag spots with very low expression or low quality signal. Several parameters e.g. spot uniformity, signal to background ratio or spot shape regularity can be defined (DRAGHICI *et al*., 2003).

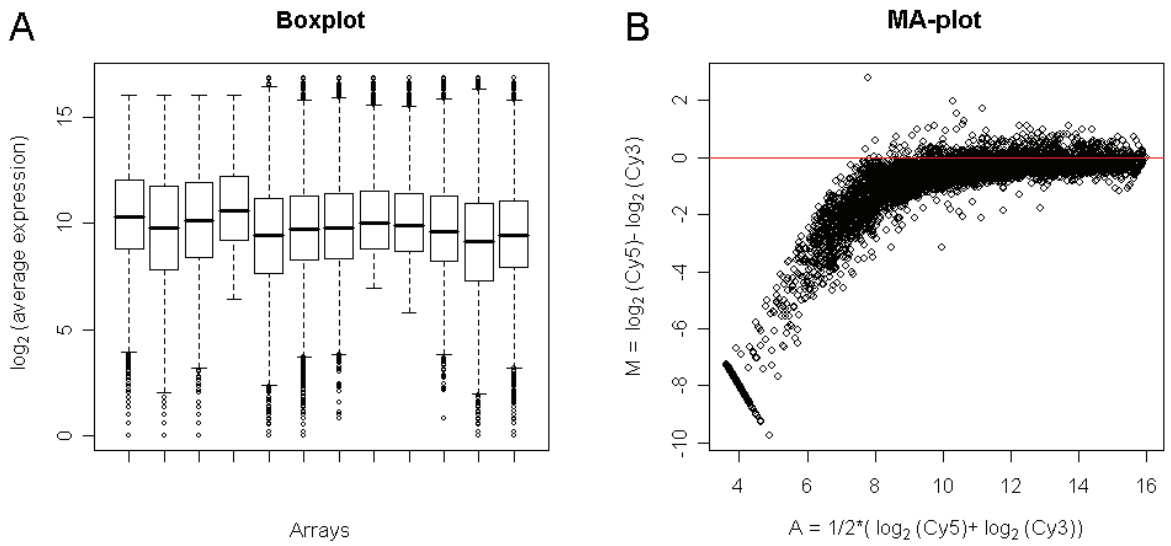

Figure 5(A+B): Example of a boxplot (A) and a MA-plot (B) of microarray expression values

Box-plots (see Figure 5A) show the mean of all raw $\log_2$ values for each array (mostly one color arrays). They can be used to identify and exclude outlier arrays of low quality. Generating MA-plots (see Figure 5B) by plotting the log-ratio “M” (the log to the ratio between the two “dye”-channels is a measure of relative expression) against the average intensity “A” for each spot, allows the detection of intensity dependant deviations of the log-ratios due to dye effects (BENDIXEN *et al.*, 2005).

Data pre-processing and normalization of two color microarrays

A first pre-processing step is the background correction of the intensity signal. The idea behind is that the signal of a spot includes the fluorescence of the background due to unspecific binding and the fluorescence due to the labeled RNA. Consequently, in order

to obtain values proportional to the amount of RNA, the value of the background needs to be subtracted from intensity signal (DRAGHICI *et al*., 2003). Background values are mostly calculated from the area around the spot. The correction approach is therefore named local background correction. Sub-grid or global background correction are also used. They calculate the background from sub-grid sections or build the average across the whole array. Moreover, a filtering of hybridization intensities, which are only slightly above the background intensity, can be done because these spots are the most imprecisely measured and therefore most likely to be of poor or questionable quality. One possible approach is to exclude signals that differ from their background signal in less than x (usually a factor of 2 is used) standard deviations. As an alternative a fixed minimum intensity can be applied as threshold to exclude poor signals (CAUSTON *et al.*, 2003).

A common step in data pre-processing is logarithmic transformation of either original intensity values or of the ratios (fold change factor between two experimental groups). The log transformation makes data more symmetrical especially the ratios and evens out highly skewed distributions. In addition it makes variation of intensities and ratios more independent of absolute magnitude and thus reduces the influence of single outliers with extreme intensities (SPEED, 2000).

As a next step between-array and color (dye) normalization is performed. Normalization is done to adjust the intensity values for effects which arise from technological variation rather then from biological differences between the samples. Differences between arrays may be due to variation in print quality, differences in ambient conditions when the slides were processed or simply from changes in scanner settings (SMYTH & SPEED, 2003). This bias can be corrected for instance by global normalization which measures the mean of intensities for all genes of each array and calculates a scaling factor (between the arrays) to correct the individual ratios (YE & DAY, 2003; DRAGHICI *et al.,* 2003). This simple scaling is based on the assumption that the total intensity on each array should be the same (BENDIXEN *et al*., 2005). For imbalances between the two dyes several approaches have been described e.g.: linear regression, log centering, Chens´ ratio statistics and rank invariant methods. However, none of these approaches takes into account systematic dependence of dye bias on intensity. Therefore, a locally weighted linear regression (lowess) analysis has been proposed to normalize for intensity-dependant effects in the $\log_2$(ratio) values (QUACKENBUSH, 2002; see Figure 6). A brief description of lowess is given by GAROSI *et al.* (2005): “Lowess normalization uses locally weighted linear regression to smooth data. The smoothing process is

considered local because each smoothed value is determined by neighboring data points defined within the span. The process is weighted because a regression weight function is defined for the data points contained within the span".

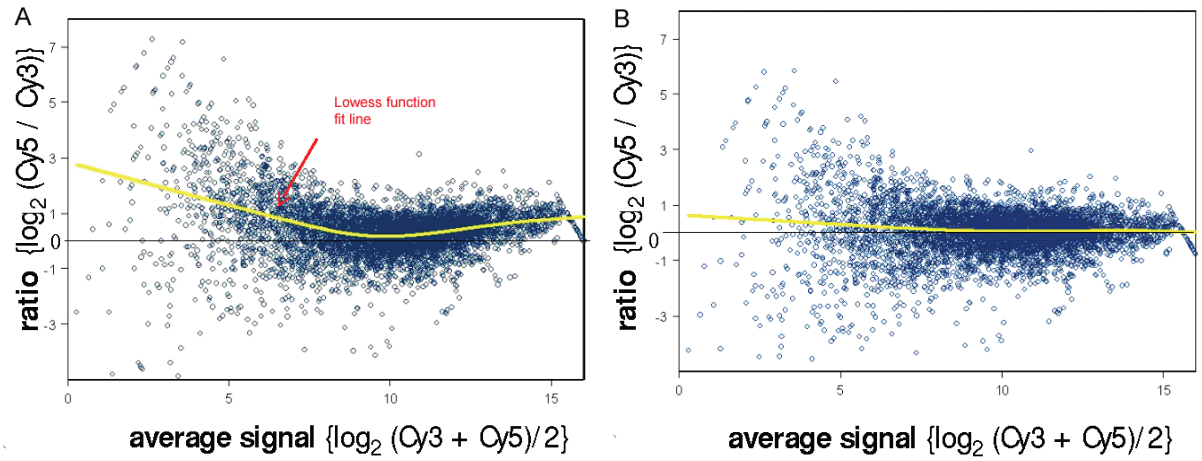

Figure 6(A+B): Analyzing two color microarrays. A: before normalization, B: after lowess normalization. (Source: http://www.umanitoba.ca/faculties/afs/plant_science/COURSES/bioinformatics /lec12/ lec12.2.html)

Defining differentially expressed genes

The purpose of most microarray experiments is to find genes which are differentially expressed between two or more experimental groups. Thus, the calculation of fold change factors (ratio) of the two intensity signals give a first hint which genes are differentially regulated. To define "differential expression" numerous methods are used in microarray experiments including (DRAGHICI, 2002):

Fold change: Any gene having a ratio above an arbitrary (often 2) threshold between two experimental groups is defined as being differentially expressed

Unusual ratio: This method involves selecting genes for which the ratio of the control and experiment values is a certain distance from the mean control/experiment ratio (typically this distance is to be taken ± 2 standard deviation)

Univariate statistics: This approach uses classical hypothesis testing e.g. t-test.

ANOVA: The idea behind defining differentially expressed genes by ANalysis Of VAriance (ANOVA) is to build an explicit model about the sources of variance that affect the measurements and use the data to estimate the variance of each individual in the model.

SAM — The software SAM calculates a modified t-statistic (SAM score) for each gene on the basis of changes in gene expression relative to the standard deviation of repeated measurements. Significant genes are those greater than the adjustable score. SAM also includes a calculation of the False Discovery Rate based on permutation of the measurements of each gene (TUSHER *et al.*, 2001; BOOTH *et al.*, 2004).

When using hypothesis testing one needs to consider the fact that many genes are analyzed at one time. Therefore, when assuming significance at $p \leq 0.05$ many false positive genes will appear being significantly different just by chance (e.g. 5 % out of 10 000 genes = 500 significantly different genes just by chance). Thus, a correction for multiple testing has to be applied (MURPHY, 2002; DRAGHICI *et al*., 2003; GAROSI *et al.*, 2005). Some correction approaches used in microarray studies, as for instance "Bonferroni", control the familiy-wise error rate which is the probability of making false discoveries, or type I errors among all the hypotheses (false positive rate). Nevertheless, they are regarded as being too conservative for microarray experiments where usually thousands of tests are done (BENDER & LANGE, 1999; DRAGHICI *et al.,* 2003). In their work, BENJAMINI & HOCHBERG (1995) introduced a new multiple-hypothesis testing error measure called False Discovery Rate (FDR). The FDR is the expected proportion of false positive findings among all rejected hypothesis. For example, by applying a False Discovery Rate of 5% as threshold in a microarray experiment, the largest subset of genes to be classified as differentially expressed, that has an expected percentage (5 %) of false positives, will be found (STOREY & TIBSHIRANI, 2003; DRAGHICI *et al*., 2003; WIT & MCCLURE, 2004). This approach is less stringent and more suitable for microarray data than e.g. "Bonferroni" procedure since the amount of estimated not differentially expressed genes is excluded from the correction procedure. A commonly used method to control the FDR and to correct for multiple testing was proposed by STOREY & TIBSHIRANI (2003). It calculates a corrected p-value described as q-value. For defining significantly differentially expressed genes a threshold of $q \leq 0.05$ is often applied (ALLISON *et al*., 2006).

Gene Ontology annotation - functional classification of differentially expressed genes

The above described steps in microarray data analysis were focusing on the identification of differentially expressed genes that accompany alterations in

physiological state. However, in many cases the ultimate purpose of the gene expression experiment is to produce biological knowledge and to interpret the results biologically. Thus, the researcher needs to translate the list of differentially expressed genes into a better understanding of the biological phenomena underlying these changes.

Extracting the important biological facts from results of such experiments is of crucial importance, but is difficult for experimental biologists. A first step can be the generation of a functional profile by using a systemized annotation vocabulary describing functional knowledge. This approach may help to detect coherent changes in the expression of groups of genes. The Gene Ontology (GO; THE GENE ONTOLOGY CONSORTIUM, 2000) annotations are an example for systems that provide sets of terms describing biological entities organized in hierarchical structures. Lists of differentially expressed genes, as obtained from microarray experiments, can be associated with certain GO terms according to the biological function they perform or represent.

The GO database provides ontologies to describe attributes of gene products in three non-overlapping domains of molecular biology (THE GENE ONTOLOGY CONSORTIUM, 2004):

Molecular Function – describes activities such as catalytic or binding activities at the molecular level

Biological Process – describes biological goals accomplished by one or more ordered assemblies of molecular function

Cellular Component – describes locations at the levels of sub cellular structures and macromolecular complexes

A functional profile can be created by recognizing statistically enriched terms in GO annotated lists of differentially expressed genes (DRAGHICI *et al*., 2003; ZHOU & SU, 2007). It enables the scientist to identify physiological alterations in terms of pathways rather than changes in single genes. Software, web servers and methods which have been developed for this purpose are summarized by KHATRI & DRĂHICI (2005) and by KIM *et al.* (2007).

2.3.2 Quantitative measurement of transcript level with real-time RT-PCR

Quantitative real-time polymerase chain reaction (PCR) is a technique that was developed at the beginning of the 1990s. This technology combines DNA amplification and the detection and quantification of the accumulated product. During PCR each DNA molecule becomes a target template for synthesis of its own new complementary copy within one cycle. To facilitate the reaction a DNA polymerase enzyme and sequence

specific oligonucleotides (primers) are needed. Moreover, a precisely set temperature regime is applied to enable the primer annealing, elongation and denaturation within each cycle.

During the log linear phase the accumulation of the PCR product can be measured via fluorescent dyes which bind to the PCR product. The emitted fluorescent signal correlates with the amount of target DNA produced and increased during every cycle. The number of amplification cycles required to obtain a particular amount of DNA molecules is registered and the initial amount of DNA template can be calculated (TICHOPAD, 2003; HIGUCHI, 1993; KUBISTA *et al.*, 2006). For quantification of PCR product generally two approaches are used: absolute and relative quantification. The absolute quantification is based on a standard curve of the corresponding PCR product or plasmid with known concentration. Relative quantification relates the measured quantity to another “non-regulated” housekeeping gene (PFAFFL, 2004). Relative quantification is also often used to normalize the quantification results in order to correct for variance among the samples in the starting amount of the template.

For labeling several fluorescent dyes or probes can be used. A commonly used dye is SYBR Green I which binds to any dsDNA available in the reaction tube (MORRISON *et al.,* 1998). Molecular beacons (TYAGI & KRAMER, 1996) or TaqMan-probes (HOLLAND *et al.*, 1991) in contrast are target sequence specific oligonucleotides which are bound to a fluorescent dye.

Real-time PCR is also frequently applied to quantify gene expression or more precisely mRNA amount in target samples. Because mRNA cannot be used as template, it is reversely transcribed into cDNA, typically by using a poly(dT)-primers and then followed by its exponential amplification in a PCR reaction. The method is referred to as real-time RT-PCR. It can be either carried out in one assay or it can be done in two separate steps: reverse transcription and PCR reaction. The advantage of the two step approach is the generation of a stable cDNA pool that can be stored and used several times (BUSTIN, 2000).

3 MATERIALS AND METHODS

3.1 Materials

All feeding experiments were conducted and supervised by the research group "nutritional physiology" of the Research Institute for the Biology of Farm Animals.

Animals

The effect of feeding a genetically modified feed additive, more precisely a recombinant baculovirus, on gene expression was studied in a first feeding experiment with rats (see Table 9). In a second experiment the impact of a feeding of two different genetically modified potatoes, was examined (see Table 10).

For both feeding experiments Wistar rats were purchased from Charles River Laboratories (Germany), which weighed about 100 g at the beginning of the experiment. They were housed individually under standardized humidity (60 ± 5 %) and temperature (21.5 ± 1 °C) in metabolism cages with a 12/12 hours light regime.

Experimental groups

Table 9: Experimental groups used in the feeding experiment with recombinant baculovirus

Experimental groups	**wtBV**	**rBV-VP60**
Diet	Commercial basic diet + 15 % lyophilized potatoes	Commercial basic diet + 15 % lyophilized potatoes
Feed additive	Wildtype baculovirus	Transgenic baculovirus
Inserted gene	-	VP60
Animals / group	6	7

Table 10: Experimental groups used in the feeding experiment with recombinant potatoes

Experimental groups	**wtAlb**	**rAlb-VP60**	**rAlb-nptII**
Diet	Semi-synthetic diet	Semi-synthetic diet	Semi-synthetic diet
Feed additive	Conventional potato	Transgenic potato	Transgenic potato
Inserted gene	-	VP60, CtxB, nptII	nptII
Animals / group	8	8	8

Tissues

Samples were obtained from the liver (left lateral hepatic lobe), the spleen (cranial) and from small intestine epithelium (jejunum). The Liver was analyzed because of its central role in metabolism especially in nutrient conversion and storage. The spleen was selected due to its involvement in immune related processes, which should be considered when a potential bioactivity of the transgenic feed additive cannot be excluded. By investigating expression in small intestine epithelium it was intended to gain

insight into impacts taking place during nutrient resorption processes which are of specific relevance when studying effects of specific feeds.

3.1.1 Feeding experiment with rats fed recombinant baculovirus

Diets and experimental conditions

For the experiment the rats were divided into two groups (wtBV and rBV-VP60; see Table 9). A commercial basic diet (ALTROMIN® Standard-Diet 1310) supplemented with 15 % lyophilized potatoes was fed to both groups. All animals received 10 g feed per 100 g body weight and per day. In addition, both groups received 20 ml solution of an additive consisting of corresponding amounts (approximately $10^{6.0}$ TCID 50 per ml) of lyophilized and ethylenimin-inactivated baculovirus. The baculovirus additives were fed on days 0, 2, 4 and 21, 23, 25. The wtBV group was fed a non-transgenic wildtype baculovirus and the rBV-VP60 group was given the recombinant rBV-VP60 (~1 µg VP60/ml) which was propagated and handled according to HAMMER (2006). VP60 is the major structural protein of a calicivirus that causes the rabbit hemorrhagic disease (RHD; PARRA & PRIETO*,* 1990; OHLINGER *et al.,* 1993). The VP60 expressing baculovirus rBV-VP60 was proven to induce production of the VP60 antigen and by this means protection against RHD virus (SCHIRRMEIER *et al.,* 1997, 2000; KADEN *et al.*, 1998). The VP60 baculovirus additive was obtained from the Friedrich-Loeffler-Institute on Riems (Institute of Diagnostic Virology). Both diets were isoenergetic and isonitrogenous and the rats had free access to the food and water. The experiment was conducted over a period of 42 days. In the following the feed additives will be named equally as the feeding groups (rBV-VP60, wtBV).

Tissue collection

At the end (day 42) of the experiment animals were exsanguinated under inhalant isofluran (IsoFlo®) anesthesia, 12 hours after the last food intake, to measure gene expression with minimized interference by food intake. Tissue samples of the liver, the spleen and of small intestine epithelium were collected, immediately frozen in liquid nitrogen and stored at -80 °C until isolation of RNA.

3.1.2 Feeding experiment with rats fed recombinant potatoes

Diets and experimental conditions

Three feeding groups (see Table 10 wtAlb, rAlb-VP60, rAlb-nptII) were used for the experiment. A half-synthetic basis diet was fed. Casein was given as main protein source

(supplying around 85 % of the total protein offered). The amount of potatoes was calculated to supply about 15 % of the protein in the diet (see Table 11, Table 12, Table 13). rAlb-VP60 group was fed a transgenic potato of the cultivar Albatros with the inserted genes VP60, CtxB and nptII. rAlb-nptII group was given a transgenic Albatros potato where only the nptII gene had been introduced. wtAlb group received the non-transgenic near-isogenic potato of the cultivar Albatros. Per 100 g body weight and day, 10 g feed were offered which comprised 150 mg N/100 g body weight.

The nptII gene codes for an aminoglycoside phosphotransferase conferring resistance to antibiotics such as kanamycin, neomycin or paromomycin etc. and is used as a selection marker in genetically modified plants (EFSA, 2008). CtxB is the non-toxic B subunit of cholera toxin and has adjuvant action by stimulating mucosal and other immune responses (CZERKINSKY *et al.*, 1989; HOLMGREN *et al*., 1993). The non-transgenic conventional potatoes were provided by NORIKA GmbH (Groß Luesewitz, Germany). Transgenic tubers were obtained from the University of Rostock (Faculty for Agricultural and Environmental Sciences, Agrobiotechnology and Applied Research in Bio- and Gene-Technology). VP60 protein presence could not be detected (see Table 14) with ELISA but mRNA expression was shown (MIKSCHOWSKY, personal communication).

Table 11: Composition of semi-synthetic diet fed to rats in g/kg DM (JANCZYK, personal communication)

Feeding groups	**wtAlb**	**rAlb-VP60**	**rAlb-nptII**
Corn starch	79	68	79
N-free mixture*	700	700	700
Casein**	85	85	85
Potato	136	147	136

*N-free mixture consisted of 18.5% cellulose, 37.1% sugar, 14.8% oil, 7.4% vitamins (Vitamin A – 750 IE, B1- 1 mg, B2 – 1 mg, B6 – 0.5 mg, B12 – 0.5 mg, C – 1 mg, D3 – 25 IE. E – 2.5 mg, K3 – 0.1 mg, Panthotenic acid – 1 mg, Nicotine acid amide – 2.5 mg, Choline hydrochloride – 100 mg, Folic acid – 0.1 mg, Biotin – 0.01 mg, Inositol – 12.5 mg, p-Aminobenzoic acid – 5 mg, fulfilled ad. 1 g with wheat starch). 14.8% minerals (CaCO3 - 6.86%, Ca-citrate – 30.83%, CaHPO4·H20 – 11.28, K2HPO4 – 21.88, KCl – 12.47%, NaCl – 7.71%, MgSO4 – 3.83%, MgCO3 – 3.52%, Fe-III ammoncitrate – 1.53%, MnSO4·H2O – 0.02%, CnS04·H20 – 0.0078%, KJ - 0.0041%, NaF – 0.051%, AlNH4(S04)2·12 H20 – 0.009%, ZnCO3 – 0.006%) and 7.4% wheat starch; corn starch was added to obtain 100%.

**3% methionine was added

Table 12: Nutrient composition of potatoes used in feeding experiments (JANCZYK, personal communication)

Parameter	**wtAlb**	**rAlb-VP60**	**rAlb-nptII**
DM (%)	93.02	93.20	93.13
cA (%DM)	3.74	3.57	3.73
cP (%DM)	8.12	7.58	8.18
cF (%DM)	2.23	2.26	2.50
EE (%DM)	0.28	0.26	0.26
NfE (%DM)	85.63	86.33	85.33
Starch (%)	77.40	77.51	Not analyzed

DM: dry matter; cA: crude ash; cP: crude protein; cF: crude fiber; EE: ether extract; NfE: nitrogen free extract

Table 13: Amino acid profile of potatoes used in feeding experiments (JANCZYK *et al*., 2007 and personal communication)

Amino acids (g/16 g N)	wtAlb	rAlb-VP60	rAlb-nptII
asparagine	7.13	15.71	14.95
threonine	4.35	3.84	3.84
serine	4.67	3.57	3.5
glutamic acid	16.14	10.39	10.92
glycine	1.79	3.08	3.06
alanine	3.31	3.23	3.14
valine	4.84	4.14	4.14
isoleucine	4.24	3.18	3.2
leucine	8.06	5.79	5.74
tyrosine	3.03	1.77	1.51
phenylalanine	3.91	3.73	3.76
histidine	2.21	1.53	1.58
lysine	7.36	5.12	5.18
arginine	2.92	4.05	4.26
proline	7.06	3.95	3.44
cysteine	1.56	1.58	1.61
methionine	1.18	1.22	1.26
tryptphan	0.83	0.91	1.11

All diets were isoenergetic and isonitrogenous and were fed once a day (at 8.00 a.m.). Water was provided ad libitum. Free access to food was allowed from 8.00 to 20.00. The experiment was conducted over a period of 14 days.

In the following the feed components will be named equally as the feeding groups (rAlb-VP60, rAlb-nptII, wtAlb).

Table 14: Content of VP60, CtxB and NPTII in freeze-dried potato tuber material, prepared for feeding experiments according to MIKSCHOWSKY (personal communication)

Parameters	wtAlb	rAlb-VP60	rAlb-nptII
VP60 µg/mg TSP	-	not detectable	-
CtxB µg/mg TSP	-	not detectable	-
NPTII µg/mg TSP	-	0.03 ± 0.01	0.05 ± 0.00

Tissue collection

After 14 days animals were exsanguinated and slaughtered as described above. Tissue samples of the liver, the spleen and of small intestine epithelium were collected, immediately frozen in liquid nitrogen and stored at -80 °C until isolation of RNA.

3.2 Methods

3.2.1 RNA isolation and quantification

As a first step frozen tissues of spleen, liver and small intestine epithelium were ground with a mortar in liquid nitrogen to avoid unfreezing and degradation of RNA. Extraction of total RNA was done with the RNeasy Mini Kit® (Qiagen, Hilden). DEPC-treated water, RNase-free reagents and vessels were used for all isolation and processing steps. According to manufacturers´ instructions 30 mg tissue powder were mixed with 1.5 ml RLT lysis buffer and homogenized with the "Ultra TurraxT25® basic" (11 000 - 24 000 rpm). The lysate was then centrifuged at 10 000 rpm for 30 min and the supernatant was removed by pipetting and transferred into a new microcentrifuge tube with 1.5 ml ethanol (70%) to clear the lysate. After mixing the solution it was transferred to an RNeasy spin column, placed in a 2 ml collection tube and centrifuged for 15 sec at 10.000 rpm. The flow-through was discarded. RNA was now bound to the membrane of the spin columns and was washed with RW1 washing buffer. A DNAse I digestion (Qiagen, Hilden) was performed on the membrane for 1 hour at 37 °C. After an additional washing with RW1 and RPE buffers, the columns were centrifuged (10.000 rpm for 30 sec) to dryness. As an ultimate step RNA could then be eluted in about 30 µl RNAse-free water by centrifugating at 10.000 rpm. Total RNA was stored at -80 °C.
Concentration and purity of the RNA were determined with a NanoDrop® ND-1000 spectrophotometer (NanoDrop technologies Ltd, USA) by measuring the optical density of each sample at 230, 260 and 280 nm. The 260/280 and 260/230 (protein-contamination-value) ratios should be above 1.8 to ensure high purity of RNA. The quality of the RNA was further confirmed by gel electrophoresis on a 2.2 M formaldehyde gel. On the gel, the bands of the 18S and 28S rRNA band should be clearly visible. To control fragment sizes, the Alu I Maker was used.

3.2.2 Microarray hybridization

For genome wide expression profiling the MWG "rat 10K array" (MWG Biotech AG, Ebersberg) was used, which allows the detection of expression levels of 9802 transcripts. 5787 probes/spots of the array were annotated to known geneIDs (http://www.ncbi.nlm.nih.gov/sites/entrez and http://rgd.mcw.edu/genes/). Additional probes could not be assigned to any known genes. They represented mostly ESTs from an in-house MWG EST-sequencing project. Probes consisted of 50mer oligonucleotides. A dual labeling system was used. For DNA chip hybridization the samples of control

group and test group were pooled and labeled with Cy3 (control group) and Cy5 (test group) dyes, respectively and co-hybridized on the same array. Each hybridization was carried out in triplicate. Probe preparation and chip hybridization were carried out essentially according to manufacturers´ protocol (MWG, 2002).

3.2.2.1 First and second strand synthesis

Synthesis of first strand cDNA was performed with "cDNA Synthesis System®" (Roche, Mannheim, Germany), the corresponding AMV Reverse Transcriptase and Oligo[$(dT)_{24}$-T7promotor]$_{65}$ primers using 15 µg of pooled total RNA. Primer annealing was done at 70 °C for 10 min. For synthesis reaction 19 µl master mix were added to the RNA and the solution was incubated for 60 min at 42 °C.

Master mix:	5 x Reverse Transcription buffer	8 µl
	0.1 M DDT	4 µl
	AMV Reverse Transcriptase	2 µl
	RNase Inhibitor (25U/µl)	1 µl
	dNTP Mix (10 mM each nucleotide)	4 µl

Second strand synthesis was done at 16 °C for 2 hours using 110 µl of the reaction mix.

Reaction mix:	5 x second strand buffer	30 µl
	dNTP mix (10 mM each nucleotide)	1.5 µl
	second strand enzyme mix	6.5 µl
	RNase-free water	72 µl

Moreover T4 DNA polymerase was added for removing overhangs (16 °C for 5 min). This reaction was stopped with 17 µl EDTA and remaining RNA was eliminated with 1.5 µl RNase I (37 °C for 30 min). Protein contamination was cleared with 5 µl of Proteinase K (37 °C for 30 min) and dsDNA was dried in a vacuum concentrator.

3.2.2.2 cRNA synthesis, labeling and fragmentation

After these steps dsDNA was transcribed into cRNA with a T7 transcription and during this step Cy3-UTPs or Cy5-UTPs were incorporated into the newly synthesized cRNA (MEGAscriptTM T7 Kit®, Ambion). 20 µl of the reaction mix was added to the dried dsDNA and incubated at 37 °C for at least 4 hours.

Reaction mix:	10 x reaction buffer	2 µl
	ATP/CTP/GTP-mix stock solution (25 mM each)	6 µl
	U-nucleotide stock solution (50 mM)	2 µl
	RNase-free water	1 µl
	Enzyme mix, incl. T7 RNA polymerase	4 µl
	Cy3-UTP or Cy5-UTP (5 mM)	5 µl

Labeled cRNA was purified and fragmented with 5 x fragmentation buffer (12 µl, incubation at 94 °C for 15 min). After an additional purification step the labeled cRNA of both groups was merged, dried and used for hybridization.

3.2.2.3 Hybridization of cRNA on the rat array

The labeled cRNA was re-dissolved in 35 µl hybridization buffer, heated for 3 min at 94 °C and immediately placed on ice. The hybridization mix was pipetted onto the array, covered with a cover slip and sealed with rubber cement. The arrays were placed for incubation in a dark, humid chamber at 42 °C for 16 – 24 hours. After hybridization, the arrays were washed for 5 min in washing solutions A, B, and C (A: 2 x SSC, 0.1 % SDS; B: 1 x SSC; C: 0.5 x SSC) at 32 °C to remove unspecifically bound molecules. After washing, arrays were centrifuged to dryness.

Hybridization buffer:	Na-Phosphate (1 M, pH 8.0)	50 µl
	Formamide (99.8 %)	500 µl
	20 x SSC	300 µl
	50 x Denhardts Solution	100 µl
	10 % SDS	50 µl

3.2.3 Microarray data analysis

3.2.3.1 Image processing

Cyanine-3 and Cyanine-5 fluorescence can be detected using a confocal scanning instrument. With two tuned lasers, Cyanine dyes were excited at appropriate wavelengths (Cy5: 651 nm; Cy3: 552 nm). After drying, all slides were scanned immediately (428TM Scanner®; Affymetrix, Inc., Santa Clara, USA). Scans were done at various photo multiplier tube (PMT) settings (gains), above and below saturation of the most intense fluorescent spots on each array. The images were saved as TIFF files. Files for Cy3 and the corresponding Cy5 scans were produced. The images were then

transformed into data matrixes using Imagene™ 5 software (BioDiscovery Inc., Los Angeles, USA). These matrices included both spot and background intensities in order to be able to correct for background specific noise.

3.2.3.2 Quality filtering and data pre-processing

Moreover, a first visual quality inspection was carried out to check for major contamination (e.g. dirt or dust) on the image and to manually flag the affected area. Additionally, Imagene™ 5 offers the choice to automatically flag spots according to several quality criteria:

- Bad quality spots (shape irregularity, contamination, offset from expected position)
- Empty spots
- Negative spots (background > signal)
- Weak spots: if for some spots this ratio

$$R = \frac{\text{Signal mean} - \text{background mean}}{\text{Background standard deviation}}$$

appeared to be lower than the threshold “two”, the spot was flagged.

Data was further processed with MAVI Pro 2.6.0 software (MWG Biotech AG, Ebersberg). The software intends to increase the dynamic range, while at the same time avoiding saturation problems. This was achieved by linear regression analysis on the intensity data of the images from three different scanner PMT gain settings. MAVI also subtracted unspecific background signals and corrected intensity signals could be exported for further analyses.
Moreover a fixed minimum intensity threshold of ≤ 150 (as recommended by MWG, personal comment) in both experimental groups was applied to exclude signals of poor or questionable performance.

3.2.3.3 Normalization

For further data analysis GeneSight 3.5 (Biodiscovery Inc., Los Angeles, USA) and TIGR MeV 4.0 (http://www.tm4.org/mev.html; SAEED *et al.*, 2003) software were used. Firstly, a logarithmic transformation of intensity values was accomplished. Then color (dye) normalization was performed. Normalization was done to adjust the intensity values for

effects which are due to technological variation i.e. dye bias. This bias can be corrected using various normalization methods. Several of them were tested in 4.1.1:

- Global normalization (QUACKENBUSH, 2002)
- Linear regression (FINKELSTEIN, 2002)
- Log centering (CAUSTON, 2003)
- Chens` ratio statistic (CHEN *et al.*, 1997)
- Lowess (QUACKENBUSH, 2002)

For subsequent analysis lowess (locally weighted linear regression) normalization was applied. MA-plots were drawn with "R" software (GENTLEMAN *et al.*, 2003; http://www.bioconductor.org/) using the following formula:

$$\mathbf{M = \log_2(Cy5) - \log_2(Cy3) \quad and \quad A = 0.5 * (\log_2 (Cy5) + \log_2 (Cy3))}$$

A lowess curve was created using the "lowess.smooth" command. Correlation analysis was done with "cor.test" command both included in "R" software.

3.2.3.4 Statistical analysis

Single gene analysis: To identify genes which are significantly differentially regulated a t-test was carried out (TIGR MeV 4.0; SAEED *et al.*, 2003). P-values were then adjusted for high numbers of expected false positives. This was done by correcting the False Discovery Rate using the concept of STOREY & TIBSHIRANI (2003). For the ordered p-values the corresponding q-values (= corrected p-values) were calculated using the software QVALUE (http://genomics. princeton.edu/storeylab/qvalue/) which has implemented the above mentioned statistical concept. A threshold of $q \leq 0.05$ was applied to define significantly differentially expressed genes (DEGs) between two feeding groups for experiments described in 4.3. Moreover a fold change/ratio was calculated and fold changes of ≥ 1.5 were considered as relevant and thus used as an additional filter for defining expression changes between the feeding groups.

$$\mathbf{Fold\ change = \log_2 (intensity\ Cy5\text{-}signal) - \log_2 (intensity\ Cy3\text{-}signal)}$$

Threshold-based *enrichment analysis of* ***functional GO processes***: Based on Gene Ontology (GO) database (http://www.geneontology.org), DEGs were functionally assigned to biological processes in which they play a role. Processes with a significantly increased number of associated DEGs were defined using the EASE Score (ES ≤ 0.05)

as implemented in the DAVID software (http://david.abcc.ncifcrf.gov/home.jsp). The software calculates the over-representation of DEGs within a GO category (gene set) with respect to the total number of genes assayed and annotated within the classification system. The EASE Score is a conservative adjustment of the fisher exact probability, taking into account the over-estimation of processes assigned with only few genes (HOSACK *et al.*, 2003). Not for all genes a functional annotation was available. Thus, for the identification of diet-affected biological processes only GO annotated molecules could be included. Starting point of this approach is a pre-defined threshold-based list of DEGs. In the following this approach is referred to as individual gene analysis (**IGA**).

***Threshold-free** gene set analysis of **functional GO processes** (**GSA**):* For applying a threshold-free gene set analysis, the software tool GAZER (http://expressome.kobic.re.kr /GAzer/index. faces; KIM *et al.*, 2007) was employed. This tool identifies significantly altered gene set categories based on GO annotation and uses all available ranking statistics (e.g. p-values, fold changes) without pre-selection according to a certain threshold. As recommended for small sample sizes, fold changes were chosen as ranking statistics and a z-test (PAGE algorithm; KIM & VOLSKY, 2005) was selected as statistical test.

3.2.4 Real-time RT-PCR

3.2.4.1 Primer design

To obtain quantitative real-time measurements of mRNA expression of several selected DEGs a real-time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was carried out with the LightCylcer® 2.0 (Roche Diagnostics) system. Using Primer3 software (http://frodo.wi.mit.edu/; ROZEN & SKALETSKY, 2000) sequence specific primers of 100 – 250 bp length were designed for the selected genes from the corresponding cDNA sequence in NCBI database (http://www.ncbi.nlm.nih.gov/sites/ entrez). Primers used within this work are given in Table 15.

As a first step primers were tested in a PCR reaction and checked in gel electrophoresis in order to examine the temperature at which they were annealing best. Moreover, a PCR product for creation of a standard curve was produced and concentration of PCR product was determined with the Nano Drop® instrument (Nanodrop Technologies, Delaware, USA). PCR was performed according to the following PCR protocol with 2.5 µl cDNA template (containing 2 µg) and within 40 cycles:

Master mix	H_2O	36 µl
	10 x PCR buffer	5 µl
	dNTPs (each, 10mM)	1 µl
	Forward primer (10 pmol/µl)	1 µl
	Reverse primer (10 pmol/µl)	1 µl
	Taq Polymerase	0.5 µl

For measuring mRNA level of specific molecules with real-time RT-PCR the corresponding RNA Pools of the microarray assay were used.

3.2.4.2 LightCycler®-PCR Protocol

Synthesis of cDNA (reverse transcribed RNA) was performed with M-MLV reverse transcriptase (Promega, Mannheim, Germany) and Oligo$(dT)_{12}$ primers using 2 µg of total RNA. The RNA was dissolved in 6 µl water, 2 µl primers (100 µM) were added and the tubes were incubated at 70 °C for 5 min for primer annealing. 17 µl of master mix were added and reverse transcription was carried out at 42 °C for 1 hour with and additional step at 95 °C (5 min) to destroy the enzyme and stop transcription process.

Master mix:	H_2O	5.4 µl
	5 x MMLV RT-buffer	5.0 µl
	dNTPs (each, 10mM)	1.25 µl
	RNasin (25 U/µl)	0.60 µl
	M-MLV reverse transcriptase	1.0 µl

A cDNA yield of 80 ng/µl was assumed. cDNA was frozen at -20°C until further use in real-time RT-PCR assay.
Real-time RT-PCR was carried out in the LightCycler instrument according to manufacturers´ recommendation and an optimized protocol essentially as described by SCHWERIN *et al.* (2002). For LightCycler reactions, a master mix of the following reaction components was prepared using the LightCylcer-FastStart DNA Sybr Green 1 kit:

Master mix:	H_2O	10.6 µl
	MgCl	2.4 µl
	Forward primer (10 pmol/µl)	1.5 µl
	Reverse primer (10 pmol/µl)	1.5 µl
	Enzyme Mix	2.0 µl

Samples of 18 µl of LightCycler master mix were filled in the LightCycler glass capillaries and 10 ng of reverse transcribed RNA, dissolved in 2 µl water, was added as PCR template. A protocol with 45 cycles was applied. Based on the melting curve analysis of the PCR products, a fluorescence acquisition temperature (see Table 15) was estimated and included in the amplification cycle program. High-temperature fluorescence acquisition melts unspecific PCR products, eliminates the non-specific fluorescence signal derived from primer dimers, and ensures an accurate quantification of only the desired specific sequence.

For all the assays a standard curve was generated using DNA standard dilutions (10^2, 10^3, 10^4, 10^5 and 10^6 copies) of the corresponding PCR product. The standard curve was used for calculation of transcript copies per 10 ng total mRNA. Fluorescence signals, which were recorded on-line during amplification, were subsequently analyzed using the "Second Derivative Maximum" method of the LightCycler data analysis software. The copy number of the housekeeping gene Gapdh was measured to normalize for equal RNA amounts. The mRNA abundance was analyzed in triplicate.

3.2.4.3 Statistical analysis

The average mRNA expression level (copies/10 ng total mRNA) of the selected DEGs was determined for the corresponding test and control groups of the feeding experiments. Differences between the experimental groups were assigned with t-test (SAS®, 2002). Significance was assumed at $p \leq 0.05$.

Table 15: Primers and corresponding reaction conditions used for real-time RT-PCR amplification

Gene symbol[a]	Accession No.	Primer sequences (5´-3´)	AT[b] [°C]	Length of PCR product [bp]	FT[c] [°C]
Acox2 for	NM_145770	cagcagcagaaactccttcc	63	165	85
Acox2 rev		aaacgtggccttcataccag			
Add2 for	NM_012491	gcttcctccatgaacttctcc	60	189	87
Add2 rev		gtcctgctccttgctcactc			
Alad for	NM_012899	ctggatatggtgcaggaggt	61	197	88
Alad rev		agctggggtgcaaagtaggt			
Cebpb for	NM_024125	caagctgagcgacgagtaca	63	157	90
Cebpb rev		cagctgctccaccttcttct			
Cpa1 for	NM_016998	cagctcctgctctaccccta	65	198	85
Cpa1 rev		gtacttgatgccctggctgt			
Ctrb1 for	NM_012536	caaatacaatgccctcaagacc	65	245	89
Ctrb1 rev		caggagtggaagtggaacaga			
Ela1 for	NM_012552	catctgctccagctcctctt	60	204	87
Ela1 rev		gtgaagactgtgggcttcct			
F3 for	NM_013057	gctgggaactgtggagtttg	63	200	83
F3 rev		cctgggcaacctagtgtctt			
Fbp1 for	NM_012558	gtctgttttgatcccctcga	60	218	85
Fbp1 rev		tccagcatgaagcagttgac			
Flt1 for	NM_019306	gacgtgtggtcctacggagt	65	190	84
Flt1 rev		tttctttggggtctttgtgc			
Gapdh for	NM_017008	catggccttccgtgttccta	65	205	86
Gapdh rev		cccagcatcaaaggtggaag			
Gp5 for	NM_012795	agggaccttgaccaaaacct	63	134	81
Gp5 rev		cgcgataaatccaacacctt			
Hsd17b2 for	NM_024391	cctcttatcggtgtcctgct	55	219	86
Hsd17b2 rev		cgctcagagcagtttttcct			
Pap1 for	NM_053289	actccatgaccccactcttg	60	136	82
Pap1 rev		tgacaagctgccacagaatc			
Pc for	NM_012744	caagcctacctgcacattcc	58	182	86
Pc rev		tccaccttgtctcccatctt			
Pemt for	NM_013003	tccagagtgaccacatttcc	63	159	87
Pemt rev		ttcaaacaggagagcaacca			
Pla2g1b for	NM_031585	tcctcatcgacaacccctac	60	217	86
Pla2g1b rev		acggcatagacaggaagtgg			
Pnlip for	NM_013161	aaccttacttccagggcaca	60	206	83
Pnlip rev		gtcaacgatttgggaaagga			
Prkaa2 for	NM_023991	caccaccggtctctgtcttt	55	155	83
Prkaa2 rev		ggccagatttctgtgtttcc			
Rara for	AJ002940	ccctacgccttcttctttcc	60	159	87
Rara rev		agggctgggcactatctctt			

[a]Acox2: acyl-Coenzyme A oxidase 2, branched chain; Add2: adducin 2 (beta); Alad: aminolevulinate, delta-, dehydratase;.Cebpb: CCAAT/enhancer binding protein, beta; Cpa1: carboxypeptidase A1; Ela1: elastase 1, pancreatic; F3: coagulation factor III; Fbp1: fructose-1,6-biphosphatase 1; Flt1: FMS-like tyrosine kinase 1; Gapdh: glyceraldehyde-3-phosphate dehydrogenase; Gp5: glycoprotein 5, platelet; Hsd17b2: hydroxysteroid (17-beta) dehydrogenase 2; Pap1: pancreatitis-associated protein; Pc: Pyruvate carboxylase; Pemt: phosphatidylethanolamine N-methyltransferase; Pla2g1b: phospholipase A2, group IB; Pnlip: pancreatic lipase; Prkaa2: protein kinase, AMP-activated, alpha 2 catal. subunit; Rara: retinoic acid receptor, alpha

[b]annealing temperature; [c]fluorescence acquisition temperature

4 RESULTS

4.1 Pre-screening of different microarray analysis parameters to select an appropriate analysis approach for subsequent investigations

Gene expression profiling was done in the liver, the spleen and in small intestine epithelium of rats fed a diet including the genetically modified (GM) feed additive rBV-VP60 (VP60 expressing baculovirus). Expression profiles were compared to rats fed a non-GM additive (wtBV). For expression studies the “10K rat oligonucleotide array”, representing about 10 000 transcripts of the rat genome, was used. As a prerequisite for further studies, a pre-screening of different analysis parameter settings was performed in order to select an appropriate analysis approach for the identification of diet effects. This was done because microarray data evaluation includes many different steps of data processing and analysis and it is known that they can have a profound impact on the results and even on the conclusion drawn of them.

4.1.1 Trimming of low quality and low intensity signals and normalization of dye specific bias

As a first step of microarray data analysis a background correction of the intensity signals was performed. This step corrects for most background noise. MA-plots of all arrays were drawn in order to evaluate the need for further filtering due to low quality signals and to study the influence of different dyes on the data (dye bias). The latter was necessary because the experiment was based on a two color platform where the two experimental groups were labeled with different dyes (Cy3, Cy5) which have different physical properties and incorporation rates. MA-plots show the log ratio between each intensity signal of the two experimental groups and its log geometric mean. As can be seen from array signals presented in Figure 7A, the variability among the fold changes was high at low intensities as it was likewise observed in all other arrays (for Figure 7 a single array from liver tissue was chosen at random to give an example). This could be caused by low quality signals due to a weak signal intensity arising from low transcript levels that may be masked or biased by noise (non-specific hybridization). Consequently, a further filtering was performed. Signals with a distance of ≤ 2 SD (standard deviations) to the respective background value were excluded. Moreover, a fixed minimum intensity (≤ 150 as recommended by MWG, personal communication) in both experimental groups was applied as quality criterion in order to remove signals of poor or questionable performance.

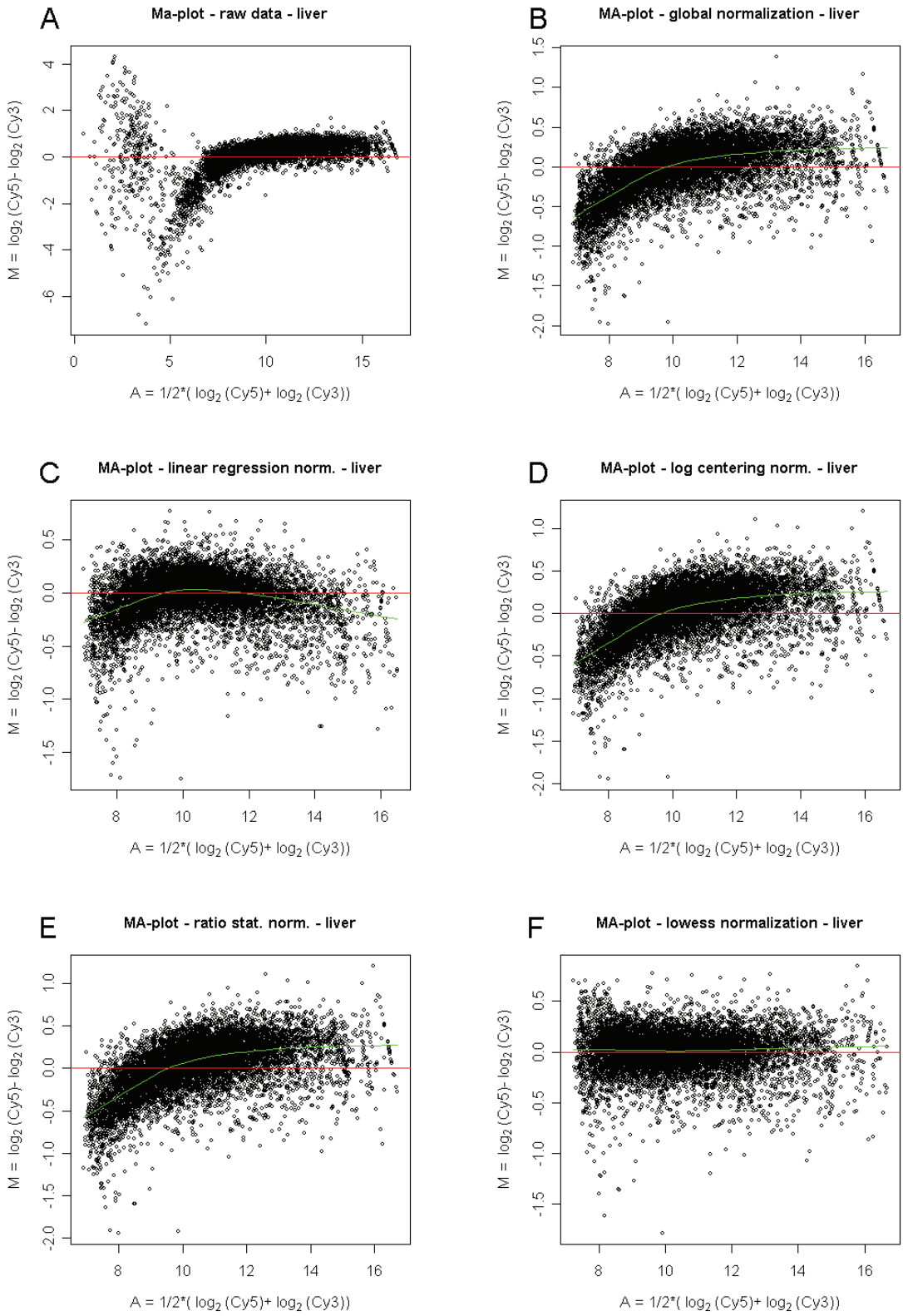

Figure 7(A-F): Fold changes of transcript levels measured in the liver of rats (fold change between animals fed the rBV-VP60 (Cy5) and the wtBV (Cy3) diet). A: background corrected, un-normalized data without quality filtering; B: quality filtered and background corrected data, globally normalized; C: quality filtered and background corrected data, normalized with linear regression method; D: quality filtered and background corrected data, normalized with log centering method; E: quality filtered and background corrected data, normalized with Chens´ ratio statistic method; F: quality filtered and background corrected data, normalized with lowess method.

The effect of the proposed data trimming as well as the influence of a global normalization on the distribution of fold changes can be seen from Figure 7B. Global normalization adjusts the mean over all genes as to be equal between the two groups (Cy3-labelled and Cy5-labelled). The quality filtering did successfully exclude low intensity and possibly unreliable signals. Though, a distinct intensity-dependant dye bias could not be diminished. The filtering had no effect on the “banana-like” shape of the plot as visualized by adding a smooth curve (green line) computed with the lowess function (locally weighted regression). Consequently other common normalization methods were tested on the dataset:

- Linear regression
- Log centering
- Chens´ ratio statistic
- Lowess

Linear regression, log centering, Chens´ ratio statistics did likewise not compensate for the intensity-dependant bias (see Figure 7C-E). Best results were obtained using lowess normalization which uses locally weighted linear regression to smooth the data. Figure 7F shows an appropriate dye bias smoothing of the expression fold changes which can be seen from the good agreement of the green lowess curve and the red “zero” line. This tendency was generally observed in all other arrays processed within this study, regardless of the array or tissue that was analyzed (data not shown). In consequence lowess normalization was used for any further experiments.

4.1.2 Application of False Discovery Rate correction and fold change thresholds

In this section of the study it was investigated whether and how the use of varying thresholds for defining significant expression changes can influence the number of differentially expressed genes (DEG). In addition, it was analyzed how threshold choice affected the pattern of identified diet-affected biological processes, which were based on a significant enrichment of associated DEGs.

First, a t-test was performed and p-values were computed. Second, the False Discovery Rate (FDR) was corrected to adjust for multiple testing and the corresponding q-values were calculated. DEGs were defined based on different significance thresholds. Significance was assumed for q-values ≤ 0.2 / 0.1 / 0.05 and for p-value ≤ 0.05 and resulting numbers of DEGs were compared. Besides, the use of a minimum FC (Fold Change) in combination with a q ≤ 0.05 was tested. The FC filter was set to FC ≥ 1.5 / 2. The results were also compared to a variant without FC criterion.

4.1.2.1 Effects of threshold choice on the number of differentially expressed genes

Table 16 shows the number of analyzed signals (= genes) after pre-processing the data which comprises genes that had detectable signals and that passed the quality criteria. From the spleen 8800, from the liver 9320 and from small intestine epithelium 8339 signals were included in further analyses. Results varied between the three tissues. 2377 (spleen), 2754 (liver) and 1608 (small intestine epithelium) genes, respectively were differentially expressed with a p-value ≤ 0.05. As outlined in chapter 1 the high amount of tests carried out simultaneously led to a very high number of expected false positives. 440 (19 %) out of the 2377 significant DEGs in the spleen are expected to be false positives (liver: 466 = 17 %; small intestine epithelium: 417 = 26 %). Even when applying a very stringent p-value threshold (≤ 0.001) a large proportion, namely 9 out of 41 DEGs in the spleen and 9 out of 43 DEGs in the liver would expected to be false positive (false positives in small intestine epithelium: 8 out of 10). These results emphasize the need for a multiple testing correction as described above.

Table 16: Number of expressed, differentially expressed (p-value ≤ 0.05) and expected false positive genes in the liver, the spleen and in small intestine epithelium of rats fed rBV-VP60 in comparison to rats fed wtBV.

Tissue	Number of analyzed genes	Number differentially expressed genes[1] (100%)	Expected false positives
Liver	9320	2754	466 (17%)
Spleen	8800	2377	440 (19%)
Small intestine epithelium	8339	1608	417 (26%)

[1] p-value ≤ 0.05

q-value threshold

A FDR correction was applied and DEGs were now defined according to different q-value thresholds (q ≤ 0.2, q ≤ 0.1, and q ≤ 0.05). When assuming significance at q ≤ 0.05, a stringency that is commonly used in microarray studies, the number of DEGs was reduced in comparison to the DEG list without FDR correction (compare Table 16 and Table 17). In the liver only a slight reduction from 2754 to 2620 was observed. The other tissues revealed a more distinct effect. In the spleen the number of DEGs dropped from 2377 to 917 and in small intestine epithelium from 1608 to 10. Loosening threshold stringency (e.g. q-value ≤ 0.1) led to a distinctively higher number of DEGs which

exceeded the results of gene selection without a multiple testing correction. In agreement with the results in Table 16, the liver seemed to be affected the most by the diet in that the number of DEGs was largest in this tissue; not matter which stringency level of the threshold was chosen.

Table 17: Number of differentially expressed genes identified at different "False Discovery Rate" (FDR) thresholds in the liver, the spleen and in small intestine epithelium of rats fed rBV-VP60 in comparison to rats fed wtBV.

Tissue	q-value threshold (FDR)	Number of differentially expressed genes (100%)	Expected false positives among the differentially expressed genes
Liver	q ≤ 0.2	7759	1552 (20%)
	q ≤ 0.1	5241	524 (10%)
	q ≤ 0.05	2620	131 (5%)
Spleen	q ≤ 0.2	6241	1248 (20%)
	q ≤ 0.1	3667	337 (10%)
	q ≤ 0.05	917	46 (5%)
Small intestine epithelium	q ≤ 0.2	4560	912 (20%)
	q ≤ 0.1	355	35 (10%)
	q ≤ 0.05	10	1 (5%)

Referring to the tissues spleen and liver, a conservative threshold of q ≤ 0.05 appeared to be most reliable for the present data. The availability of only few replicates (n = 3) and thus a low statistical power may produce high false positive rates. It was intended to reduce the number of expected false positives amongst the DEGs to an acceptable and commonly applied level of significance. In consequence, a **q ≤ 0.05** was used to define significant changes in gene expression **for continuative investigations** as described in 4.1.2.3 and 4.3 (see appendix Table 34, Table 35 and Table 36: entire list of DEGs).

Fold change filter in combination with a q ≤ 0.05

When applying fold change filters of ≥ 1.5 or ≥ 2 to the list of significant DEGs defined with a q ≤ 0.05, the number of DEGs was reduced considerably in all examined tissues. In the liver 6 % (see Table 18) of the significant DEGs remained when filtering with a FC ≥ 1.5. A FC filter of ≥ 2 even reduced the amount of DEGs to 1 %. In the spleen a FC filter application diminished the number of DEGs to 17 % (FC ≥ 1.5) and 5 % (FC ≥ 2). In small intestine epithelium only very few genes had a q ≤ 0.05. A FC filter reduced the amount of DEGs to two (FC ≥ 1.5) and one gene (FC ≥ 2).

Table 18: Number of differentially expressed genes identified with different “fold change” (FC) filters in the tissues liver, spleen and in small intestine epithelium of rats fed rBV-VP60 in comparison to rats fed wtBV.

Tissue	Fold change filter[1]	Number of differentially expressed genes[2]
Liver	No FC	2620 (100%)
	FC ≥ 1.5	149 (6%)
	FC ≥ 2	24 (1%)
Spleen	No FC	917 (100%)
	FC ≥ 1.5	157 (17%)
	FC ≥ 2	49 (5%)
Small intestine epithelium	No FC	10 (100%)
	FC ≥ 1.5	2 (20%)
	FC ≥ 2	1 (10%)

[1]fold change (rBV-VP60 group/wtBV group); [2]only genes with q ≤ 0.05 were included

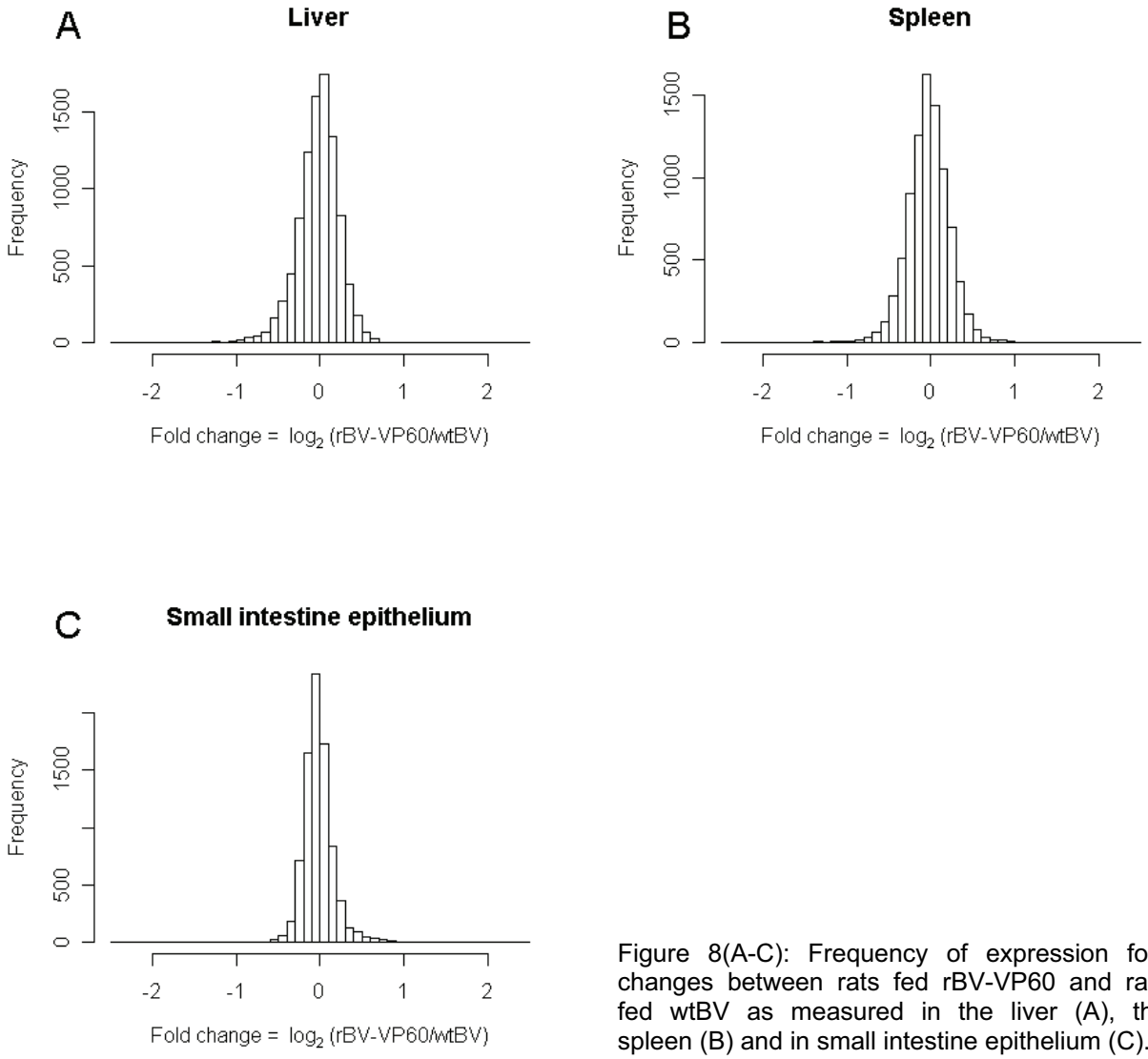

Figure 8(A-C): Frequency of expression fold changes between rats fed rBV-VP60 and rats fed wtBV as measured in the liver (A), the spleen (B) and in small intestine epithelium (C).

From Figure 8(A–C) it becomes clear that the frequency of high fold changes (i.e. ≥ 2) was very low, thus applying such a stringent filter might be not appropriate for this data. Moderate **FC filters of ≥ 1.5** are probably more suitable in the present case and were regarded as **most appropriate** for further investigations within this study (4.1.2.3 and 4.3). Moreover, it can be seen that small intestine epithelium had the least expression changes between the two groups and small fold changes, respectively.

4.1.2.2 Effects of threshold choice on biological processes, exhibiting a significant enrichment of differentially expressed genes

Results of 4.1.2.1 had shown that using different significance thresholds or fold change filters can have a profound influence on the number of differentially expressed genes. Whether the varying lists of DEGs also impact on the pattern of diet-affected biological processes, was additionally tested.

To define affected diet-biological processes the DEGs were annotated to biological processes in which they are known to be involved. This was done using gene ontology (GO) database (http://www. geneontology.org/). GO annotation was not available for the entire number of genes/probes represented on the “10K rat oligo array”. On the one hand, this is due to a lack of functional knowledge meaning that for many genes the specific function is simply not known yet. On the other hand, it is by reason that a considerable amount of the probes, presented on the array, were deduced from EST (expressed sequence tag) sequences. ESTs are sequences known to be expressed but are not yet annotated to genes. Thus, it was not possible to assign a GO annotation for these ESTs (GO annotated genes on array 4217 out of 9802). Nevertheless, in the liver 4022 out of 9320, in the spleen 3738 out of 8800 and in small intestine epithelium 3600 out of 8339 probes/genes could be GO annotated and associated to biological processes in which they play a role. For the remaining genes no GO annotation was available and they were consequently excluded from further GO analysis. For defining a significant enrichment of DEGs within a biological process an enrichment score (EASE Score) was calculated and significance was assumed for an EASE Score of ≤ 0.05. Findings from liver tissue were chosen as example and are presented in Table 19. Results from spleen and small intestine epithelium are given in Table 27 and Table 29 (appendix).

q-value threshold

Table 19 shows, that the decision whether to apply a FDR correction or not and which stringency (q ≤ 0.2 / 0.1 / 0.05) to use can strongly influence the results. In liver tissue

only few processes (e.g. “nucleic acid metabolism”, “regulation of Wnt receptor signaling pathway”) showed partial consistency, with respect to significance, when applying different thresholds. In general the pattern of significant processes varied strongly and non-predictably with the number of DEGs included in the analysis. Comparable results were observed in the spleen and in small intestine epithelium (see Table 27 and Table 29, appendix). Highest agreement in the liver was observed between applying no FDR correction and a q ≤ 0.05; which is due to the similar quantities of DEGs annotated to the specific processes and to comparable lengths of DEG lists (see row: All).

Table 19: Influence of an application of different “False Discovery Rate” (FDR) thresholds on identified biological processes exhibiting a significantly increased number of differentially expressed genes. Different gene expression refers to the liver of rats fed rBV-VP60 in comparison with rats fed wtBV. Grey color marks significantly affected biological processes with ES ≤ 0.05.

Liver	**Number of GO annotated genes on the array**	**Number of differentially expressed GO annotated genes**							
Biological process (GO term)		**p ≤ 0.05**		**q ≤ 0.2**		**q ≤ 0.1**		**q ≤ 0.05**	
		n[1]	**ES[2]**	**n[1]**	**ES[2]**	**n[1]**	**ES[2]**	**n[1]**	**ES[2]**
Nucleic acid metabolism	760	248	0.05	628	0.01	441	0.08	239	0.04
Regulation of Wnt receptor signaling pathway	14	9	0.03	12	0.67	10	0.35	9	0.02
Macromolecule catabolism	124	47	0.05	99	0.56	75	0.20	45	0.06
Spermatid development	16	11	0.01	14	0.56	12	0.21	9	0.06
Aldehyde metabolism	11	8	0.02	10	0.59	10	0.07	7	0.06
Germ cell development	19	12	0.01	18	0.21	17	0.01	10	0.06
Epidermis morphogenesis	13	7	0.16	12	0.47	12	0.03	6	0.31
Transport	1120	339	0.44	915	0.01	650	0.03	329	0.30
Organelle organization and biogenesis	275	86	0.38	237	0.00	160	0.23	80	0.51
DNA repair	50	17	0.43	46	0.03	30	0.42	16	0.47
Sensory perception of smell	50	13	0.86	46	0.03	30	0.42	13	0.81
All	4022	1205		3183		2234		1153	

[1] n = number of differentially expressed genes in process

[2] ES = calculated EASE Score based on modified Fishers´ exact test; significance was assumed at ES ≤ 0.05

Some processes, e.g. “regulation of Wnt receptor signaling pathway”, did not exhibit significance at lower stringencies (q ≤ 0.1) but did meet the criteria at a more stringent threshold (q ≤ 0.05) although the number of significant genes in process was nearly the same (10 vs. 9). It would be expected that with larger DEG lists as obtained e.g. at a q ≤ 0.1, the number of significant processes would be larger as well and would diminish but overlap along with raising threshold stringency and a reduced number of DEGs. As to be seen from Table 19 the results are different. Less processes appeared to be significant with an increased stringencies but the agreement of processes was very

low or not existent. For calculating whether a process is significantly affected by a diet, the following question is generally asked (e.g. “regulation of Wnt receptor signaling pathway” at q ≤ 0.1 and q ≤ 0.05):

Are e.g. **10 out of 2234** or **9 out of 1153** genes significantly more than **14 out of 4022** genes, or is it just random chance?

q ≤ 0.1	q ≤ 0.05*	q ≤ 0.1 and q ≤ 0.05
DEGs in process / all DEGs	DEGs in process / all DEGs	all genes in process / all genes
10 / 2234 = 0.004	**9 / 1153 = 0.008**	**14 out of 4022 = 0.003**

* the process “regulation of Wnt receptor signaling pathway” had an EASE Score ≤ 0.05

This example shows that the ratio between DEGs within the process and all DEGs increased with a more stringent q-value (0.004 vs. 0.008). The ratio between all genes within the process and the entire list of analyzed genes, which was used as reference, remained the same (0.003). Thus, the calculated EASE Score changed and the process that was not significant at a q ≤ 0.1 became significant at a q ≤ 0.05. This was due to the distinctively modified “n” of the complete DEG list at the particular threshold, whereas the number of DEGs in a specific process did hardly change. Genes of “regulation of Wnt receptor signaling pathway” tended to have high q-values, the distribution was not even.

Fold change filter in combination with a q-value ≤ 0.05

In addition to selecting DEGs by FDR criterion (q ≤ 0.05), FC filters were applied and the impact on the biological interpretation was likewise investigated. The number of significantly diet-affected processes in the liver of rats was reduced to zero with a FC filter ≥ 2 and q ≤ 0.05 (see Table 20) due to a drastically reduced list of DEGs (compare Table 18). When filtering with a FC ≥ 1.5, “nucleic acid metabolism” was the only category that was significant in agreement with results when no FC filter was applied. The fact that threshold choice and significance are not linked linearly and that results varied non-predictably with the number of DEGs gives reason to the assumption that the distribution of FCs is likewise not even between the DEGs in process and all DEGs. Besides, a filter of FC ≥ 2 seemed to be very stringent. In the spleen only one and in the liver and in small intestine epithelium no biological processes had EASE Scores ≤ 0.05 (see Table 20 and Table 28). This is in agreement with results from 4.1.2.1 where it was shown that the frequency of high fold changes (≥ 2) was very low in the data used within this work (Figure 8A-C). Thus, setting a FC filter of ≥ 2 strongly reduced the DEG list and accordingly the number of biological processes with a significant enrichment of DEGs.

Table 20: Influence of an application of different fold change filters (no FC filter; FC ≥ 1.5; FC ≥ 2) on the identified biological processes exhibiting a significant over-representation of differentially expressed genes. Different gene expression refers to the liver of rats fed rBV-VP60 in comparison to rats fed wtBV and to DEGs with q ≤ 0.05. Grey color marks significantly affected biological processes with ES ≤ 0.05.

Liver		**Number of differentially expressed GO annotated genes**					
Biological process (GO term)	**Number of GO annotated genes on the array**	**no FC**		**FC ≥ 1.5**		**FC ≥ 2**	
		n[1]	**ES[2]**	**n[1]**	**ES[2]**	**n[1]**	**ES[2]**
Regulation of Wnt receptor Signaling pathway	14	9	0.02	-	-	-	-
Nucleic acid metabolism	760	239	0.04	25	0.01	-	-
Metanephros development	18	6	0.62	4	0.01	1	1.00
Positive regulation of metabolism	173	51	0.50	10	0.01	-	-
Regulation of cellular metabolism	566	174	0.15	21	0.01	1	1.00
Kidney development	22	6	0.80	4	0.01	1	1.00
Intracellular signaling cascade	495	142	0.55	17	0.04	2	0.82
Alcohol metabolism	164	54	0.16	8	0.05	1	1.00
All	4022	1153		83		14	

[1] n = number of differentially expressed genes in process

[2] ES = calculated EASE Score based on modified Fishers´ exact test; significance was assumed at ES ≤ 0.05

4.1.2.3 Regulation of genes involved in biological processes, exhibiting a significant enrichment of differentially expressed genes

In the spleen and the liver significant expression changes were monitored when applying selected threshold settings. Biological processes presented in Table 20 and Table 28 (see appendix) with q ≤ 0.05 and FC ≥ 1.5 were regarded as being affected by the rBV-VP60 diet. Small intestine epithelium did not exhibit any significant effects on that condition. Regulation of genes involved in these diet-affected biological processes is given in Figure 9, Figure 10(A-B) and Figure 11(A-D). In the spleen an enrichment of DEGs was observed in lipid catabolism. Four of the five associated genes had a decreased expression level in rBV-VP60 group compared to wtBV group (see Figure 9). One gene had a increased expression. In liver several biological processes including

- nucleic acid metabolism
- metanephros and kidney development (comprises two processes)
- intracellular signaling cascade
- alcohol metabolism
- regulation of cellular metabolism
- positive regulation of metabolism

were significantly affected by the diet.

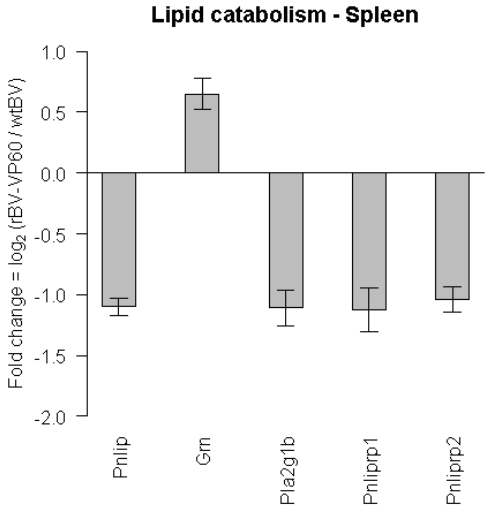

Figure 9: Regulation of differentially expressed genes as revealed by microarray technology. Genes are involved in lipid catabolism, a diet-affected biological process (GO annotation) that was identified in the spleen of rats fed rBV-VP60 compared to wtBV group. (Abbreviations: Pnlip: pancreatic lipase; Grn: granulin; Pla2g1b: phospholipase A2, group IB, pancreas; Pnliprp1: pancreatic lipase related protein 1; Pnliprp2: pancreatic lipase-related protein 2)

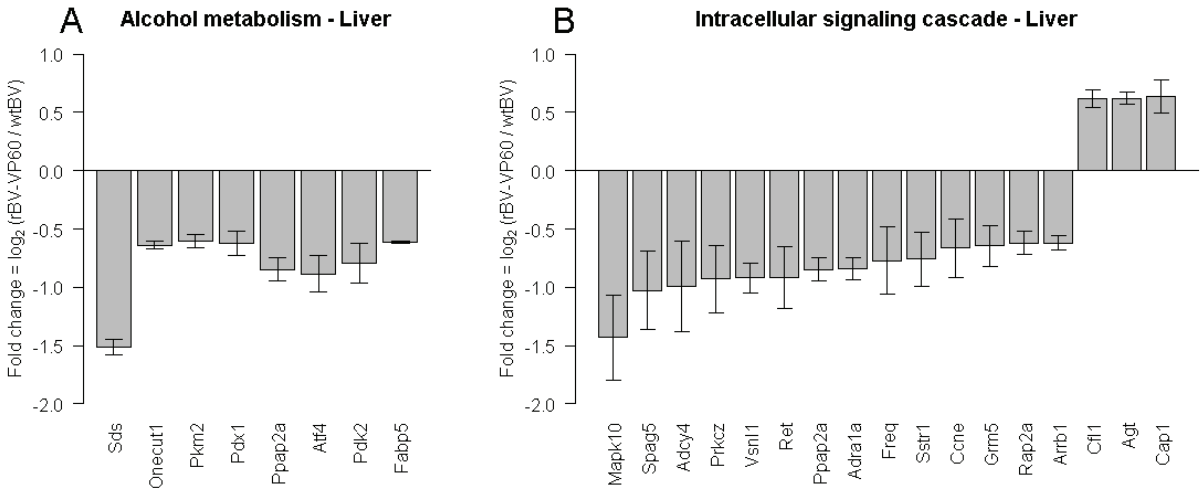

Figure 10(A-B): Regulation of differentially expressed genes involved in several diet-affected biological processes (GO annotation) as revealed by microarray technology. Different expression refers to the liver of rats fed rBV-VP60 compared to wtBV group. (Abbreviations: Sds: serine dehydratase; Onecut1: one cut domain, family member 1; Pkm2: pyruvate kinase, muscle; Pdx1: pancreatic and duodenal homeobox 1; Ppap2a: phosphatidic acid phosphatase 2a; Atf4: activating transcription factor 4; Pdk2: pyruvate dehydrogenase kinase, isoenzyme 2; Fabp5: fatty acid binding protein 5, epidermal; Mapk10: mitogen activated protein kinase 10; Spag5: sperm associated antigen 5; Adcy4: adenylate cyclase 4; Prkcz: protein kinase C, zeta; Vsnl1: visinin-like 1; Ret: ret proto-oncogene; Adra1a: adrenergic receptor, alpha 1a; Freq: frequenin homolog; Sstr1: somatostatin receptor 1; Ccne: cyclin E1; Grm5: glutamate receptor, metabotropic 5; Rap2a: RAS related protein 2a; Arrb1: arrestin, beta 1; Cfl1: cofilin 1, non-muscle; Agt: angiotensinogen (serpin peptidase inhibitor, clade A, member 8); Cap1: CAP, adenylate cyclase-associated protein 1).

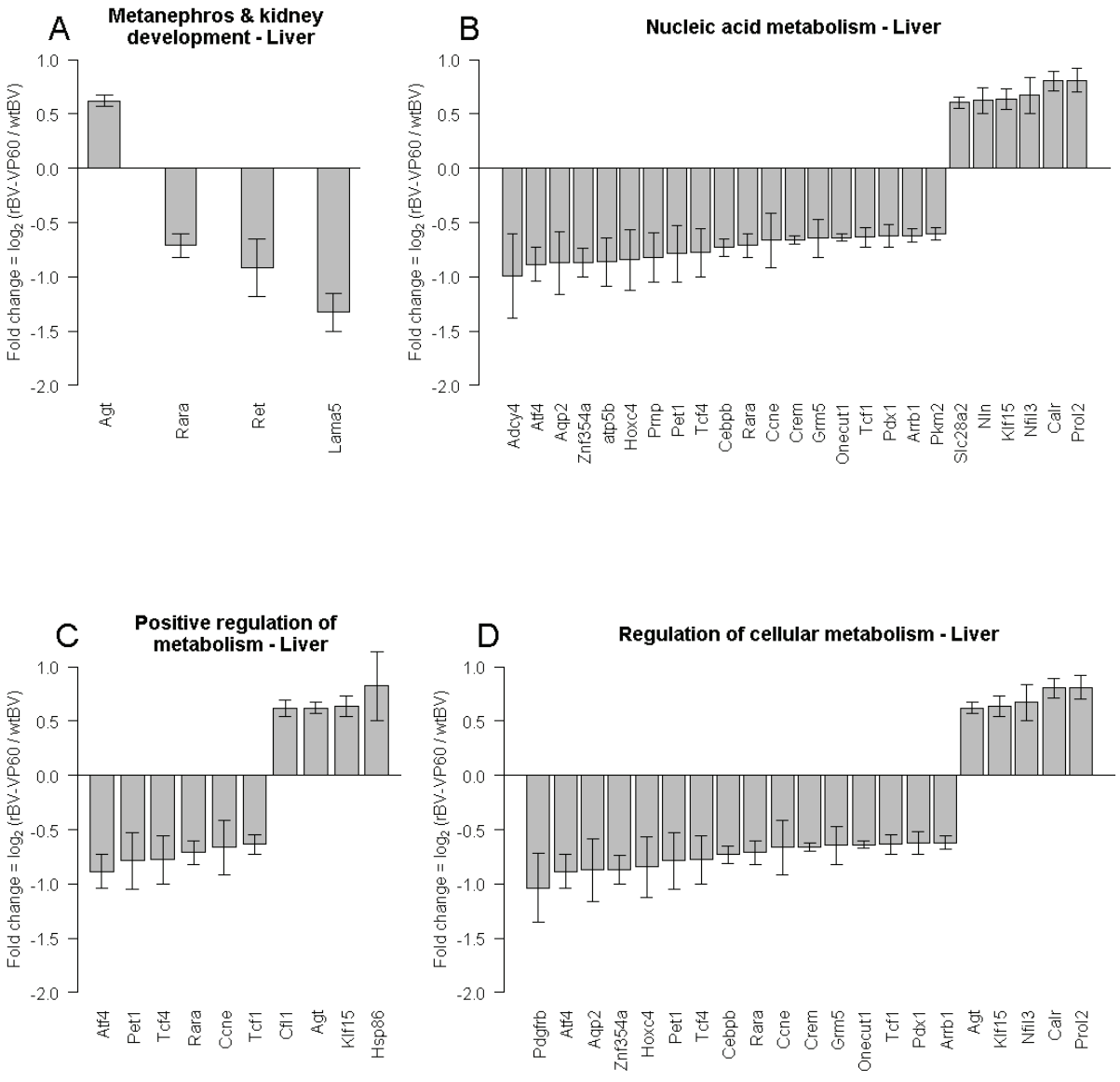

Figure 11(A-D): Regulation of differentially expressed genes involved in several diet-affected biological processes (GO annotation) as revealed by microarray technology. Different expression refers to the liver of rats fed rBV-VP60 compared to wtBV group. (Abbreviations: Agt: angiotensinogen (serpin peptidase inhibitor, clade A, member 8); Rara: retinoic acid receptor, alpha; Ret: ret proto-oncogene; Lama5: laminin, alpha 5; Adcy4: adenylate cyclase 4; Aqp2: aquaporin 2; Znf354a: zinc finger protein 354A; Hoxc4: homeo box C4; Prnp: prion protein; Crem: cAMP responsive element modulator; Grm5: glutamate receptor, metabotropic 5; Onecut1: one cut domain, family member 1; Pdx1: pancreatic and duodenal homeobox 1; Arrb1: arrestin, beta 1; Pkm2: pyruvate kinase, muscle; Slc28a2: solute carrier family 28 (sodium-coupled nucleoside transporter), member 2; Nln: neurolysin (metallopeptidase M3 family) ; Nfil3: nuclear factor, interleukin 3 regulated; Prol2: proline-rich nuclear receptor coactivator 1; Atf4: activating transcription factor 4; Pet1: Pet1; Tcf4: transcription factor 4; Ccne: cyclin E1; Tcf1: HNF1 homeobox A; Cfl1: cofilin 1, non-muscle; Klf15: Kruppel-like factor 15; Hsp86: heat shock protein 90, alpha (cytosolic), class A member 1; Pdgfrb: platelet derived growth factor receptor, beta polypeptide).

All molecules involved in “alcohol metabolism” had a reduced expression level after feeding rBV-VP60 in comparison to the wtBV fed rats (see Figure 10A). Genes playing a role in “intracellular signaling cascade”, in “metanephros and kidney development”, in “nucleic acid metabolism” and in “regulation of cellular metabolism” showed a predominant down-regulation in rBV-VP60 group (Figure 10B + Figure 11(A, B, D). Molecules attributed to the GO process “positive regulation of metabolism” were both down- and up-regulated after feeding the GM feed additive (see Figure 11C).

4.2 Comparison of microarray and real-time RT-PCR measurements

In addition to the DNA chip hybridization measurements (microarray), gene expression of several randomly selected molecules was determined by using real-time RT-PCR – a method that is often used to validate microarray findings. These genes were chosen from the microarray experiment described in 4.1 and had revealed significant expression changes ($q \leq 0.05$ and $FC \geq 1.5$) in the tissues liver or spleen (see Table 21). Transcript levels of the housekeeping gene Gapdh (glyceraldehyde-3-phosphate dehydrogenase) were measured in the spleen and the liver in order to normalize for equal RNA amounts (see Figure 12). As to be expected, Gapdh did not show significant differences between the two feeding groups when analyzed with t-test. It could be used for normalization in further analyses.

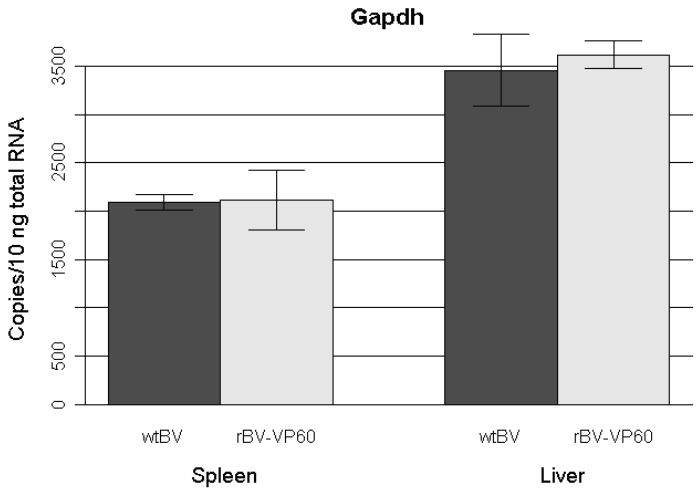

Figure 12: Transcript level of housekeeping gene Gapdh (Glyceraldehyde-3-phosphate dehydrogenase) measured via real-time RT-PCR in the spleen and the liver of rats fed either a wtBV or a rBV-VP60 feed additive (n = 3).

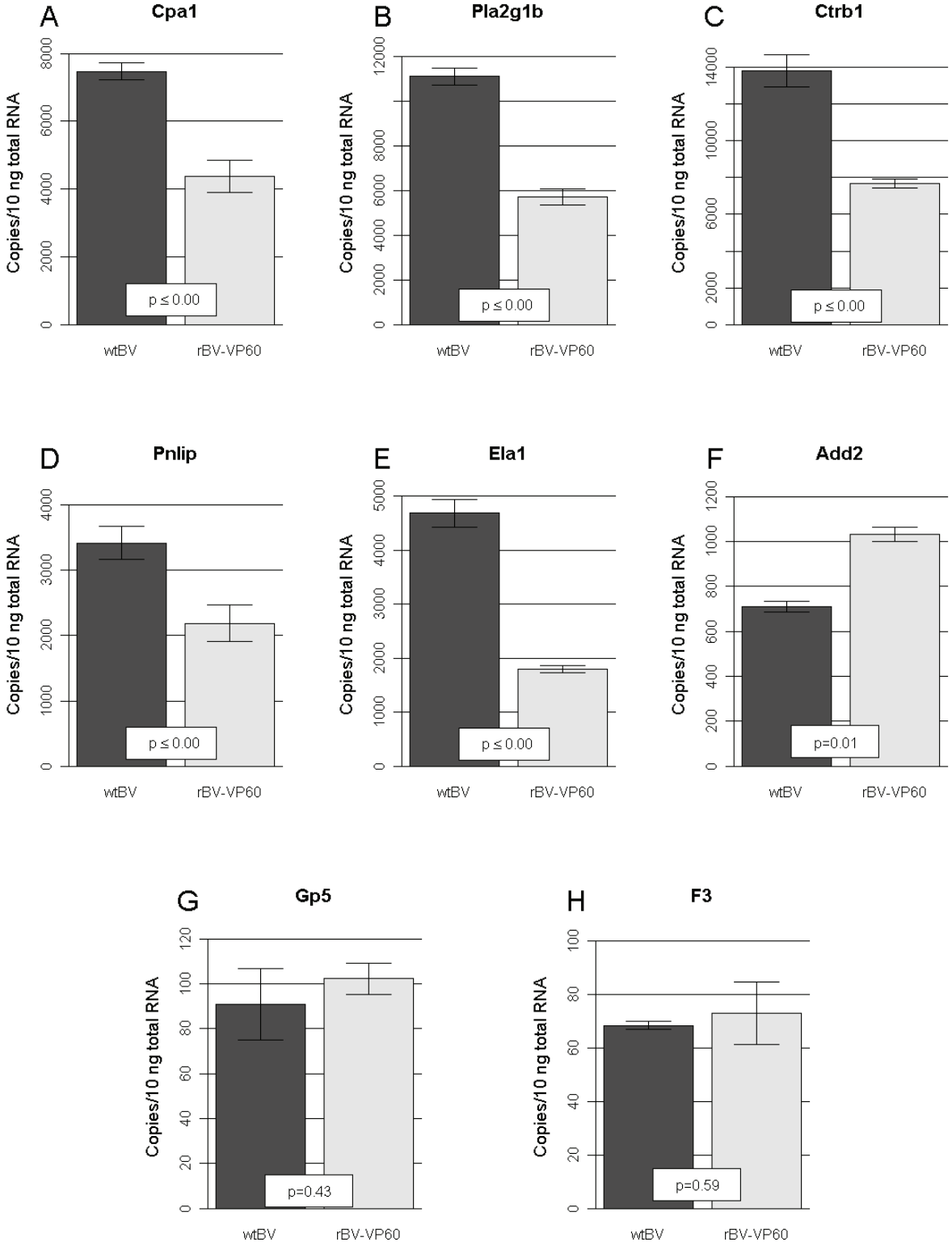

Figure 13(A-H): Transcript levels of selected molecules (A: carboxypeptidase A; B: phospholipase A2, group IB; C: chymotrypsinogen B1; D: pancreatic lipase; E: elastase 1, pancreatic; F: adducin 2 (beta); G: glycoprotein 5, platelet; H: coagulation factor III) measured via real-time RT-PCR in the spleen of rats fed either a wtBV or an rBV-VP60 feed additive (n = 3).

In general real-time RT-PCR results were in agreement with the results of the microarray study. Most studied genes that had shown significant expression differences between the feeding groups in microarray investigations also exhibited significant differences in real-time RT-PCR approach. In spleen five genes (Ela1: elastase 1, pancreatic; Pla2g1b: phospholipase A2, group IB; Pnlip: pancreatic lipase; Ctrb1: Chymotrypsinogen B1; Cpa1: carboxypeptidase A1) were down-regulated in rBV-VP60 group (see Figure 13A-H) and significance of the difference was proven in real-time RT-PCR approach. One gene (Add2: adducin 2 (beta) was significantly up-regulated in rBV-VP60 group when studied by real-time RT-PCR as well as by chip hybridization. Direction of regulation coincided with results obtained from microarray study. For two genes (Gp5: glycoprotein 5, platelet; F3: coagulation factor III) significant differences in transcript level could not be proved by real-time RT-PCR although it was demonstrated using microarray technology. In the liver both genes (Rara: retinoic acid receptor, alpha; Cebpb: CCAAT/enhancer binding protein (C/EBP), beta) investigated by real-time RT-PCR showed a down-regulation in rBV-VP60 group compared to wtBV group; as it was likewise observed with microarray approach (see Figure 14A+B). Moreover significance of these differences could be proved.

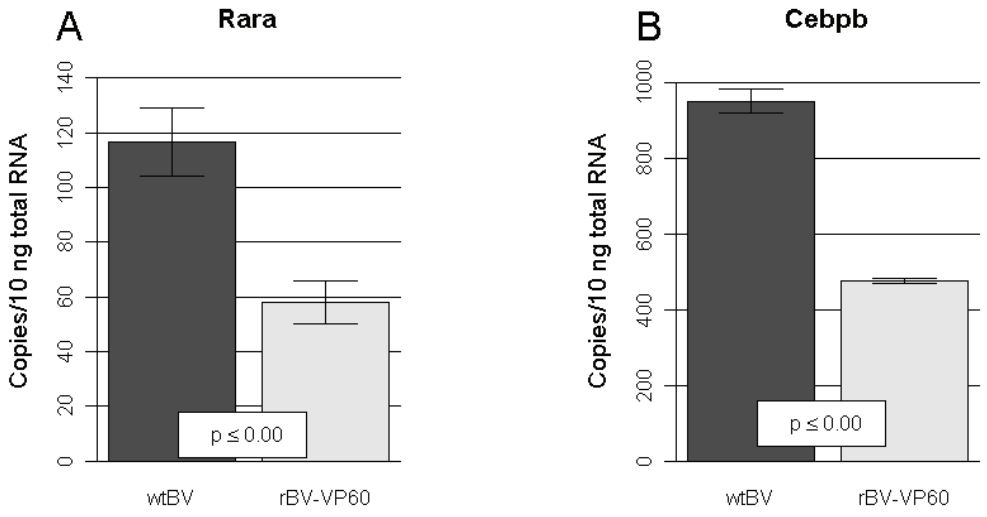

Figure 14(A+B): Transcript levels of selected molecules (A: retinoic acid receptor, alpha; B: CCAAT/enhancer binding protein (C/EBP), beta) measured via real-time RT-PCR in the liver of rats fed either the wtBV or the rBV-VP60 feed additive (n = 3).

Correlation of expression values between microarray and real-time RT-PCR measurements was high (r = 0.72) and significant (p-value= 0.0004, see Figure 15) with a confidence interval (95 %) of 0.40 – 0.88.

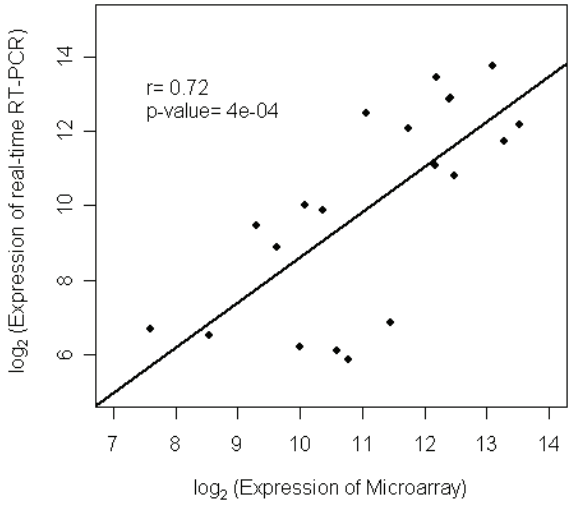

Figure 15: Correlation between expression values measured with real-time RT-PCR and microarray technology. Measurements of both groups (wtBV and rBV-VP60) and of tissues spleen and liver are included and comprise the following genes: Ela1: elastase 1, pancreatic; Pla2g1b: phospholipase A2, group IB; Pnlip: pancreatic lipase; Ctrb1: Chymotrypsinogen B1; Cpa1: carboxy-peptidase A1; Add2: adducin 2 (beta); Rara: retinoic acid receptor, alpha; Cebpb: CCAAT/enhancer binding protein (C/EBP), beta; Gp5: glycoprotein 5, platelet; F3: coagulation factor III.

Table 21: Selected differentially expressed genes identified by microarray technology in rats fed rBV-VP60 compared to rats fed wtBV

Tissue	Gene	Fold change	q-value
Spleen	Pnlip	-1.10	0.03
	Pla2g1b	-1.11	0.05
	Ctrb	-0.67	0.05
	Cpa1	-0.77	0.05
	Ela1	-1.07	0.00
	Add2	0.78	0.03
	Gp5	-0.94	0.05
	F3	-0.61	0.03
Liver	Rara	-0.61	0.04
	Cebp	-0.71	0.03

4.3 Characterization of physiological effects of genetically modified potatoes in rats using expression profiling

The aim of the present part of the study was to characterize the potential physiological effects of two specific feed components on the animal organism by means of expression profiling. The components consisted of two different genetically modified potato lines (rAlb-VP60 and rAlb-nptII). For this purpose a feeding experiment with laboratory rats was carried out and a comparative gene expression study using a “rat 10k oligo array” was conducted. This technology was shown to be useful for detecting diet-related physiological changes in rats in 4.1. Expression was studied in the spleen, the liver and

in small intestine epithelium of rats which ingested the genetically modified material.
For defining significant expression differences between the two feeding groups, a q-value of ≤ 0.05 was accepted according to results of 4.1.2. Moreover, a fold change filter was applied and set to FC ≥ 1.5 since the histograms of the fold change distribution had shown a very low occurrence of high FCs (see Figure 23A+B and Figure 24, appendix).

4.3.1 Comparative expression analyses in the spleen, the liver and in small intestine epithelium of rats fed recombinant rAlb-VP60 potatoes

rAlb-VP60 was one of the transgenic potatoes that were studied regarding their influence on animals' expression profile and therewith on physiology. Expression profiles of the rAlb-VP60 fed rats were then compared to profiles of animals that were given the non-transgenic near-isogenic line of these potatoes (wtAlb). rAlb-VP60 line expresses VP60 (capsid protein of RHD virus, which is able to induce immunity) and CtxB (Cholera toxin subunit B, which is known to have adjuvant activity). Additionally nptII, a marker gene used for plant selection (nptII confers resistance to antibiotics e.g. canamycin), is produced. Effects on physiology of the animals which ingested this feed component can possibly be caused by altered composition or nutrient value of the potatoes due to transgenesis or may occur due to a potential bioactivity of the "foreign" genes.

4.3.1.1 Identification of differentially expressed genes in rats fed rAlb-VP60 potatoes

From the microarray analysis 7021 (spleen), 8058 (liver) and 8398 (small intestine epithelium) evaluable signals were obtained (total probes/genes on array: 9802). These signals had passed quality filtering and could be included in further statistical analysis (see Figure 16A).
Microarray analysis revealed a significantly altered gene expression pattern between rats fed rAlb-VP60 and rats fed wtAlb. Distinct changes were observed in the spleen and in small intestine epithelium whereas only minor changes were found in the liver. The number of significant differentially expressed genes varied widely between the tissues. In the spleen 117 genes succeeded the significance criteria (see Figure 16B). 30 genes showed an elevated transcript level and 87 genes were down-regulated in rAlb-VP60 group in comparison to wtAlb group. In the liver two genes showed significant expression differences; one gene was up-regulated and one gene was down-regulated. In small intestine epithelium 27 DEGs were found, whereof 13 genes had an increased and 14 a

decreased level expression (see appendix Table 30, Table 31 and Table 32: list of all differentially expressed genes).

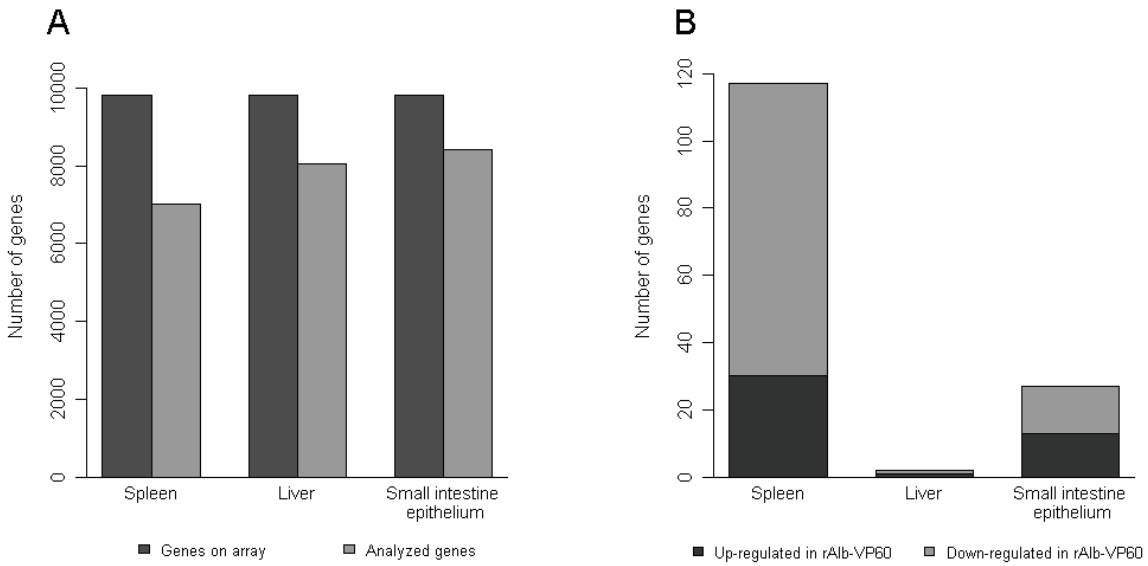

Figure 16(A+B): A: Number of genes on the microarray and number of genes included in statistical analysis. B: number of differentially expressed genes which had a q ≤ 0.05 and passed an additional FC filter of ≥ 1.5. Data is referring to the comparative gene expression profiling in different tissues of rats fed the rAlb-VP60 compared to rats fed the wtAlb potato.

4.3.1.2 Identification of biological processes with a significant enrichment of differentially expressed genes in rats fed rAlb-VP60 potatoes

As reported in 4.3.1.1, gene expression in the spleen and in small intestine epithelium differed significantly between the two feeding groups rAlb-VP60 and wtAlb. DEGs from these tissues were used for further identification of diet-affected biological processes. The amount of only two DEGs as observed in the liver does not meet the requirements of statistical analysis for identifying GO annotation-based physiological effects. These genes were therefore excluded from further analysis. A GO annotation was available for 67 of the 117 DEGs in the spleen. Out of 27 DEGs found in small intestine epithelium nine could be annotated based on the GO database.

In the spleen the GM diet had a significant impact on rats' physiology. An over-representation of DEGs in several biological processes was demonstrated by means of an EASE score (≤ 0.05, see Table 22). For condensed illustration in figures, several biological processes shown in Table 22 were abstracted because they are represented by the same genes and are functionally very closely related. In the following "**lipid catabolism**" embodies genes playing a role in "lipid catabolism" and "membrane lipid catabolism". The term "**pigment/porphyrin metabolism**" summarizes genes of "porphyrin biosynthesis", "pigment biosynthesis", "heme metabolism", "porphyrin

metabolism", "pigment metabolism" and "pigmentation". "Macromolecule catabolism" as well as "proteolysis and peptidolysis" associated genes are included in "**protein catabolism**". Genes of GO process "catabolism" are also represented by the terms "lipid catabolism" and "protein catabolism". This process was therefore not separately displayed as figure.

As shown in Figure 17(A, B, D, E)), the application of rAlb-VP60 diet caused mainly a significant down-regulation of genes associated with "developmental processes", "protein catabolism", "digestion" and "lipid catabolism" in the spleen of rats. In contrast, a diet-associated up-regulation of gene expression was mainly to be found in genes related to "pigment/porphyrin metabolism" (see Figure 17C). Genes playing a role in "developmental processes" showed primarily a reduced transcription level in the rAlb-VP60 feeding group, though up-regulation of some molecules was observed.

In small intestine epithelium only minor but nevertheless significant diet-related changes were observed. rAlb-VP60 feeding led to a down-regulation of two genes playing a role in "acute phase response" (see Table 23).

Table 22: Biological processes exhibiting significantly increased numbers of differentially expressed genes, as revealed by microarray technology. Different expression refers to the spleen of rats fed rAlb-VP60 potatoes in comparison with wtAlb group. Significance of expression changes was assumed for $q \leq 0.05$ and a $FC \geq 1.5$ was accepted. Significant over-representation of differentially expressed genes in biological processes was defined by means of an EASE Score ≤ 0.05.

Spleen **Biological process (GO term)**	**Number of GO annotated genes in process**	**Number of differentially expressed GO annotated genes**	
		n[1]	**ES**[2]
Lipid catabolism	31	7	0.00
Digestion	13	5	0.00
Catabolism	253	17	0.00
Protein catabolism	168	10	0.02
Proteolysis and peptidolysis	164	10	0.01
Macromolecule catabolism	171	10	0.02
Development	402	17	0.02
Heme biosynthesis	5	3	0.01
Membrane lipid catabolism	5	3	0.01
Pigment biosynthesis	6	3	0.01
Porphyrin biosynthesis	6	3	0.01
Heme metabolism	7	3	0.01
Porphyrin metabolism	8	3	0.01
Pigment metabolism	9	3	0.02
Pigmentation	9	3	0.02
All	2781	67	

[1] n = number of differentially expressed genes in process

[2] ES = calculated EASE Score based on modified Fishers exact test; significance is assigned at ES ≤ 0.05

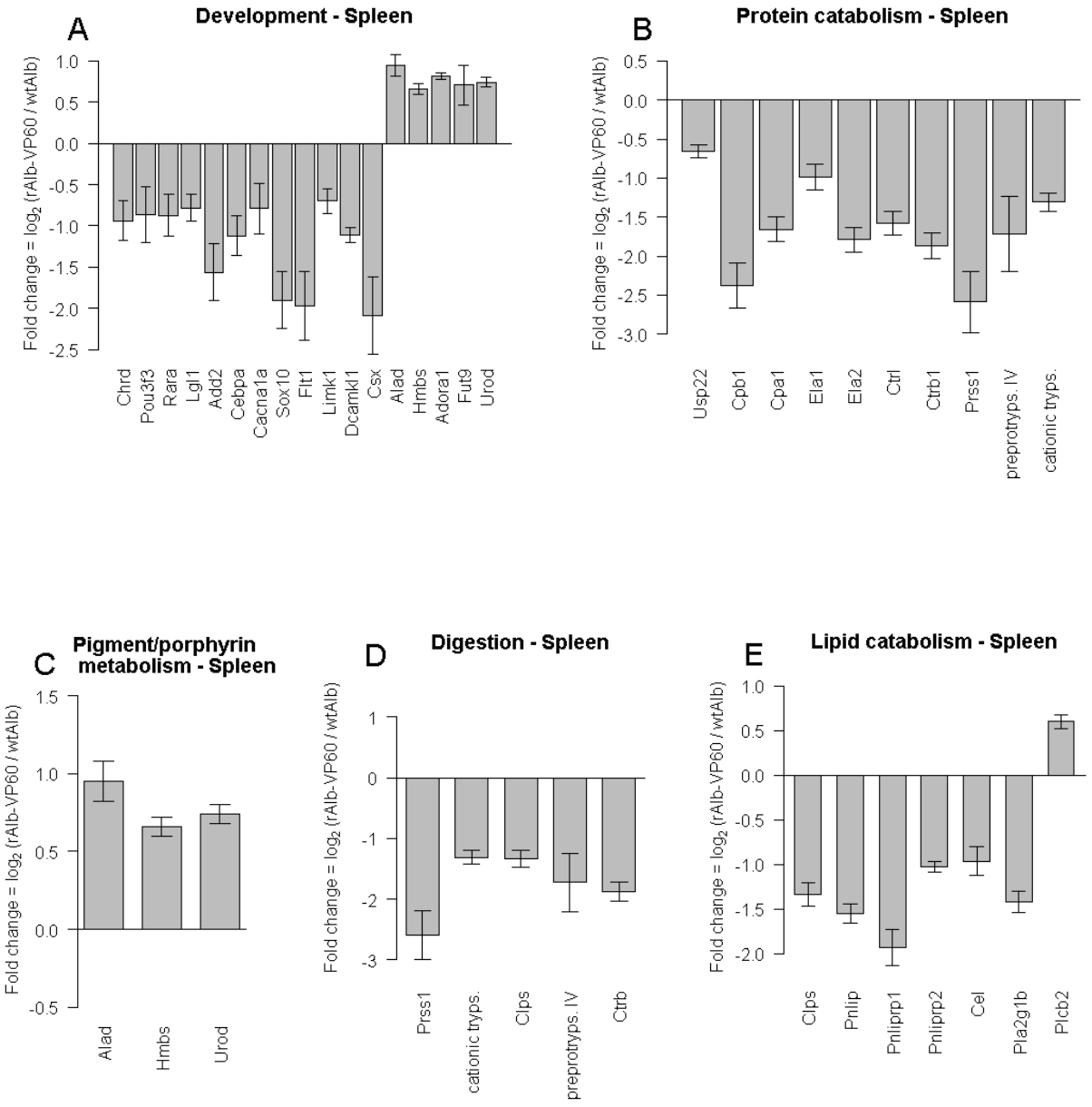

Figure 17(A-E): Regulation of differentially expressed genes, involved in several diet-affected biological processes (GO annotation) as revealed by microarray technology. Different expression refers to the spleen of rats fed rAlb-VP60 potatoes in comparison to wtAlb group. (Abbreviations: Chrd: chordin; Pou3f3: POU domain, class 3, transcription factor 3; Rara: retinoic acid receptor, alpha; Lgl1: cysteine-rich secretory protein LCCL domain containing 2; Add2: adducin 2 (beta); Cebpa: CCAAT/enhancer binding protein (C/EBP), alpha; Cacna1a: calcium channel alpha 1A; Sox10: SRY-box containing gene 10; Flt1: FMS-like tyrosine kinase 1; Limk1: LIM domain kinase 1; Dcamkl1: doublecortin-like kinase 1; Csx: NK2 transcription factor related, locus 5; Adora1: adenosine A1 receptor; Fut9: ucosyltransferase 9; Urod: uroporphyrinogen decarboxylase; Usp22: ubiquitin specific peptidase 22; Cpb1: carboxypeptidase B1; Cpa1: carboxypeptidase A1; Ela1: elastase 1, pancreatic; Ela2: elastase 2A; Ctrl: chymotrypsin-like; Ctrb1: chymotrypsinogen B1; Prss1: protease, serine, 1 (trypsin 1); preprotryps. IV: preprotrypsinogen IV; cationic tryps.: cationic trypsinogen; Alad: aminolevulinate, delta-, dehydratase; Hmbs: hydroxymethylbilane synthase; Clps: colipase, pancreatic; Pnlip: pancreatic lipase; Pnliprp1: pancreatic lipase related protein 1; Pnliprp2: pancreatic lipase related protein 2; Cel: carboxyl ester lipase; Pla2g1b: phospholipase A2, group IB; Plcb2: phospholipase C, beta 2).

Table 23: Biological process exhibiting significantly increased number of differentially expressed genes, as revealed by microarray technology. Different expression refers to small intestine epithelium of rats fed rAlb-VP60 potatoes in comparison to wtAlb group. For defining significant gene expression changes, a q of ≤ 0.05 and a FC ≥ 1.5 was accepted. Significant over-representation of differentially expressed genes in biological processes was defined by means of an EASE Score ≤ 0.05.

Small intestine epithelium	**Number of GO annotated genes in process**	**Number of differentially expressed GO annotated genes**	
Biological process (GO term		**n[1]**	**ES[2]**
Acute phase response	15	2	0.04
All	3366	9	

[1] n = number of differentially expressed genes in process

[2] ES = calculated EASE Score based on modified Fishers' exact test; significance is assigned at ES ≤ 0.05

4.3.1.3 Quantitative gene expression analysis of selected genes using real-time RT-PCR

Eight DEGs found in the spleen (see Figure 17A-D and Table 30) and one DEG found in small intestine epithelium (see Table 32), representing significant biological processes as described in 4.3.1.2, were selected. Transcript levels were determined using real-time RT-PCR in order to underline results of the microarray study. Results are shown in Figure 18(A-I).

In agreement with microarray results, two genes playing a role in "lipid catabolism" (Pla2g1b, Pnlip) and three genes, which are associated with "protein catabolism" (Cpa1, Ela1 and Ctrb1, that is also associated with "digestion"), showed a significant ($p \leq 0.05$) down-regulation in rAlb-VP60 feeding group. The biological GO process "development" was represented by the molecules Rara and Flt1 which equally exhibited a significant down-regulation and thus confirmed microarray results. Alad ("pigment/porphyrin metabolism"; "development") revealed a significantly increased expression level in real-time RT-PCR analysis as well as in microarray examination. Expression of Pap1, an "acute phase response" related gene, was reduced in small intestine epithelium of rAlb-VP60 feeding group. Significant differences were demonstrated with real-time RT-PCR and with microarray technology.

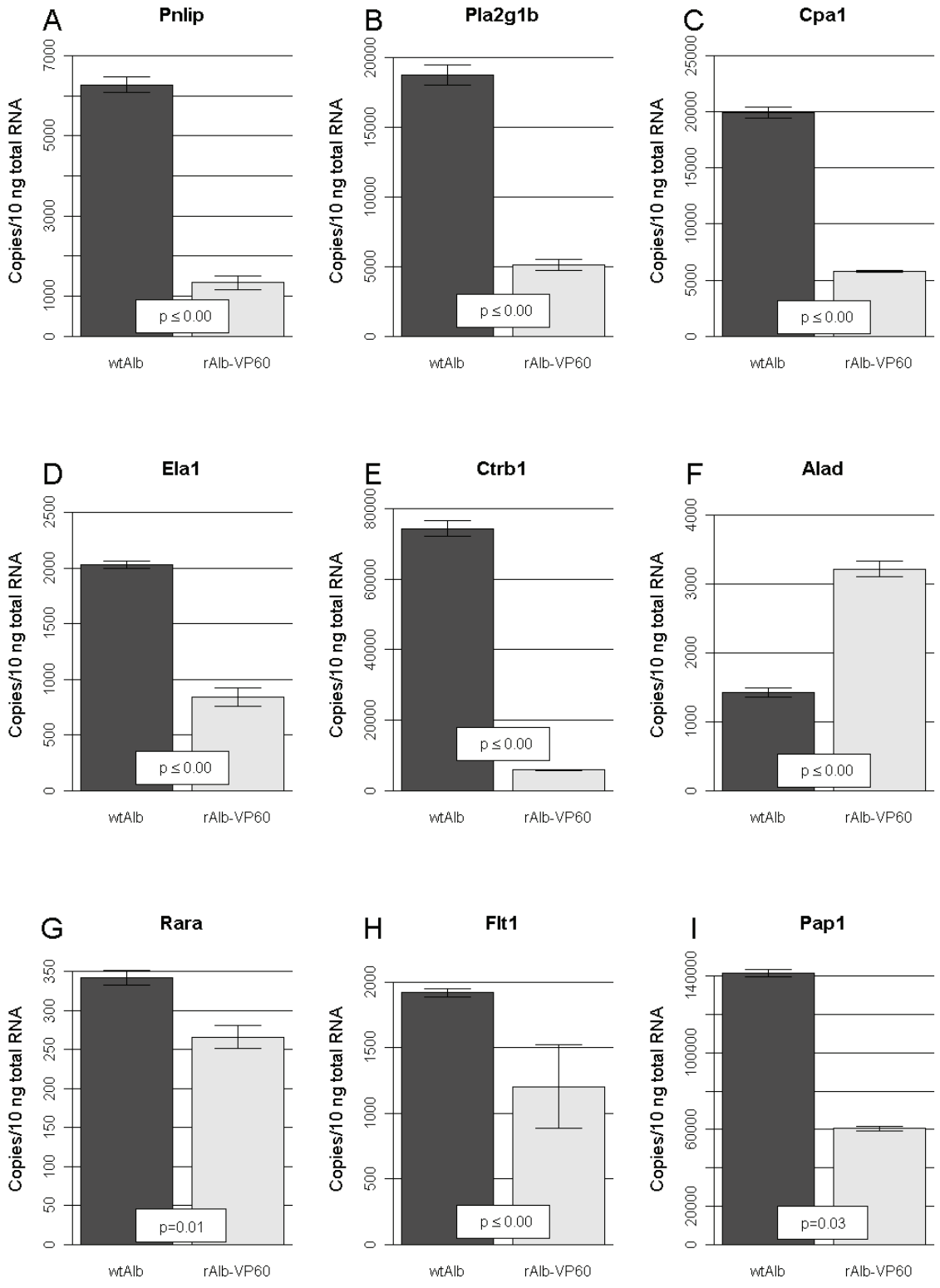

Figure 18 (A-I): Transcript level of selected genes (A: pancreatic lipase; B: phospholipase A2, group IB; C: carboxypeptidase A1; D: elastase 1, pancreatic; E: Chymotrypsinogen B1; F: aminolevulinate, delta-, dehydratase; G: retinoic acid receptor, alpha; H: FMS-like tyrosine kinase 1; I: pancreatitis-associated protein) measured via real-time RT-PCR in the spleen (A-H) and in small intestine epithelium (I) of rats fed either wtAlb or rAlb-VP60 potatoes (n = 3).

4.3.2 Comparative expression analysis in the spleen, the liver and in small intestine epithelium of rats fed rAlb-nptII potatoes

In a further experiment, the physiological effects of feeding recombinant rAlb-nptII potatoes were investigated. Expression profiles were compared to the corresponding control group that was given the near-isogenic non-GM potatoes (wtAlb). The transgenic tubers fed in this experiment were only expressing the nptII marker gene, but no VP60, CtxB or any other additional molecules. This marker is solely used for selecting plants that have successfully integrated the “foreign” DNA sequence of interest. It has no intended trait-improving effect on the plant. By investigating physiological effects of these potatoes it was intended to be able to identify effects that are caused due nptII gene insertion. The NPTII protein has no known bioactivity but its integration into the plant genome can possibly lead to an unexpectedly altered plant composition or nutritional value. Likewise to 4.3.1 this transgenic potato was fed to rats and gene expression was studied in the tissues spleen, liver and in small intestine epithelium.

4.3.2.1 Identification of differentially expressed genes in rats fed rAlb-nptII potatoes

The microarray investigations revealed 6707 (spleen), 6083 (liver) and 5959 (small intestine epithelium) signals, respectively that passed quality checking and could be further analyzed (see Figure 19A). Low quality signals (see 4.1.1) were not included in advanced data evaluation.

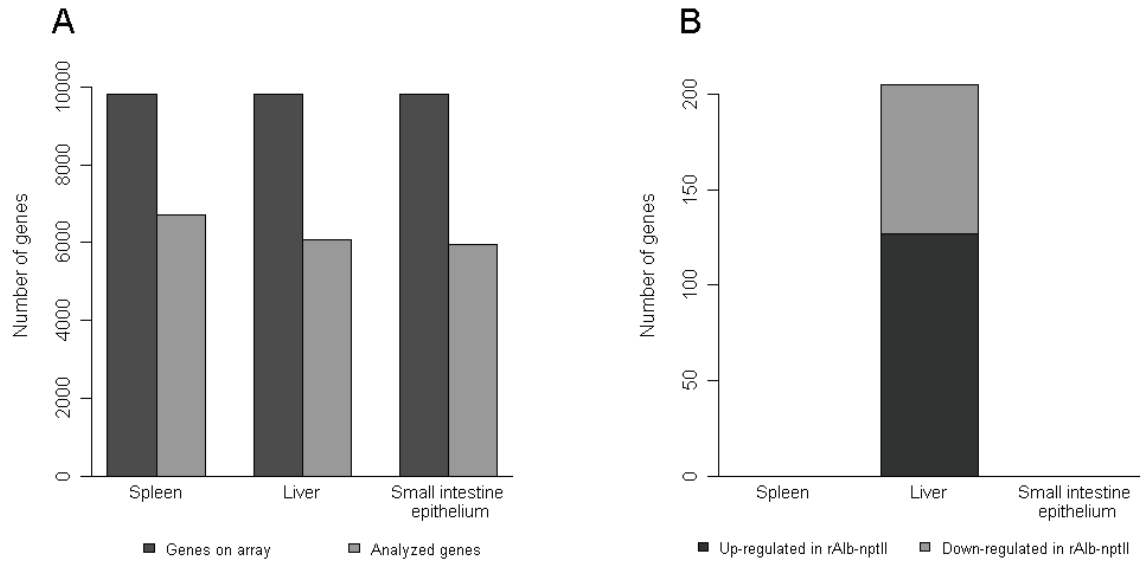

Figure 19(A+B): A: Number of genes on the microarray and number of genes included in statistical analysis. B: number of differentially expressed genes which had a q-value ≤ 0.05 and passed an additional FC filter of ≥ 1.5. Data is referring to the comparative gene expression profiling in different tissues of rats fed rAlb-nptII compared to rats fed wtAlb potato.

Only in the liver significant effects of rAlb-nptII feeding on gene expression in rats were observed. 205 genes were differentially expressed when compared to the expression level of wtAlb fed rats (see Figure 19B). 127 of these molecules had a higher transcript level in rAlb-nptII fed animals compared to the wtAlb group. 78 genes were down-regulated after rAlb-nptII feeding (see appendix Table 33: list of all differentially expressed genes in the liver). In the spleen and in small intestine epithelium no gene showed significant expression differences between the two feeding groups.

4.3.2.2 Identification of biological processes with a significant enrichment of differentially expressed genes in rats fed rAlb-nptII potatoes

For an identification of diet-affected biological processes DEGs found in the liver could be used because the remaining tissues did not exhibit significant effects. 123 out of the 205 DEGs could be GO annotated and thus be included in the further analysis.

By means of a GO analysis several biological processes with a significant enrichment of DEGs were identified. Feeding of rAlb-nptII potatoes led to significant changes in expression level of genes involved in “electron transport”, “lipid metabolism” and several “monosaccharide” and “alcohol metabolism”-related processes (see Table 24) in comparison to wtAlb potato feeding. “Monosaccharide biosynthesis”, “gluconeogenesis”, “hexose biosynthesis”, “alcohol biosynthesis” and “alcohol metabolism” will be referred to as “**alcohol/monosaccharide metabolism**” in that they are functionally very closely related according to the GO annotation structure and comprise the same genes.

Table 24: Biological processes exhibiting significantly increased numbers of differentially expressed genes, as revealed by microarray technology. Different expression refers to the liver of rats fed rAlb-nptII potatoes in comparison to wtAlb group. For defining significant gene expression changes a q-value of ≤ 0.05 and a FC ≥ 1.5 was accepted. Significant over-representation of differentially expressed genes in biological processes was defined by means of an EASE Score ≤ 0.05.

Liver Biological process (GO term	Number of GO annotated genes in process	Number of differentially expressed GO annotated genes	
		n[1]	ES[2]
Electron transport	116	16	0.00
Lipid metabolism	174	16	0.02
Monosaccharide biosynthesis	5	3	0.02
Gluconeogenesis	5	3	0.02
Hexose biosynthesis	5	3	0.02
Alcohol biosynthesis	5	3	0.02
Alcohol metabolism	88	9	0.05
All	2570	123	

[1] n = number of differentially expressed genes in process

[2] ES = calculated EASE Score based on modified Fishers´ exact test; significance is assigned at ES ≤ 0.05

As shown in Figure 20(A-C) nearly all genes, involved in “electron transport”, “alcohol/monosaccharide metabolism” as well as “lipid metabolism”, exhibited an up-regulation of gene expression in the rAlb-nptII group in comparison to the wtAlb group. Only few genes showed a down-regulation.

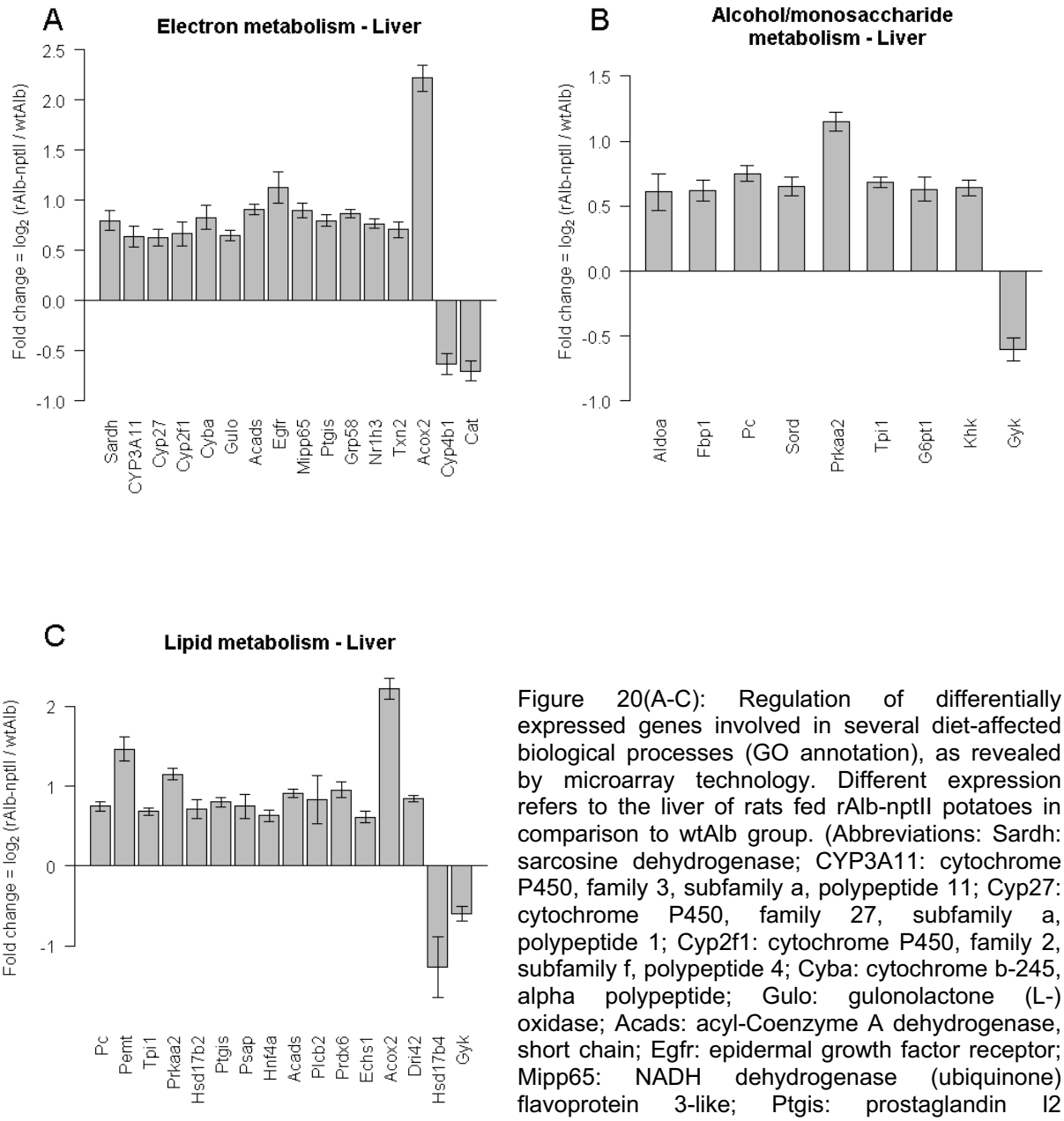

Figure 20(A-C): Regulation of differentially expressed genes involved in several diet-affected biological processes (GO annotation), as revealed by microarray technology. Different expression refers to the liver of rats fed rAlb-nptII potatoes in comparison to wtAlb group. (Abbreviations: Sardh: sarcosine dehydrogenase; CYP3A11: cytochrome P450, family 3, subfamily a, polypeptide 11; Cyp27: cytochrome P450, family 27, subfamily a, polypeptide 1; Cyp2f1: cytochrome P450, family 2, subfamily f, polypeptide 4; Cyba: cytochrome b-245, alpha polypeptide; Gulo: gulonolactone (L-) oxidase; Acads: acyl-Coenzyme A dehydrogenase, short chain; Egfr: epidermal growth factor receptor; Mipp65: NADH dehydrogenase (ubiquinone) flavoprotein 3-like; Ptgis: prostaglandin I2 (prostacyclin) synthase; Grp58: protein disulfide isomerase associated 3; Nr1h3: nuclear receptor subfamily 1, group H, member 3; Txn2: thioredoxin 2; Acox2: acyl-Coenzyme A oxidase 2, branched chain; Cyp4b1: cytochrome P450, family 4, subfamily b, polypeptide 1; Cat: catalase; Aldoa: aldolase A; Fbp1: fructose-1,6- biphosphatase 1; Pc: pyruvate carboxylase; Sord: sorbitol dehydrogenase; Prkaa2: protein kinase, AMP-activated, alpha 2 catalytic subunit; Tpi1: triosephosphate isomerase 1; G6pt1: solute carrier family 37 (glucose-6-phosphate transporter), member 4; Khk: ketohexokinase; Gyk: glycerol kinase; Pemt: phosphatidylethanolamine N-methyltransferase; Hsd17b2: hydroxysteroid (17-beta) dehydrogenase 2; Psap: prosaposin; Hnf4a: hepatic nuclear factor 4, alpha; Plcb2: phospholipase C, beta 2; Prdx6: peroxiredoxin 6; Echs1: enoyl Coenzyme A hydratase, short chain, 1, mitochondrial; Dri42: phosphatidic acid phosphatase type 2B; Hsd17b4: hydroxysteroid (17-beta) dehydrogenase 4).

4.3.2.3 Quantitative gene expression analysis of selected molecules using real-time RT-PCR

In addition to the microarray investigations, real-time RT-PCR was used to determine transcription levels of six selected molecules in the liver of rats fed rAlb-nptII or wtAlb potatoes. For these molecules a significant expression change was observed in microarray analysis (see Figure 20A-C or Table 33) and they are known to be involved in the biological processes “electron transport” (Acox2), “lipid metabolism” (Pemt, Hsd17b2, Prkaa2) and “alcohol/monosaccharide metabolism” (Fbp1, Pc, Prkaa2). These processes were identified as being diet-affected in 4.3.2.2. Transcript levels detected via real-time RT-PCR were partly consistent with microarray results (see Figure 21). A significant ($p \leq 0.05$) increase of expression levels in rAlb-nptII fed rats was shown for the molecules Acox2 and Hsd17b2.

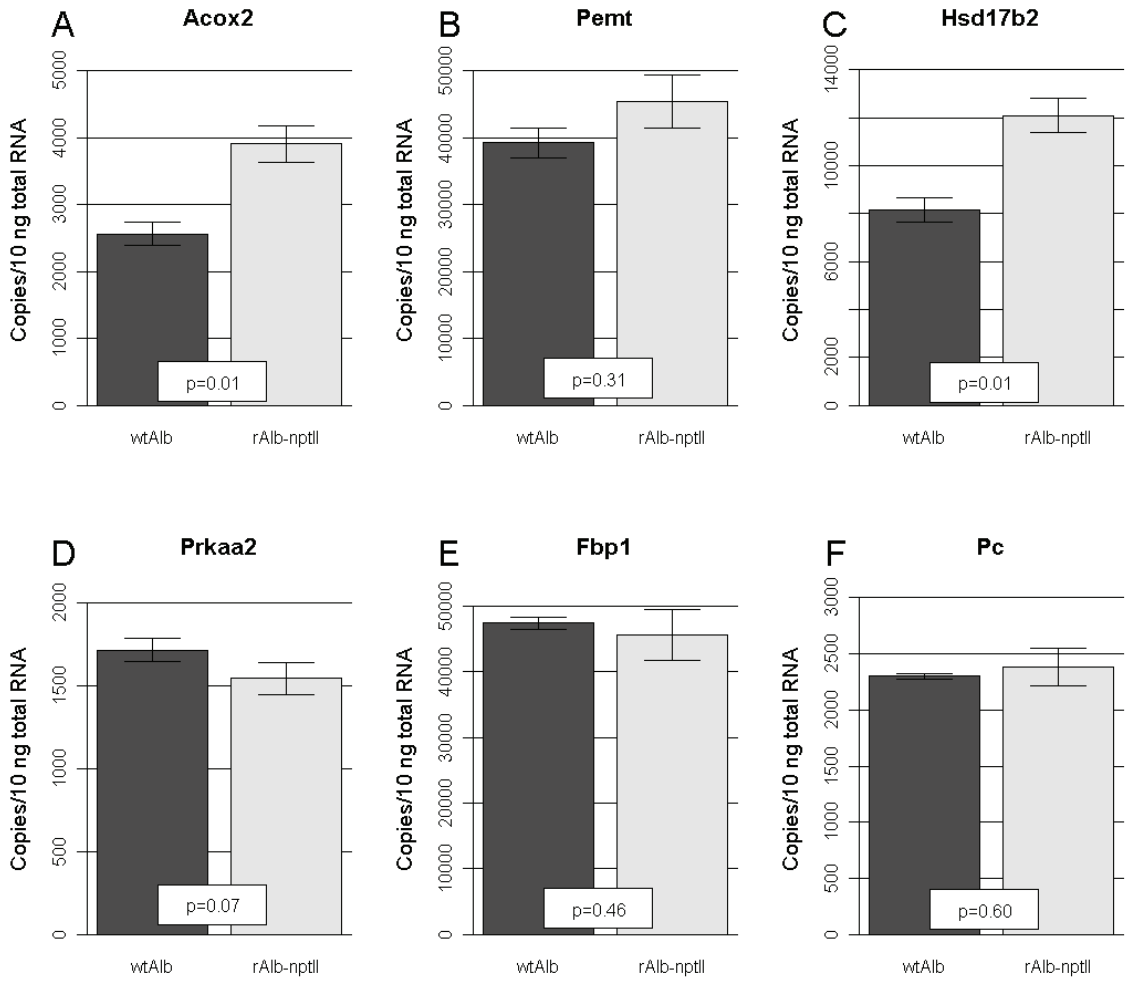

Figure 21(A-F): Transcript level of selected genes (A: acyl-Coenzyme A oxidase 2, branched chain; B: phosphatidylethanolamine N-methyltransferase; C: hydroxysteroid (17-beta) dehydrogenase 2; D: protein kinase, AMP-activated, alpha 2 catalytic subunit; E: fructose-1,6- biphosphatase 1; F: Pyruvate carboxylase) measured via real-time RT-PCR in liver of rats fed rAlb-nptII or wtAlb potatoes (n = 3).

For the four remaining genes Pemt, Prkaa2, Fbp1 and PC no significant expression changes between the two feeding groups were observed, although this was shown using microarray technology.

4.4 Comparative analysis of physiological effects observed in rats fed rBV-VP60 and in rats fed rAlb-VP60 components

Results (i.e. lists of DEGs) obtained from comparative expression profiling in different tissues of rats fed the VP60 potatoes (rAlb-VP60 vs. wtAlb) were compared with DEGs identified in the experiment with rats fed VP60 baculovirus additive (rBV-VP60 vs. wtBV). Both investigations (4.3 and 4.1.2.3) had revealed physiological changes due to a feeding of the particular transgenic dietary component. Though, an interpretation of the results of rAlb-VP60 experiment remained difficult because it could not be concluded whether the observed effects were due to the foreign genes (VP60/CtxB/nptII) or due to compositional alterations or changes in the nutritional value of the potatoes. Thus, a cross-comparison of DEGs and significantly affected biological processes identified in the two experiments should facilitate a further interpretation of the findings. The only concordant feed component given to the rats in both feeding trials (rBV-VP60 and rAlb-VP60) was the VP60 molecule. VP60 was expressed by the potato tubers as well as by the baculovirus expression system. Consequently, coinciding findings might be attributed to the bioactive or immunogenic properties of VP60.

In the spleen the transcript levels of 13 molecules were found to be significantly affected by feeding rAlb-VP60 as well as by feeding rBV-VP60 (see Figure 22). These genes showed a down-regulation in both groups (rAlb-VP60 and rBV-VP60) when comparing them to the corresponding control group (wtAlb and wtBV); though the effects observed in the rAlb-VP60 group were generally stronger. Functionally these genes are mainly associated with “lipid catabolism” (Clps, Pnlip, Pnliprp1, Pnliprp2, Pla2g1b) and “protein catabolism” (Cpb1, Ctrb1, Cpa1, Ela1, Ela2, Ctrl). Consequently in both investigations “lipid catabolism” had a significant enrichment of DEGs and was identified as being affected by the GM diets (rAlb-VP60 and rBV-VP60). “Protein catabolism” did show a significant over-representation of DEGs in rats fed the rAlb-VP60 potatoes but not in rBV-VP60 group, although genes associated with this process were congruently down-regulated (Figure 22). Further agreements of results from the tissues liver or small intestine epithelium were not detected.

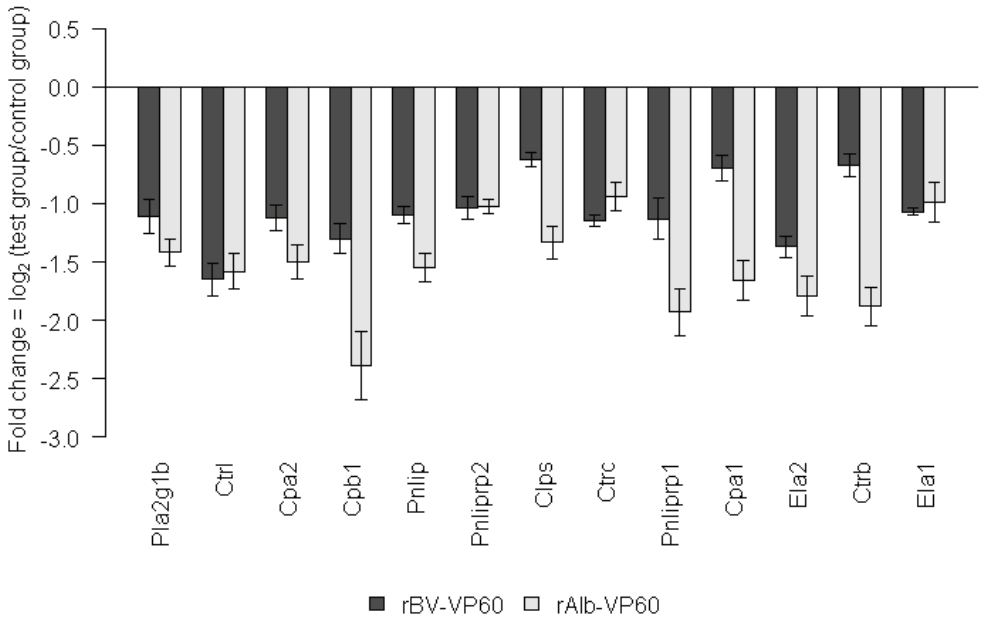

Figure 22: Regulation of significantly differentially expressed genes identified in the spleen of rats fed rAlb-VP60 (test group) potatoes as well as in the spleen of rats fed rBV-VP60 (test group). Difference in gene expression refers to a comparison with the corresponding non-GM control group (wtAlb or wtBV).

4.5 Comparative analysis of physiological effects observed in rats fed rAlb-VP60 and in rats fed rAlb-nptII components

A further cross-comparison of DEGs identified in the liver of animals fed rAlb-VP60 (experiment: rAlb-VP60 vs. wtAlb) and animals fed rAlb-nptII (experiment: rAlb-nptII vs. wtAlb) did not reveal any consistent results. In this case the coincident component was the nptII gene which was expressed in rAlb-VP60 as well as in rAlb-nptII potatoes.

4.6 Comparison of diet-affected biological processes identified with either individual gene analysis or gene set analysis

Commonly a two-step approach is used to identify treatment-affected processes. First, expression measurements between two groups are compared. Lists of altered genes are defined from significance or cut-off thresholds (IGA: individual gene analysis); as it was done in foregoing presented results. In a second step, these DEG lists are then compared to biologically defined gene sets (e.g. GO processes) and an enrichment of DEGs in specific processes is assessed. The strong dependency of such approaches on threshold parameter-settings has been demonstrated in 4.1.2. Thus, part of the data was re-analyzed with a more recently suggested analysis method (PAGE algorithm) which

belongs to the “gene set analysis” (GSA) approaches. IGA and GSA results were compared. The term GSA summarizes several methods which also focus on gene sets but use for instance ranked FCs or q-values of all genes analyzed, without limitation to threshold-based lists. For small sample sizes of less than five, the use of fold changes rather than t-statistics or other parameters is recommended. Consequently, the entire lists of fold changes were used as measurement of expression difference between the GM (rAlb-VP60 or rAlb-nptII) and the non-GM (wtAlb) feeding group and explored in GAZER (GSA-tool).

Table 25: Comparison of significant biological processes (GO) identified with either individual gene analysis (IGA) or with gene set analysis (GSA) in the spleen and in small intestine epithelium of rats fed rAlb-VP60 potatoes compared to rats fed wtAlb. X refers to significance ($q \leq 0.05$) of the process.

Tissue	Significant biological processes	IGA	GSA
Spleen	**Lipid catabolism**	**X**	**X**
	Digestion	**X**	**X**
	Protein catabolism	X	
	Pigment/porphyrin metabolism	X	
	Development	X	
	Lymph gland development		X
Small intestine epithelium	Acute phase response	X	
	Protein biosynthesis		X
	Ribosome biogenesis		X
	Ubiquitin-dependent prot. Catab./ Ubiquitin cycle		X
	RNA processing		X
	DNA replication		X

Re-analyzing expression data from the spleen and from small intestine epithelium using GSA revealed a significant effect of the rAlb-VP60 diet on several biological processes (see Table 25). In the spleen “lipid catabolism”, “digestion” and “lymph gland development” exhibited a significant enrichment of up- or down-regulated genes. These results are partly in agreement with results from IGA analysis. Processes “lipid catabolism” and “digestion” exhibit significance in both analysis approaches. Nevertheless some categories e.g. “protein catabolism” and “pigment/porphyrin metabolism” or “lymph gland development” did not coincide. In small intestine epithelium no consistency between the results of both analyses was observed. With the IGA approach, only “acute phase response” revealed a diet effect whereas with GSA analysis

"protein biosynthesis", "ribosome biogenesis", "ubiquitin related processes", "RNA processing" and "DNA replication" were identified.

In a comparative gene expression study in the liver of rats fed rAlb-nptII and wtAlb only "lipid biosynthesis" was identified as diet-affected when using GSA (see Table 26). In contrast IGA approach had shown effects in "electron transport", "alcohol/ monosaccharide metabolism" and "lipid metabolism". However, some consistency can be concluded in that "lipid metabolism" and "lipid biosynthesis" are closely related and comprise to some extent the same molecules.

Table 26: Comparison of significant biological processes (GO) identified with either individual gene analysis (IGA) or with gene set analysis (GSA) in the liver of rats fed rAlb-nptII potatoes compared to rats fed wtAlb. X refers to significance ($q \leq 0.05$) of the process.

Tissue	Significant biological processes	IGA	GSA
Liver	**Lipid metabolism**	**X**	
	Lipid biosynthesis		**X**
	Electron transport	X	
	Alcohol/monosaccharide metabolism	X	

5 DISCUSSION

The present study was conducted to evaluate the sensitivity and adequacy of expression profiling for the characterization of potential physiological side effects of genetically modified (GM) feed components. The application of this method for studying such effects of GM feeds in animal feeding trials could be promising since sensitivity of conventional methods has been criticized recently. Within the scope of this work, effects of several specific GM components on rats' physiology were investigated. For this purpose feeding experiments with laboratory rats were conducted and a comparative expression profiling was done using microarray technology in the tissues liver, spleen and in small intestine epithelium. The GM feed components consisted either of recombinant potatoes with the inserted genes VP60, CtxB and nptII (rAlb-VP60) or of recombinant potatoes that had introduced only the nptII gene (rAlb-nptII). Expression profiles of animals fed these potatoes were compared to profiles of rats fed the corresponding non-transgenic near-isogenic potatoes (wtAlb). In order to identify VP60-specific effects, expression was also investigated after feeding an additive of inactivated, recombinant baculovirus with the inserted gene VP60 (rBV-VP60). The profiles were then compared to rats fed the corresponding additive consisting of wildtype baculovirus (wtBV). Based on the identified differentially expressed genes (DEG) and on their gene ontology (GO) annotation, biological processes with a significantly enrichment of associated DEGs were identified in order to characterize physiological alterations in response to these specific GM feeds and GM feed additives.

5.1 Application of different analysis parameter settings in microarray experiments conducted for the identification of diet effects

As a pre-requisite for reliable results and interpretation of microarray investigations, data pre-processing steps as well as different thresholds for defining gene expression differences were evaluated regarding their appropriateness for the present study. This pre-study was based on the feeding experiment with the rBV-VP60 and its corresponding wildtype (wtBV) additive.

Data pre-processing and normalization

Generally, a considerable proportion of typical microarray data sets is composed of signals with low intensities that cause variability or impair of reproducibility due to their low transcript levels. These signals may be masked or biased by noise and non-specific

hybridization, respectively (BILBAN *et al.*, 2002). In the present study analogue findings, namely strong noise at low intensity levels, emphasized the need for removing data with a low signal to noise ratio. The applied quality filtering eliminated most of the potentially unreliable signals. Such filtering has been discussed in literature and is often applied (EPSTEIN *et al.*, 2001; LEUNG & CAVALIERI, 2003; CAUSTON *et al.*, 2003). In contrast, GAO *et al.* (2003) point out that weak signals do contain valuable information and should be kept for further analysis. Thus, by correcting for noise, a loss of information is always accepted. Nevertheless, DRAGHICI *et al.* (2006) state that today's technology might still be insufficient to detect relevant changes in low abundance genes, such as transcription factors.

However, quality filtering does not adjust for a bias which occurs in two-color approaches. Normalization procedures are applied to correct for a dye bias due to differences related to incorporation and detection of the two dyes. Out of the five algorithms tested in this work, only lowess normalization did adjust satisfactorily, not only for general dye bias, but also for intensity-dependant dye differences and thus seemed to be the most appropriate. The use of the lowess algorithm has been proposed by various authors as for instance by QUACKENBUSH (2002) or YANG *et al.* (2002a). Nevertheless, there has also been criticism. According to SANCHEZ-CABO *et al.* (2003) lowess is not suitable for "low-density" microarray experiments though this is kind of consequential because lowess, like other normalization methods as well, is based on the assumption that most of the genes do no differ in their expression between the experimental groups. Besides, the authors report a poor reproducibility of replicated measurements after lowess correction and propose the use of self normalization. However, within the range of given design options and accessible analysis software tools and therewith the available normalization algorithms, lowess still performed best for the data used within this work.

Effect of threshold choice on the number of differentially expressed genes and on the biological processes with a significant enrichment of DEGs

The application of varying thresholds for defining significant gene expression differences was evaluated as a further parameter. The identification of differentially expressed genes is a crucial step in microarray data analysis as it determines strongly the following biological interpretation. In the data used within this study, the application of solely a t-test with an assumed significance at a $p \leq 0.05$ led to high false positive rates. This is due to the large amount of tests carried out in such microarray analyses. Between 17 %

and 26 % expected false positives were observed among the significant differentially expressed genes. It became clear that a correction for multiple testing was essential. There is consensus about the use of a False Discovery Rate (FDR) correction as an alternative to conventional correction approaches for multiple-testing (ALLISON *et al.*, 2006). However, the decision about the stringency of the threshold, above which gene expression of two groups is defined as significantly different, is left of course to the scientist. A threshold of q ≤ 0.05 (FDR ≤ 5 %) is commonly used and suggested for instance by authors of the EADGENE network (JAFRÈZIC *et al.*, 2007). Nevertheless, in some studies less stringent q-values of ≤ 0.1 or ≤ 0.2 were applied (RUIZ-BALLESTEROS *et al.*, 2005; ZHU *et al.*, 2005; PONSUKSILI *et al.* 2007).
Referring to the present investigations the use of varying thresholds (q ≤ 0.05 / 0.1 / 0.2) indicated a strong impact of threshold choice on the number of identified DEGs and thus on the biological conclusion drawn from the experiment. The application of a FDR correction and the use of a threshold of q ≤ 0.05 reduced the number of DEGs in comparison to the DEG list obtained without FDR correction (p ≤ 0.05). 95 % of genes remained significant in the liver despite this stringent q-value threshold. In small intestine epithelium only 1 % of the genes exceeded the FDR threshold. This diversity is attributed to a different distribution of p-values in these tissues. A high occurrence of small p-values, as observed in the liver, results in a higher number of genes with q-values below the threshold. In small intestine epithelium in contrast, a lesser amount of small p-values was found. Consequently, the estimated fraction of truly nulls (not differentially expressed) - the key parameter that determines FDR characteristics - was higher (PAWITAN *et al.*, 2005). As a result, very few genes with small q-values arose. When applying lower stringencies (q ≤ 0.1 or 0.2), increased numbers of DEGs were found. They were exceeding in most cases the amount of DEGs after t-test without FDR correction. Nevertheless, is has to be kept in mind that a q ≤ 0.2 means that the expected rate of false positives among the DEGs is raised to 20 %.

DEG lists, as determined by different threshold levels, were used for the identification of significantly diet-affected biological processes. A biological process was regarded as being influenced by the diet when a significant enrichment of associated DEGs was demonstrated. It was shown that the size of the DEG input list considerably influenced the output. Increased proportions of expected false positives due to less stringent thresholds led to varying results with respect to the enriched biological processes. For instance, in the liver the pattern of significant biological processes changed completely

when raising the q-value threshold applied to the DEG list from q ≤ 0.05 to q ≤ 0.1. No overlapping processes were found. Moreover, the relationship between threshold choice and significance of the processes did neither seem to be linear nor predictable. This effect may be due to the possible non-linearity of the ratio between the total number of identified DEGs and the number of DEGs belonging to a specific process. PAN *et al.* (2005) showed similar effects in their study. Their work was related to the identification of DEGs that comprise “gene sets” which are then associated with tumor types and functional categories. With varying thresholds they produced gene sets of different sizes and exhibited strong effects on the identification of functional categories.

These results emphasize that caution that has to be taken when choosing the threshold as it strongly influences the biological conclusion. The general aim of gene selection is clearly to identify a list of DEGs as accurately as possible (CHEN *et al.*, 2007b). Nevertheless, choice of threshold stringency should depend on the objective of the experiment (CHEN *et al.*, 2007a). An optimal threshold that at the same time minimizes both false positives and false negatives does not exist. Threshold choice will always be a compromise. When intending to select few candidate genes for instance, procedures with few false positives might be desirable. For pathway analysis a certain size of DEG list is requisite. Thus, more relaxed parameters, at the prices of higher a FDR, could possibly be applied.

For the data used within this work it was concluded that a FDR threshold of q ≤ 0.05 was most appropriate because it was aimed for a high reliability of the results. The fact that only few replicates (n = 3) were available supported this decision. WEI *et al.* (2004) pointed out that a small number of replicates, resulting in low statistical power for detecting differentially expressed genes, may produce high false positive rates. The application of less stringent thresholds (q ≤ 0.1 / 0.2) and consequently higher numbers of expected false positives might be more suitable for experimental designs that allow a higher statistical power.

As an additional criterion to define DEGs the application of a fold change (FC) filter was tested in combination with a q-value ranking. According to ALLISON *et al.* (2006), fold differences are a reasonable measure of effect size. In the history of microarray studies they were the first method used to evaluate whether genes differed in their expression level. Today it is common to apply fold change (FC) filters to a gene list that has passed the p-value or q-value thresholds and use this approach for DEG identification; as it was suggested by the Microarray Quality Control Consortium (MAQC Consortium, 2006). SHI

et al. (2004) mentioned that gene lists obtained by fold change ranking performed well since they found a large overlap when comparing data produced with different microarray platforms. They argue that this overlap was reasonable a surrogate for assessing the accuracy of microarray data. According to CHEN *et al.* (2007a) fold change rankings for producing gene lists provide better ranking than t-statistics when the sample size is small. GUO *et al.* (2006) also showed a higher reproducibility of results generated by fold change ranking than those obtained by t-test. Nevertheless, using solely a FC is not advisable, as it does not account for variance (MURPHY *et al.*, 2002; CHEN *et al.*, 2007a). The choice of applicable filter stringencies is again left to the investigator, but according to QUACKENBUSH *et al.* (2001) most studies have used a cut-off of two-fold increase or decrease in measured expression level. Nevertheless, fold differences of 1.3 (BOOTH *et al*., 2004) and 1.5 (HAN *et al.*, 2006) can also be found in literature.

Therefore, the effect of different FC filters (without, FC ≥ 1.5 and FC ≥ 2.0) on the number of identified DEGs and ultimately on significantly affected biological processes, was studied as an additional parameter. The combined application of a $q \leq 0.05$ and a fold change thresholds (FC ≥ 1.5, FC ≥ 2.0) in data analysis did significantly reduce the number of DEGs in all tissues in comparison to the DEG lists obtained without FC filter.

The resulting DEG lists were used to identify diet-affected biological processes with a significant enrichment of associated DEGs. These analyses resulted in a similar conclusion as above. The use of varying fold change filters produced diverse results. Consistency between the information obtained at changing FC levels - when referring to diet-affected biological processes - was low. Nevertheless, it became clear that a FC filter of ≥ 2 was probably too stringent as no significant biological process was identified due to a low frequency of high fold changes and consequently an extremely reduced number of DEGs. BEN-SHAUL *et al.* (2005) argues that the organisms' reaction could involve smaller adjustments of large gene sets, rather than extreme changes in fewer genes which would be disregarded by a very conservative fold change cut-off. Again, the decision whether to apply a fold change filter and which stringency to use, has to be taken in the context of the experiment. For this study a FC of ≥ 1.5 was regarded as most appropriate. A FC of ≥ 2 was excluded for the above mentioned reasons. Skipping a fold change cut-off seemed to produce high numbers of false positives as indicated by the large amount of DEGs that remained at all FDR levels. In studies with the aim of discovering "high response" genes, a higher FC filter might be more suitable.

5.2 Characterization of physiological effects of genetically modified feed components in rats

In addition to the methodological investigation discussed in 5.1, a further aim of the study was to investigate unintended physiological effects of an ingestion of specific genetically modified potatoes in feeding experiments with rats.

Physiological effects of a feeding of rAlb-VP60 potatoes

The effect of an oral intake of the rAlb-VP60 potatoes on gene expression was studied and compared to a feeding of its near-isogenic non-GM potato (wtAlb). Gene expression profiling in rats fed the rAlb-VP60 potatoes revealed significant expression differences mainly in the spleen and slight changes in small intestine epithelium. Hepatic gene expression seemed to be un-affected by the diet. In the spleen several biological processes exhibited a significant enrichment of DEGs. The transcription level of genes associated with “lipid” (e.g. Pnlip, Pla2g1b) and “protein catabolism” (e.g. Ela1, Ctrb1, Cpa1) were mainly down-regulated, whereas genes of “pigment/porphyrin metabolism” (e.g. Alad, Urod) showed a significant up-regulation in animals fed rAlb-VP60 compared to wtAlb group. Expression of molecules involved in different “developmental processes” had both an elevated (e.g. Urod, Alad) and reduced (e.g. Flt1, Add2) expression level. In small intestine epithelium few genes related to acute phase response (e.g. Pap1) showed significant down-regulation in response to rAlb-VP60. In addition to the microarray measurements, transcription levels of several genes were determined using quantitative real-time RT-PCR. The recorded expression of the molecules Pnlip, Pla2g1b, Cpa1, Ctrb1, Ela1, Alad, Rara, Flt1 and Pap1, representing the different diet-affected biological processes, correlated well with microarray results. The good correlation underlines the reliability and importance of the results.

Particularly the down-regulation of genes involved in proteolysis and lipolysis indicate reduced catabolic activities in the spleen of rats fed the rAlb-VP60 potatoes. Molecular causes for these expression changes are difficult to explain. Effects on physiology of the animals which ingested this potato can possibly be caused by altered nutrient composition or nutrient value of the potatoes due to transgenesis. Such effects may occur as a result of genetic rearrangements, gene disruption or sequence changes as a consequence of the recombination event (ILSI, 2007) or could be caused by somaclonal variation or pleiotropic effects of the newly introduced gene (MOHAN JAIN, 2001; SHEPARD, 2006). Though, the potential bioactivity of the introduced genes, especially VP60, may also have affected rats’ physiology.

For coming up to the question whether an altered nutrient profile or the bioactivity of the introduced genes caused the observed changes some issues have to be taken into account. According to JANCZYK *et al.* (2007), who investigated nutritional parameters of the rAlb-VP60 and the wtAlb potatoes, most contents of crude nutrients did not differ between the two potato lines, though slight changes in protein content were detected (wtAlb: 8.12 %DM; rAlb-VP60: 7.58 %DM). Moreover, they showed that parameters recorded in the animals, as for instance nitrogen balance and other protein value/digestibility traits, remained mainly equal between the feeding groups. Nevertheless, contents of several amino acids (e.g. asparagine, glycine, glutamic acid, leucine) differed explicitly between the two lines. Besides, the digestibility quotients of these amino acids showed significant changes. These alterations can be implicated with the reported expression changes since it is known that amino acids can act as dietary signals and thereby influence transcription (KIMBALL & JEFFERSON, 2004).

Expression differences in rats that were related to proteolytic processes could also point at physiological effects that were caused by variation in amino acid profiles or protein content of the GM potatoes. Alad for instance is discussed to be a critical factor for the inhibition of protein degradation via the proteasome (GUO *et al.*, 1994). It was up-regulated after rAlb-VP60 feeding and acts in this way correspondingly to the down-regulated catabolic molecules of protein metabolism. However, only 15 % of the protein was offered via the potatoes and all diets were isonitrogenous. Thus it can be argued whether this amount is enough to cause distinctive effects. Besides, an extensive analysis of classical parameters was carried out by JANCZYK *et al.* (2007) in the same animals. Neither, growth, blood, serum parameters nor histopathological investigations revealed any diet effects. But still, a possible implication of variations in the metabolic profile of the potato cannot be excluded. Compositional alterations, which have possibly not been detected with the methods used in this work, should be considered.

A possible involvement of the VP60/CtxB molecules in the observed expression changes appears comprehensible, when looking at results of small intestine epithelium where acute phase molecules showed a significant down-regulation in rAlb-VP60 fed rats. In addition, several DEGs identified in the major immune relevant organ spleen, can be implicated in immune processes or the preservation of normal cell function. Some enzymes, including pancreatic lipase (Pnlip), are known to be involved in the induction of macrophages to produce the pro-inflammatory cytokine TNF-alpha (JAFFRAY *et al.*, 2000; ORDAS *et al.*, 2007). Besides, Pla2g1b and Ctrb1 have been associated with immune relevant functions (WANG *et al.*, 1998; KOSHIKAWA *et al.*, 1998; MANDAL *et*

al., 2001; PERRIN-COCON *et al.*, 2004). Rara is involved in transcriptional regulation, but anti-apoptotic properties against oxidative stress induced apoptosis may be an additional function of this molecule (HEGELE *et al.*, 2001; XU *et al.*, 2002). In addition, Flt1 is suggested to be a useful marker for monocyte-macrophages in humans (SAWANO *et al.*, 2001). Furthermore, FOLCH-PUY *et al.* (2006) speculated that Pap1 could play a similar role to that of IL-10 in epithelial cells. However, most of these molecules were down-regulated and it remains unclear why the antigen VP60 or the adjuvant CtxB should induce a down-regulation of immune related molecules rather than an up-regulation.

It should be also mentioned that a VP60 molecule was proven to induce IgG production when expressed in baculovirus and administered to rats via intramuscular injections. In contrast, when determining IgG production after an oral application of the VP60 baculovirus or the rAlb-VP60 potato, no antibodies could be detected in a feeding study with rabbits (HAMMER *et al.*, 2006). This could be explained with the biological active concentration of VP60 that is needed to induce an immune reaction. The amount of VP60 might have been too low after passing the gastrointestinal tract of the animals. In fact, VP60 could not be detected in the rAlb-VP60 tubers with the applied methods (MIKSCHOWSKY, personal communication) although expression was proven. These last-mentioned findings in turn argue against a VP60 involvement in the expression changes. Though it is not clear whether gene mRNA expression profiling might be more sensitive and able to detect slight responses of the organism, which are not reflected by a major IgG production and are consequently not sensed with ELISA techniques.

Physiological effects of a feeding of rAlb-nptII potatoes

To gain more insight into expression modifications that are most likely caused by an unexpected altered plant composition without interfering effects that have to be ascribed to the immunogenic properties of the VP60/CtxB molecules, a further GM potato was studied in a feeding experiment with rats. A potential bioactivity of nptII cannot be completely excluded but has never been proven. Moreover, FUCHS *et al.* (1993) and EFSA (2008) concluded that the ingestion of genetically engineered plants, which are expressing the nptII protein, do not pose safety concerns.

Nevertheless, in the present study it was shown that a feeding of potatoes, which are solely expressing the nptII marker gene, did change gene expression in rats in comparison to wtAlb fed animals. However, in this case the liver, the most important organ for nutrient metabolism, was the only affected organ. A significant enrichment of

mostly up-regulated DEGs was observed in GO processes “electron transport”, “lipid metabolism” and “alcohol/monosaccharide metabolism”. Microarray results of six selected molecules partly correlated with findings of real-time RT-PCR measurements. A significant increase of expression levels in rAlb-VP60 fed rats was shown for the molecules Acox2 (electron transport) and Hsd17b2 (lipid metabolism). For the four remaining genes Pemt, Prkaa2, Fbp1 and PC, which were associated with “lipid metabolism” (Pemt, Prkaa2) and “alcohol/monosaccharide metabolism” (Fbp1, Pc, Prkaa2), no significant expression changes between the feeding groups were observed via real-time RT-PCR; although this was proven using microarray technology.

Especially the up-regulation of genes associated with alcohol/monosaccharide metabolism, which are also involved in general energy equilibrium, and an up-regulation of lipid metabolism related genes indicate a higher hepatic metabolic activity in rats fed the rAlb-nptII potatoes. Acox2 for instance is part of GO processes “electron transport” and “lipid metabolism” and encodes for the acyl-Coenzyme A oxidase 2. Acyl-CoA oxidases are known to be involved in fatty acid oxidation (LI *et al.*, 2006). Hsd17b2 is involved in steroid synthesis. Steroid hormones in turn are known to play a role in lipid metabolism and to be influenced by dietary fat intake (ACOSTA, 2001; GROMADZKA-OSTROWSKA *et al.*, 2002; LIN *et al.*, 2006). The up-regulation of molecules like Fbp1, Pc and Prkaa2 demonstrates implications with energy providing processes since these molecules are playing a role in gluconeogenesis and in regulation of energy metabolism (VELEZ & DONKIN, 2005; VAN POELJE *et al.*, 2006; TOSCA *et al.*, 2008). Moreover, the transcription of several cytochrome P450 molecules was affected. Cytochromes P450 are associated with xenobiotic metabolism but literature also suggests a function in the regulation of steroid hormones (MEYER, 2007; GEHLHAUS *et al.*, 2007). These facts underline the interaction between the organisms` response and diet composition or nutrient supply and could hint at a physiological reaction towards the observed alterations in the nutrient profile of the two potato lines.

Therefore rAlb-nptII potatoes were also screened for alterations in macro nutrients. In addition, classical parameters of digestibility and health were studied in the rats which ingested these plants. Macro-nutrients did not differ between the non-GM and the GM line. However, distinctive changes in amino acid profile (e.g. serine, glutamic acid, leucine, lysine, proline) were observed; likewise to the modification monitored in rAlb-VP60 potatoes. Consequently also digestibility of these amino acids was altered when measured in rats. General health parameters like growth, blood chemistry and histopathological examination of the animals did not reveal any differences or negative

effects of the GM potato feeding when comparing it to the wtAlb feeding group (JANCZYK, personal communication). Again, only 15 % of the protein source was supplied by the potato. This may seem little especially because there was no protein restriction. Both, the wtAlb and the rAlb-nptII diet were isonitrogenous and isoenergetic. But as discussed before, some amino acids have been shown to be significantly involved in the regulation of gene expression (JOUSSE *et al.*, 2000; KIMBALL & JEFFERSON, 2004). An altered amino acid profile of the plant could consequently result in expression changes as monitored in the animal.

Feeding of rBV-VP60, rAlb-VP60 and rAlb-nptII – agreement of results

Interestingly the results of the expression profiling discussed above are partly consistent with results of the pre-study (rBV-VP60/wtBV feeding experiment). An enrichment of differentially expressed genes of “lipid catabolism” was recorded consistently in spleen. Most genes of “lipid catabolism” were down-regulated when feeding the rBV-VP60 additive as well as when feeding the rAlb-VP60 potato. Similar findings were observed for several genes involved in “protein catabolism”. Overlapping significant molecules were down-regulated after rBV-VP60 and rAlb-VP60 feeding although this process was only significant in the rAlb-VP60 feeding study. Thus, some of our findings have to be ascribed most likely to the VP60 molecule for the reason that it was the only identical component in both feeding trials. However, some results did not coincide (e.g. “development” or “pigment/porphyrin metabolism” appeared only after rAlb-VP60 feeding) and therefore provide indication that not all changes can be explained by a potential bioactivity of the VP60 molecule. Though, it has to be kept in mind that conditions were not equal between these two experiments. Alternatively, these differences could be also attributed to the variation in nutrient profile between the recombinant and the non-GM potato line or could be assigned to the CtxB molecule or to interacting and combined effects of several causative factors. A potential bioactivity of the nptII molecule does not seem likely because no corresponding results were observed when comparing DEGs identified in the liver of animals fed rAlb-VP60 and rAlb-nptII although both potatoes expressed the nptII molecule.

5.3 The application of threshold-based and threshold-free approaches for the identification of diet-affected biological processes

Results discussed in 5.1 show some drawbacks of “classical” threshold-based, “two-step” approaches (IGA: individual gene analysis; definition according to NAM & KIMI,

2008) for functional interpretation of genome-scale experiments. Besides, these kinds of analyses have been criticized since they are based on the assumption that biological relevant changes involve a high number of transcripts showing strong expression differences above defined thresholds. However, this might not always be the case. Moderate but meaningful expression changes could be discarded by a strict cut-off-based approach. Interacting affected genes may be modified concertedly but without any member undergoing extreme modulations (KIM & VOLSKY, 2004; BEN-SHAUL *et al.*, 2005). DOPAZO (2006) criticizes the massive loss of information because a large number of false negatives is accepted in order to preserve a low fraction of false positives. More recently suggested analysis methods, which are summarized under the term gene set analysis (GSA; NAM & KIM, 2008), focus on so called gene sets (e.g. GO biological processes can be used to define gene sets) but use the ranked phenotype association information (e.g. q-values, FC etc.) of all analyzed genes; without constraint to threshold-based lists (JIANG & GENTLEMAN, 2007). The basic principle is to test the over- or under-expression of sets of functionally related genes by studying their relative position across a list of genes ranked by differential expression. That means a gene set is most likely related to the treatment/phenotype when its attributed genes tend to be ranked at the top end of the list (AL-SHAHROUR *et al.*, 2007).

Because of the mentioned shortcomings of threshold-based approaches, the data from the feeding experiments with GM potatoes was re-analyzed with a GSA algorithm (PAGE; KIM *et al.* 2005) and results were compared. According to the recommendations of KIM *et al.* (2007) for experiments with a small sample size, fold changes were used to define expression changes between the feeding groups. Although a very different concept was applied, parts of the results were in agreement. In the spleen of rats fed GM potatoes (rAlb-VP60) “lipid catabolism” and “digestion” were significantly affected by the diet and identified with both approaches. In the liver (rAlb-nptII) coincident results were observed as well, when regarding lipid metabolisms and lipid biosynthesis as strongly related categories. In small intestine epithelium, a tissue where applying the IGA approach only resulted in one significant process, GSA seemed to be advantageous; several processes could be identified. This is probably due to the low frequency of high FCs which was extreme in this tissue. These are obviously the cases where GSA performs better than IGA. In contrast, in the spleen IGA exhibited more processes. “Protein catabolism” did not appear in GSA though it has to be mentioned that most genes of this category were also involved in “digestion”. Pigment/porphyrin metabolism however did not emerge at all in GSA results. In liver “electron transport” and “alcohol/monosaccharide metabolism” were ignored with GSA.

It was concluded that in experiments/tissues where only slight changes are observed or expected, GSA might be a valuable approach and obviously performs better than IGA, though this is not conclusive. However, a general advantage of GSA could not be reasoned from these results, although the superiority of this new approach was demonstrated in literature e.g. by SUBRAMANIAN *et al.* (2005) or AL-SHAHROUR *et al.* (2005). In some cases "classical" IGA approaches might be more valuable in that they tend to find more processes in datasets with higher FCs. Though, the strong dependency of the results on the threshold remains a problem. RAGHAVAN *et al.* (2006) reported similar findings. They found that, depending on the distribution of the p-values, in some cases the IGA approach was superior to threshold-free approaches. They point out that distribution-based statistics (GSA) tend to check for a broad range of alternatives and consequently their sensitivity gets diluted for the specific alternatives of interest. But a suffering from the cut-offs applied in IGA approaches and the yield of different results depending on the thresholds was also mentioned. DAMIAN & GORFINE (2004) also criticize GSA and indicate situations when using GSA will ignore genes with highest values solely due to the size of the selected gene set. Since there has been criticism about both approaches it seems advisable to adapt the functional analysis to the particular experiment.

However, both approaches seemed to be applicable to identify diet-affected biological processes. Depending on the data one or the other approach was more informative. Both should be tested and the one that gives the more interpretable results could be chosen. Nevertheless, for the present study both approaches were valuable as they showed overlapping findings which may be regarded as indication for the correctness of the results.

5.4 The use of expression profiling in the safety assessment of genetically modified plants

The present findings demonstrate that unintended changes in terms of a modified nutrient composition in the plant may occur and can affect physiology of animals ingesting such foods. Moreover, the impact of the introduced gene and its produced protein on the organism cannot always be predicted. This emphasizes the need for a holistic safety assessment when applying genetically modified foods or food additives to animals or humans. Classical parameters (e.g. blood chemistry, nutritional parameters) studied in rats by JANCZYK *et al.* (2007 and personal communication) did not show any effect of the diet on the animals' physiology. In contrast, gene expression profiling

revealed effects although interpretation of the results remained difficult and effects could not be explained thoroughly from this study. As pointed out in 2.2.3 so far only very few indications for unintended effects of feeding genetically modified plants to animals were found. Detailed overviews are given by FLACHOWSKY *et al*. (2005, 2007) and EFSA (2008). In general they conclude, that genetically modified plants without substantial changes in their composition do not significantly differ in their nutritional value from those of their nearest isogenic varieties and do not have negative physiological effects on the organism that ingested the plant. Consequently, the present results may raise the question whether the classical approaches used until now are adequate and sensitive enough to detect possible effects. As shown in this study expression profiling methods, which are among the so called non-target approaches, provide a great sensitivity. NARASAKA *et al.* (2006) proposed and proved the usefulness of such techniques in the field of food safety. LIU-STRATTON *et al.* (2004) also suggests studying expression, proteomic and metabolomic profiles of tissues from experiments with humans or animals which have ingested such foods and to compare it to the corresponding traditional food as a reference for a complete safety assessment. Nevertheless, for a meaningful interpretation of expression results in animals' tissues an extensive knowledge about alterations in nutrient profiles of the GM plants is essential. Thus, the implementation of expression and metabolic profiling in the characterization of the plants should perhaps be the first step towards a global safety assessment of GM plant and was likewise proposed by several authors e.g. KUIPER *et al.* (2001), CELLINI *et al.* (2004), METZDORF *et al.* (2006) and EFSA (2008).

Some limitations of microarray approaches should also be considered. Although the microarray used within this work comprised expression information for about 10 000 transcripts, only about 50 % of the probe IDs could be annotated to known genes and only these genes provide useful information. Nevertheless, this technology is a fast developing field and annotation information improves quickly. The same difficulties were encountered in functional assignment of genes, which is still far from being complete but likewise developing rapidly. Furthermore, it has to be kept in mind that there are no known critical values for expression signals which could be used to define dangerous health effects; as it may be done for instance with blood chemistry parameters. Expression studies should only be seen as a screening method to provide an open-ended broad view of complex cellular reactions. They may not replace conventional analyses but could be useful to confirm and supplement other results and give indication whether further and more complete classical analyses are needed. In that way they

present a very valuable tool which could also be applicable in the field of food or feed safety.

5.5 Agreement of real-time RT-PCR and microarray results

In the course of evaluating different approaches for the analysis of microarray data, a comparison of expression measurements done by real-time RT-PCR and by microarray was performed. Expression values measured with real-time RT-PCR in the spleen and the liver of rats fed either rBV-VP60 or wtBV did significantly correlate with microarray results ($r = 0.72$, $p = 0.0004$). Eight out of 10 molecules, which revealed significance in the microarray analysis, did also show significant expression differences with the real-time RT-PCR approach. These results are comparable to the correlations observed in other studies and may prove the reliability of the present microarray findings. RAJEEVAN *et al.* (2001a,b) for instance confirmed 71 % of the genes, which were significant with microarray approach, by real-time RT-PCR. MOREY *et al.* (2006) proved a significant correlation between microarray and real-time RT-PCR results of 0.7. In a further feeding experiment with rAlb-VP60 potatoes, 100 % confirmation of significance of the expression differences obtained from the two methods was shown in the spleen and in small intestine epithelium. In contrast, when assessing significant differences of single molecules in the liver of rats fed rAlb-nptII by real-time RT-PCR, some disagreements between real-time RT-PCR and microarray results were observed.

The reasons for disagreements are manifold and widely discussed in literature. WANG *et al.* (2006) reported for instance a decrease in the overall accuracy of significance detection at low expression levels. This could be a reason for the lack of differential expression detection in two molecules (F3, Gp5) of rBV-VP60/wtBV experiment; both genes had a very low expression level (< 100) in real-time RT-PCR. ETIENNE *et al.* (2004) demonstrated the best agreement for genes with moderate expression levels indicating that high transcriptions levels, which are close to saturation point, can also be problematic. Fbp1 and Pemt which were identified in the rAlb-nptII/wtAlb experiment revealed a relatively high expression rate that could have introduce a bias into the measurement. In addition, Fbp1 and Pc (rAlb-nptII/wtAlb experiment) had FCs just above 1.5. According to DALLAS *et al.* (2005) this is a critical threshold. Below this threshold decreased correlations for expression differences have to be expected.

Another important factor influencing consistency could be the intense data processing and modification/correction steps especially in microarray analysis (MOREY *et al.*, 2006). DALLAS *et al*. (2005) mention that different strategies in oligo probe and primer design

could attribute to the poor correlation because different subsets of alternative transcripts or alternative cross-hybridizing transcripts may be recognized. Moreover, microarray probes are usually 3´-based. This may not be the case for real-time RT-PCR primers. Thus, it seems comprehensible that ETIENNE *et al.* (2004) found a good agreement for genes with PCR primers located close to the microarray probes. In the present study microarray probe sequences were unfortunately not available. Consequently, a possible distance between microarray probe and PCR primer might have introduced some bias into the measurements. In addition, gene specific variation due to the different hybridization kinetics, which are associated with the two technologies, and misleading results due to errors in GeneBank sequence data or probe set annotations have to be considered (DALLAS *et al.*, 2005).

Most of the discussed points might be effectual for the reported disagreement in the present study though it is difficult to elucidate the actual cause. Moreover, it is not clear whether to believe in real-time RT-PCR results or in microarray values since the true expression level is unknown (ROCKETT & HELLMANN, 2004); even if it is widely accepted that real-time RT-PCR constitutes the “gold standard”. However, expression levels of microarrays undergo normalization procedures which rely on the assumption that the majority of genes are not differentially expressed. Therefore many expression values can be included in normalization and correction procedures to make up for technical variability. In real-time RT-PCR only a single gene is investigated and correction methods solely rely on the use of housekeeping genes.

Moreover, microarray measurements are often based on multiple probe sets, which distributed within the gene sequence, thus the sensitivity of the measurement might be higher. In contrast real-time RT-PCR relies only on one primer pair and covers only a very limited sequence segment. ROCKETT & HELLMANN (2004) mention that all techniques used to analyze biological systems suffer some kind of bias and state that with regard to these aspects the “gold standard” status might appear arguable. ALLISON *et al.* (2006) question the general accepted need for real-time RT-PCR validation of microarray studies and conclude that it stems more from tradition than careful thought. Taking these arguments into account the present microarray measurements are not interpreted as false positive findings. In contrast, since both techniques are known to have drawbacks the results are still regarded as valuable.

6 SUMMARY

The aim of the present work was to evaluate sensitivity and adequacy of comparative gene expression profiling using microarray technology in the characterization of unexpected physiological side effects caused by a feeding of genetically modified (GM) feed components. This was studied in feeding experiments with laboratory rats which were fed different kinds of diets containing genetically modified potatoes or genetically modified baculovirus. The GM potatoes were either expressing the viral antigen VP60 and the nptII gene (rAlb-VP60) or solely nptII (rAlb-nptII). VP60 can induce an immune response against the Rabbit Hemorrhagic Disease Virus; the nptII gene transfers antibiotic resistance, which is needed for plant selection. Another genetically modified dietary component was made of inactivated, recombinant baculovirus, which also expressed the VP60 gene (rBV-VP60). Expression profiles obtained from the spleen, the liver and from small intestine epithelium of the rats were compared to profiles of animals fed a near-isogenic non-GM potato (wtAlb) or wildtype baculovirus (wtBV) additive. Differentially expressed genes were then classified, according to their function, into biological processes in order to characterize the effect of the feed components or additives on the organisms' physiology.

As a prerequisite for these expression studies, a pre-screening of different data analysis parameters was accomplished to select an appropriate analysis approach. This is crucial since microarray data evaluation includes many steps of data processing and analysis which can have a profound impact on the results and on the conclusions drawn from them. In this work the application of a False Discovery Rate threshold and a fold change filter with different stringencies was tested. The number of differentially expressed genes as well as the identified diet-affected biological processes varied considerably with changing threshold stringencies. This underlines the importance of setting thresholds carefully with respect to the aim of the study and the specific context of the experiment. As a result it was concluded that for the data, used within this work, a stringent False Discovery Rate level of ≤ 5 % would be most appropriate since a high reliability was aimed. A moderate fold change filter of ≥ 1.5 was evaluated as being adequate.

Threshold-free approaches can be considered as an alternative to such threshold-based analyses as they avoid the above mentioned strong dependency of the results on threshold choice. The application of a so called "gene set analysis" was tested on parts of the same data. This method identifies diet-affected biological processes by using

ranked lists, containing fold changes or FDR values of all analyzed genes, without constraint to any threshold. The analyses revealed results which were partially coinciding with the threshold-based findings. In addition, it was reasoned that in cases with only small expression changes, the gene set analysis performed better. Though, a general superiority could not be concluded from the present findings.

The investigation of physiological effects caused by an ingestion of specific genetically modified potatoes revealed significant effects on gene expression. A feeding of rAlb-VP60 potatoes caused expression changes mainly in the spleen of rats compared to expression profiles of animals fed the corresponding wtAlb potato. The differentially expressed genes were for instance associated with “lipid and protein catabolism” and showed a prior down-regulation in the rAlb-VP60 feeding group. Similar results, found in rats fed the rBV-VP60 additive, indicate at least a partial involvement of the VP60 molecule in expression changes. Nevertheless, some results did not correspond between the two feeding experiments (rAlb-VP60/wtAlb and rBV-VP60/wtBV). These disagreements could point to an additional influence of some variations in the nutrient profile of the used genetically modified potato. A feeding of rAlb-nptII potatoes caused an up-regulation of several genes in the liver which are known to play a role for instance in “lipid” and “alcohol/monosaccharide metabolism” and suggest an increased metabolic activity in this organ. Since the nptII molecule has no known bioactivity these changes are probably attributed to alterations in the potato composition due to genetic modification and/or somaclonal variation.

In conclusion, the feeding of different GM dietary components did significantly affect gene expression in several organs of rats. According to these results, effects that have to be ascribed to the VP60 molecule as well as to an altered nutrient composition or nutrient value of the potatoes may occur. Though, classical nutrition parameters did not indicate any physiological impact of the diet. Moreover, it has been shown that expression profiling approaches provide great sensitivity in monitoring physiological reactions of an organism to such diets. They could therefore be a valuable supplement of conventional methods used in safety assessments of GM foods or feeds.

7 ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurden Untersuchungen zur Sensitivität und Eignung von vergleichendem „Genexpressionsprofiling“ für die Charakterisierung unerwarteter physiologischer Effekte, bedingt durch eine Verfütterung genetisch veränderter (GV) Futterzusätze, durchgeführt. In Fütterungsexperimenten wurden Laborratten unterschiedliche Futterzusätze, bestehend aus genetisch veränderten Kartoffeln oder Bakuloviren, verabreicht. Die GV Kartoffeln exprimierten entweder das virale Antigen VP60 und das Gen nptII (rAlb-VP60) oder ausschließlich nptII (rAlb-nptII). VP60 kann eine Immunität gegenüber der hämorrhagischen Kaninchen-Krankheit induzieren; nptII vermittelt Antibiotika-Resistenz, die für die Pflanzenselektion notwendig ist. Ein weiterer Futterzusatz bestand aus inaktivierten, rekombinanten Bakuloviren, die ebenfalls VP60 exprimierten (rBV-VP60). Die Expressionsprofile wurden für unterschiedliche Gewebe (Milz, Leber, Dünndarmepithel) der Ratten erstellt und mit Profilen von Tieren verglichen, die den entsprechenden Futterzusatz aus Wildtyp-Kartoffel (wtAlb) bzw. Wildtyp-Bakuloviren (wtBV) erhalten hatten. Different exprimierte Gene wurden dann, gemäß ihrer Funktion, biologischen Prozessen zugeordnet, um den Effekt der Futterzusätze auf physiologische Vorgänge im Organismus zu charakterisieren.

Um einen geeigneten Ansatz für die Datenanalyse im Rahmen der Expressionsuntersuchungen zu ermitteln, bildeten Voruntersuchungen zur Stringenz verschiedener Auswertungs-Parameter die Vorraussetzung. Ein adäquater Ansatz ist unerlässlich, da die Microarray-Auswertung zahlreiche Schritte der Datenberarbeitung und -analyse beinhaltet. Diese können einen entscheidenden Einfluss auf die Ergebnisse und die daraus gezogenen Schlussfolgerungen haben. Im Rahmen dieser Arbeit wurden die Anwendung einer Korrektur der „False Discovery Rate“ (FDR: erwartete Rate von falsch Positiven unter den abgelehnten Hypothesen) und die Verwendung von Ratio-Filtern mit unterschiedlichen Stringenzen untersucht. Die Anzahl different exprimierter Gene wie auch die identifizierten biologischen Prozesse variierten beträchtlich in Abhängigkeit von dem gewählten Grenzwert. Daraus wird die Notwendigkeit deutlich, diese Grenzwerte mit Bedacht in Hinblick auf das Ziel der Untersuchungen und unter Berücksichtigung spezifischer Versuchsbedingungen festzulegen. Für die vorliegenden Daten wurde ein stringentes FDR Niveau von ≤ 5 % als adäquat eingestuft, da eine hohe Verlässlichkeit der Ergebnisse angestrebt wurde. Für den Ratio-Filter wurde ein moderater Grenzwert von ≥ 1.5 als geeignet bewertet.

Als Alternative zu solchen grenzwert-basierten Vorgehensweisen können auch Ansätze in Betracht gezogen werden, die ganz auf die Anwendung von Grenzwerten verzichten und somit den Einfluss dieser Parameter auf die Ergebnisse vermeiden. Die Nutzung der sogenannten „genes set analysis“ wurde anhand eines Teils der vorliegenden Daten getestet. Diese Methode identifiziert diät-beeinflusste biologische Prozesse mittels einer Rangliste der Ratio- oder FDR-Werte aller analysierten Gene und verzichtet dabei auf jegliche Einschränkungen durch Grenzwerte. Aus den Untersuchungen ergaben sich Ergebnisse, die zum Teil mit denen des grenzwert-basierten Ansatzes übereinstimmten. Zudem wurde geschlussfolgert, dass bei zu erwartenden geringen Expressions-unterschieden, die „genes set analysis“ geeigneter ist, obwohl eine generelle Überlegenheit aus den vorliegenden Untersuchungen nicht abgeleitet werden konnte.

Physiologische Nebeneffekte einer Verabreichung spezifischer, genetisch veränderter Futterzusätze wurden mittels „Genexpressionsprofiling“ untersucht. Die Microarray Ergebnisse zeigten, dass die Verfütterung von rAlb-VP60 Kartoffeln im Vergleich zu einer Verfütterung von wtAlb Kartoffeln hauptsächlich in der Milz der Ratten zu signifikanten Expressionsunterschieden führte. Die different exprimierten Gene spielen unter anderem im Lipid- und im Proteinkatabolismus eine Rolle und zeigten vorrangig ein niedrigeres Expressionsniveau in der rAlb-VP60 Gruppe. Vergleichbare Ergebnisse wurden in rBV-VP60 gefütterten Ratten beobachtet und deuten auf eine zumindest partielle Beteiligung des VP60 Moleküls an den veränderten Expressionsmustern hin. Allerdings waren die Ergebnisse der beiden Experimente (rAlb-VP60/wtAlb und rBV-VP60/wtBV) nur teilweise übereinstimmend. Diese Unterschiede könnten ein Indiz dafür sein, dass Veränderungen in der Nährstoffzusammensetzung der eingesetzten Kartoffellinie ebenfalls einen Einfluss hatten. Eine Verfütterung von Kartoffeln, die nur das nptII Markergen exprimierten, führte in der Leber zu einer Aufregulierung verschiedener Gene, die zum Beispiel im Lipid- und Alkohol/Monosaccharid-metabolismus eine Rolle spielen und auf eine erhöhte Stoffwechselaktivität in diesem Organ hinweisen. Da es keine Kenntnisse über eine biologische Wirksamkeit des nptII Moleküls gibt, sind diese Veränderungen vermutlich auf Unterschiede in der Kartoffelzusammensetzung zurückzuführen

Zusammenfassend ist festzustellen, dass die Verfütterung verschiedener GV Futterzusätze die Genexpression in einigen Organen von Ratten signifikant veränderte. Entsprechend dieser Ergebnisse können Effekte auftreten, die auf das VP60 Molekül,

eine veränderte Nährstoffzusammensetzung oder einen veränderten Nährwert zurückzuführen sind, obwohl klassische Ernährungsparameter nicht auf physiologische Auswirkungen der Diät hindeuteten. Weiterhin wurde gezeigt, dass „Genexpressionsprofiling“ eine genaue Erfassung von physiologischen Reaktionen eines Organismus gegenüber solchen Diäten ermöglicht. Im Rahmen von Sicherheitsuntersuchungen zu genetisch veränderter Nahrung oder Futtermitteln könnte dieser Ansatz eine wertvolle Ergänzung zu konventionellen Methoden darstellen.

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9 APPENDIX

9.1 Figures & Tables

Table 27: Influence of applying different "False Discovery Rate" (FDR) thresholds on identified biological processes exhibiting a significantly increased number of differentially expressed genes. Different gene expression refers to the spleen of rats fed rBV-VP60 in comparison to rats fed wtBV. Grey color marks significantly affected biological processes with ES ≤ 0.05.

Spleen	**Number of GO annotated genes on the array**	**Number of differentially expressed GO annotated genes**							
Biological process (GO term)		**p ≤ 0.05**		**q ≤ 0.2**		**q ≤ 0.1**		**q ≤ 0.05**	
		n[1]	**ES[2]**	**n[1]**	**ES[2]**	**n[1]**	**ES[2]**	**n[1]**	**ES[2]**
Nucleic acid metabolism	719	216	0.03	513	0.05	326	0.01	58	0.08
Central nervous system development	103	38	0.03	71	0.60	46	0.34	7	0.69
Aldehyde metabolism	10	7	0.03	8	0.61	8	0.06	2	0.50
Hyperosmotic response	6	5	0.05	5	0.72	5	0.20	3	0.06
DNA repair	51	19	0.12	44	0.01	25	0.24	5	0.44
Establishment of cellular localization	269	73	0.55	201	0.02	124	0.07	19	0.53
Cellular localization	274	74	0.57	204	0.02	125	0.09	20	0.46
Intracellular transport	265	72	0.54	197	0.03	122	0.08	19	0.50
Secretory pathway	129	35	0.59	99	0.04	57	0.34	8	0.77
Cell migration	146	39	0.63	111	0.04	64	0.35	15	0.10
Organelle organization and biogenesis	267	77	0.30	197	0.04	121	0.12	18	0.62
Regulation of protein metabolism	108	31	0.46	70	0.88	56	0.03	12	0.10
Biopolymer metabolism	673	192	0.18	461	0.56	300	0.04	44	0.65
Macromolecule catabolism	119	36	0.30	87	0.21	60	0.04	10	0.40
Response to salt stress	4	3	0.29	3	0.90	3	0.55	3	0.02
Lipid catabolism	54	16	0.49	34	0.91	19	0.91	9	0.02
Cellular lipid metabolism	265	67	0.80	187	0.30	102	0.88	27	0.03
Glycerol ether metabolism	12	6	0.20	11	0.22	7	0.37	4	0.04
All	3738	1007		2562		1544		250	

[1] n = number of differentially expressed genes in process

[2] ES = calculated EASE Score based on modified Fishers´ exact test; significance was assumed at ES ≤ 0.05

Table 28: Influence of applying different fold change filters (no FC filter; FC≥1.5; FC≥2) on identified biological processes exhibiting a significant over-representation of differentially expressed genes. Different gene expression refers to the spleen of rats fed rBV-VP60 in comparison to rats fed wtBV and to DEGs with q-values ≤ 0.05. Grey color marks significantly affected biological processes with ES ≤ 0.05.

Spleen	**Number of GO annotated genes on the array**	**Number of differentially expressed GO annotated genes**					
Biological process (GO term)		**no FC**		**FC ≥ 1.5**		**FC ≥ 2**	
		n[1]	**ES[2]**	**n[1]**	**ES[2]**	**n[1]**	**ES[2]**
Lipid catabolism	54	9	0.02	5	0.03	4	0.01
Cellular lipid metabolism	265	27	0.03	6	0.72	3	0.54
Glycerol ether metabolism	12	4	0.04	2	0.24	2	0.08
Response to salt stress	4	3	0.02	1	1.00	-	-
All	3738	250		85		26	

[1] n = number of differentially expressed genes in process

[2] ES = calculated EASE Score based on modified Fishers´ exact test; significance was assumed at ES ≤ 0.05

Table 29: Influence of applying different “False Discovery Rate” (FDR) thresholds on identified biological processes exhibiting a significantly increased number of differentially expressed genes. Different gene expression refers to small intestine epithelium of rats fed rBV-VP60 in comparison to rats fed wtBV. Grey color marks significantly affected biological processes with ES ≤ 0.05.

Small intestine epithelium	**Number of GO annotated genes on the array**	**Number of differentially expressed GO annotated genes**							
Biological process (GO term)		**p ≤ 0.05**		**q ≤ 0.2**		**q ≤ 0.1**		**q ≤ 0.05**	
		n[1]	**ES[2]**	**n[1]**	**ES[2]**	**n[1]**	**ES[2]**	**n[1]**	**ES[2]**
Nucleic acid metabolism	697	166	0.02	385	0.21	39	0.16	2	0.58
Fertilization	16	8	0.03	9	0.71	3	0.17	-	-
Ion transport	310	66	0.50	191	0.00	15	0.59	1	1.00
Response to virus	13	2	0.95	13	0.00	-	-	-	-
Regulation of signal transduction	123	34	0.06	81	0.01	9	0.21	1	1.00
Transport	1003	206	0.66	569	0.01	45	0.72	3	0.31
Lipid metabolism	306	69	0.29	182	0.02	14	0.68	-	-
Regulation of biosynthesis	72	16	0.55	47	0.05	1	1.00	-	-
Regulation of cellular metabolism	515	117	0.17	295	0.05	23	0.71	2	0.46
All	3600	751		1932		169		5	

[1] n = number of differentially expressed genes in process

[2] ES = calculated EASE Score based on modified Fishers´ exact test; significance was assumed at ES ≤ 0.05

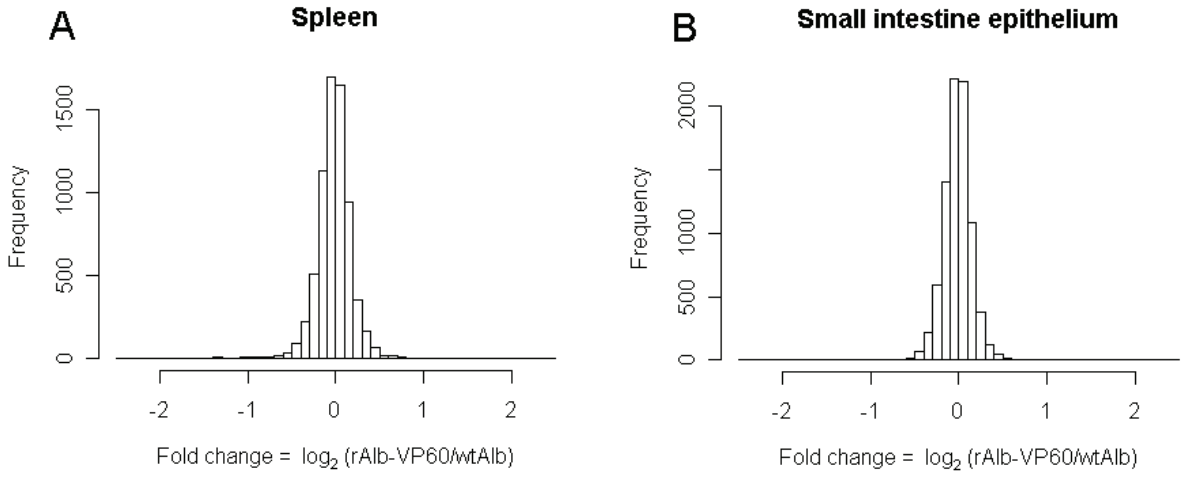

Figure 23: Frequency of expression fold changes observed between rats fed rAlb-VP60 in comparison to rats fed wtBV (A: spleen; B: small intestine epithelium).

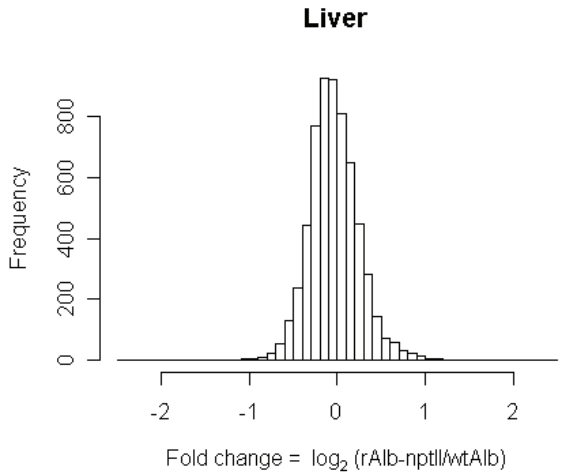

Figure 24: Frequency of expression fold changes observed between rats fed rAlb-nptII in comparison to rats fed wtBV (liver).

entified in the spleen of rats fed rAlb-VP60 in comparison with wtAlb fed animals.

change (log_2)	q-value	SD	Description
.72	0.03	0.08	cell division cycle control protein 2 cdc2a; cdc2 +
0.95	0.03	0.14	thymidine phosphorylase
0.70	0.03	0.01	chromosome 14 open reading frame 4
1.42	0.02	0.12	phospholipase a2, group ib, pancreas pla2g1b; pancreatic a-2
0.63	0.03	0.13	ragb ras-related, alternatively spliced gtpase b; gtp-binding protein
1.18	0.03	0.20	EST
.74	0.03	0.12	truncated nociceptin receptor orl1
.76	0.00	0.02	pituitary tumor transforming gene protein pttg; tumor-transforming 1 pttg1
1.02	0.04	0.33	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit
.61	0.03	0.09	prostacyclin receptor
1.57	0.04	0.35	adducin, beta add2; adducin
1.97	0.03	0.42	fms-related tyrosine kinase 1 vascular endothelial growth factor
.60	0.03	0.09	phospholipase c beta 2 plcb2
0.70	0.04	0.16	lim-domain containing, protein kinase limk1; limk-1
1.66	0.02	0.17	carboxypeptidase a1 pancreatic cpa1; a precursor; preprocarboxypeptidase
0.82	0.02	0.08	EST
1.20	0.02	0.12	immunoglobulin gamma-2b immunoglobulin gamma-2b
1.50	0.02	0.15	carboxypeptidase cpa2
0.94	0.04	0.25	chordin
.95	0.03	0.14	aminolevulinate,delta-,dehydratase alad; delta-alad aa 1-330
0.82	0.05	0.30	EST
1.11	0.03	0.13	basigin
.82	0.02	0.04	adenosine a1 receptor adora1; 326 as
0.62	0.02	0.06	nadh dehydrogenase, mitochondrial subunit 1 nd1
0.96	0.04	0.28	EST
1.65	0.03	0.29	neighbor of BRCA1 gene 1
.87	0.02	0.08	microsomal glutathione S-transferase 3
0.61	0.04	0.20	endogenous retroviral family W
0.62	0.05	0.07	transient receptor potential channel 4 beta-2 splice variant trpc4; trp4alpha
0.88	0.03	0.09	macrophage inflammatory protein-1 alpha receptor gene loc57301
0.91	0.03	0.14	s100 calcium-binding protein a9 calgranulin b s100a9; intracellular mrp14

.11	0.02	0.09	double cortin and calcium/calmodulin-dependent protein kinase-li dcamkl1
.66	0.03	0.07	spindlin 1
0.76	0.04	0.22	cyclic nucleotide phosphodiesterase
0.94	0.03	0.12	preprocaldecrin; elastase iv
2.59	0.03	0.40	trypsinogen 1 is 3rd base in codon; i trp1; ii; pancreatic trypsin prss1; prss2
0.67	0.04	0.15	neurexin iii-alpha; 3 nrxn3
0.72	0.03	0.13	EST
0.80	0.05	0.31	calcium channel alpha-1 subunit rba-i; alpha 1a cacna1a
.31	0.02	0.12	trypsinogen ec 3.4.21.4
0.90	0.03	0.19	fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase rb2k1; rb2k2
.75	0.02	0.06	uroporphyrinogen decarboxylase
.70	0.02	0.03	2-alpha globin 2-alpha-1 globin; major alpha-hemoglobin
.61	0.04	0.18	olfactory receptor; protein
.71	0.05	0.25	fucosyltransferase 9 alpha 1,3 fucosyltransferase fut9; alpha1,3
.63	0.04	0.17	putative olfactory receptor tpcr44
.71	0.03	0.11	core binding factor alpha 1 cbfa1
.91	0.03	0.17	transient receptor potential cation channel, subfamily C, member 4 asso. prot.
.34	0.03	0.18	small proline-rich protein spr
.58	0.02	0.15	chymotrypsin-like ctrl; chymopasin
0.99	0.00	0.02	trypsin v a-form; b-form
.63	0.02	0.05	carbonic anhydrase 2 ca2; ii; dehydratase
.62	0.05	0.22	retinoid x receptor gamma rxrgamma
0.88	0.04	0.26	syncollin; sip9
.24	0.02	0.11	integrin alpha e2
0.66	0.03	0.09	testis ubiquitin specific processing protease; deubiquitinating enzyme ubp45
0.67	0.03	0.14	gap junction membrane channel, protein alpha 4 connexin 37 gja4; cxn-37
.12	0.03	0.24	caat/enhancer-binding protein, dna-binding protein cebpa; c/ebp aa 1-358
.00	0.03	0.13	h-caldesmon
0.70	0.04	0.16	EST
.52	0.03	0.29	serine incorporator 2
.95	0.03	0.35	shab-related delayed-rectifier k+ channel kv9.3 kcns3; potassium channel
.33	0.03	0.25	cd28 antigen cd28
2.39	0.03	0.29	carboxypeptidase b cpb
.67	0.02	0.06	hydroxymethylbilane synthase hmbs; porphobilinogen deaminase; hemc

0.63	0.02	0.03	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1
1.09	0.04	0.25	solute carrier family 14, member 2 slc14a2; urea transporter ut4; ut-a3
1.91	0.03	0.35	sry-box containing gene 10 sox10; protein
0.62	0.03	0.08	rp59 protein rp59
0.84	0.05	0.07	transmembrane emp24 protein transport domain containing 9
0.78	0.03	0.17	late gestation lung protein 1 lgl1
1.34	0.03	0.25	Rab40b, member RAS oncogene family
0.74	0.05	0.26	chordin
0.64	0.02	0.05	epithelial sodium channel, beta subunit renac
1.55	0.02	0.12	pancreatic triglyceride lipase; pnlip
1.03	0.04	0.06	pancreatic lipase-related protein 2 pnliprp2; lipase
0.84	0.00	0.01	glycoprotein 330 lrp2; megalin
0.96	0.03	0.17	cholesterol esterase pancreatic
1.33	0.02	0.14	colipase pancreatic clps
1.61	0.05	0.20	EST
1.93	0.03	0.20	pancreatic lipase related protein 1 pnliprp1; triacylglycerol
0.84	0.03	0.18	homeodomain protein gbx2 gbx2
1.32	0.02	0.13	EST
0.86	0.03	0.10	cytidine 5'-triphosphate synthase
1.03	0.03	0.21	serologically defined breast cancer antigen ny-br-16-like protein
1.73	0.04	0.48	preprotrypsinogen iv aa -15 to 232
0.64	0.05	0.26	histone 2b h2b
1.28	0.03	0.20	brain alpha-tropomyosin tmbr-1; tmbr-3
0.62	0.05	0.21	adenylyl cyclase type ii; 2 adcy2
0.67	0.04	0.16	CXXC finger 1 (PHD domain)
0.63	0.03	0.09	gata-binding protein 1 globin transcription factor 1 gata1; gata-1
0.95	0.03	0.20	creb-binding protein cbp
1.01	0.04	0.30	interleukin-3 receptor beta-subunit ril-3r<beta>
1.00	0.05	0.37	N-terminal asparagine amidase
1.27	0.04	0.28	regulator of g-protein signaling 8 rgs8
0.73	0.04	0.16	rh blood group protein; rhesus
1.13	0.03	0.20	myotonic dystrophy kinase-related cdc42-binding kinase mrck-beta mrck-beta
0.64	0.04	0.15	serine protease bsp2
0.84	0.02	0.06	s100 calcium-binding protein a8 calgranulin a s100a8; intracellular mrp8

.30	0.03	0.03	tulip 1 loc56785; 2
.93	0.02	0.09	RUN and FYVE domain containing 1
.03	0.03	0.13	ser-thr protein kinase related to the myotonic dystrophy protein kinase pk428
.66	0.03	0.11	potassium voltage gated channel, shab-related subfamily, member 1 kcnb1
.64	0.03	0.09	d6.1a protein
.60	0.04	0.43	furosemide-sensitive k-cl cotransporter kcc1; solute carrier family 12, member 4
.79	0.02	0.17	reading frame preproelastase ii; elastase precursor; 2, pancreatic ela2
.60	0.04	0.16	EST
.10	0.04	0.48	nk2 transcription factor related, locus 5 drosophila csx
.87	0.04	0.27	retinoic acid receptor alpha1
.87	0.05	0.35	brain-1 brn-1 protein brn-1
.63	0.05	0.24	transmembrane protein 10
.60	0.03	0.07	polo-like kinase isoform
.99	0.03	0.34	cytosolic peroxisome proliferator-induced acyl-coa thioesterase
.88	0.02	0.16	chymotrypsin b chyb; ctrb
.98	0.04	0.27	serine protease inhibitor, kanzal type 1/ trypsin inhibitor-like protein, pancreatic
.99	0.03	0.17	preproelastase i; elastase; 1 ela1
.70	0.04	0.23	factor 8-associated gene A

entified in the liver of rats fed rAlb-VP60 in comparison with wtAlb fed animals.

change og_2)	**q-value**	**SD**	**Description**
.62	0.00	0.02	zinc finger protein; kidney 1 kid1
.79	0.00	0.02	EST

entified in small intestine epithelium of rats fed rAlb-VP60 in comparison with wtAlb fed animals.

change og_2)	**q-value**	**SD**	**Description**
.61	0.03	0.07	leukotriene c4 synthase ltc4s; ltc4
.63	0.04	0.12	organic anion transporting polypeptide 3 slc21a7; transporter protein
.63	0.03	0.10	chromosome 14 open reading frame 4
.62	0.04	0.11	cubilin intrinsic factor-cobalamin receptor cubn; factor-b12 precursor
.75	0.03	0.07	rtr4alpha1 tr4

.61	0.02	0.03	EST
.04	0.03	0.14	phospholipase c beta 2 plcb2
.69	0.02	0.04	hydroxysteroid sulfotransferase
.79	0.03	0.07	nemo like kinase
0.65	0.04	0.13	EST
0.87	0.04	0.16	sirtuin 5 (silent mating type information regulation 2 homolog) 5
0.62	0.04	0.15	EST
0.86	0.03	0.12	crp-ductin crpd; ebnerin
.71	0.02	0.03	transient receptor potential cation channel, subfamily C, member 4 asso. protein
0.67	0.05	0.20	pancreatitis associated protein iii papiii0; pancreatitis-associated papiii
0.93	0.03	0.02	transmembrane emp24 protein transport domain containing 9
0.82	0.02	0.04	fatty acid binding protein 6 bile acid-binding protein fabp6
.66	0.04	0.17	pleiotrophin heparine binding factor, hbnf, in the mouse ptn
0.86	0.03	0.09	EST
.89	0.02	0.05	brain alpha-tropomyosin tmbr-1; tmbr-3
0.67	0.03	0.07	zona pellucida 2 glycoprotein zp2
.66	0.03	0.10	glutathione S-transferase A3
0.82	0.05	0.28	EST
0.92	0.04	0.18	pancreatitis-associated protein precursor pap; pancreatitis associated; 1 pap1
.03	0.02	0.07	cytosolic peroxisome proliferator-induced acyl-coa thioesterase; hydrolase rbach
.60	0.02	0.03	spectrin repeat containing, nuclear envelope 1

entified in the liver of rats fed rAlb-nptII in comparison with wtAlb fed animals.

change (log_2)	**q-value**	**SD**	**Description**
0.78	0.05	0.30	recoverin rcvrn
0.61	0.03	0.12	second ltb4 receptor julf2 julf2; leukotriene b4 2 ltb4r2
0.69	0.04	0.20	atp-dependent metalloprotease ftsh1 homolog meg-4;
0.69	0.02	0.09	heat shock protein 90, alpha (cytosolic), class A member 1
0.61	0.03	0.10	eukaryotic translation initiation factor 4 gamma, 3
0.62	0.02	0.03	dopamine receptor d5 drd5; d1b
0.65	0.05	0.29	calpastatin probably multiple splicing products cast
0.69	0.02	0.04	nopp140 associated protein nap65
0.98	0.03	0.24	claudin 1 cldn1; claudin-1

0.77	0.04	0.22	rab9, member ras oncogene family rab9; small gtp binding protein
1.66	0.02	0.17	androsterone udp-glucuronosyltransferase ugt2b2
0.60	0.03	0.12	angiotensinogen pat
0.73	0.04	0.22	EST
0.76	0.02	0.06	EST
0.62	0.02	0.08	robo2
0.77	0.03	0.16	rst transporter homolog
0.83	0.03	0.14	vesicle-associated membrane protein 8 endobrevin vamp8
0.90	0.02	0.07	acidic calcium-independent phospholipase a2 aipla2
0.64	0.04	0.18	putative pheromone receptor v2r3
0.86	0.04	0.22	acidic calcium-independent phospholipase a2 aipla2
0.68	0.03	0.12	cytochrome p450, subfamily iif, polypeptide 1 cyp2f1; p4502f4 cyp4502f4
0.75	0.05	0.10	camp-regulated guanine nucleotide exchange factor ii camp-gefii
0.62	0.05	0.27	EST
1.27	0.04	0.08	EST
0.71	0.03	0.12	17-beta hydroxysteroid dehydrogenase type 2 hsd17b2
0.71	0.02	0.04	EST
0.81	0.02	0.05	EST
0.67	0.04	0.19	stress activated protein kinase beta serk2
0.91	0.00	0.03	betaine-homocysteine methyltransferase 2
0.84	0.03	0.17	lipogenic protein s14 lpgp; spot 14 peptide
0.85	0.04	0.06	EST
0.70	0.03	0.11	eukaryotic translation initiation factor 4e binding protein 1 eif4ebp1
0.75	0.02	0.06	hepsin hpn
0.84	0.05	0.31	phospholipase c beta 2 plcb2
0.63	0.05	0.06	acidic membrane protein of rat brain nap-22; basp1
0.65	0.02	0.06	ketohexokinase khk
0.72	0.02	0.06	brain lipid binding protein fabp7; fatty acid
0.90	0.02	0.07	mipp65 protein mipp65
0.60	0.02	0.07	cathepsin y
1.10	0.02	0.10	acidic calcium-independent phospholipase a2 aipla2
0.68	0.02	0.06	EST
0.64	0.02	0.08	vitamin d 3 25-hydroxylase precursor cyp27
0.75	0.03	0.11	EST

0.85	0.02	0.06	EST
0.61	0.03	0.15	syntaxin 5a stx5a; 5
0.63	0.03	0.11	cytochrome p450, subfamily ivb, polypeptide 1
0.62	0.04	0.19	aquaporin-pancreas and liver aqp8; aquaporin 8
0.94	0.03	0.20	EST
0.97	0.04	0.26	lck tyrosine kinase
0.76	0.04	0.24	d site albumin promoter binding protein dbp; transcriptional activator
0.87	0.02	0.06	Shwachman-Bodian-Diamond syndrome homolog (human)
0.63	0.02	0.08	zinc finger protein 17 dzf17
0.84	0.02	0.04	er transmembrane protein dri 42
0.77	0.02	0.04	nadh dehydrogenase, mitochondrial subunit 1 nd1
1.13	0.03	0.24	WNK lysine deficient protein kinase 4
0.65	0.03	0.11	testosterone-6beta-hydroxylase 6 beta-a cyp3a2
0.80	0.05	0.32	melanoma associated antigen (mutated) 1
1.06	0.03	0.23	cyclin d1 ccnd1; rat
0.63	0.04	0.22	type iii multi-pass transmembrane protein
1.01	0.04	0.07	mitochondrial dicarboxylate carrier
0.65	0.02	0.07	sorbitol dehydrogenase sord; l-iditol 2-dehydrogenase
0.92	0.02	0.05	short chain acyl-coa dehydrogenase precursor ec 1.3.99.2
0.77	0.02	0.06	nuclear receptor subfamily 1, group h, member 3 nr1h3; rld-1
0.95	0.02	0.05	mitogen activated protein kinase kinase kinase 1 map3k1; map mekk1
0.66	0.02	0.06	l-gulono-gamma-lactone oxidase precursor; gulo
0.74	0.02	0.09	apolipoprotein e rapoe; apoe
1.47	0.02	0.16	phosphatidylethanolamine n-methyltransferase pemt
0.62	0.04	0.21	aspartyl-trna synthetase dars; drs1
0.78	0.03	0.14	udp-glucuronosyltransferase ugt2b12
0.69	0.04	0.22	leukemia viral v-raf-1 oncogene homolog 1 3611-msv raf1; raf protein
1.27	0.04	0.38	17 beta-hydroxysteroid dehydrogenase type iv hsd iv
0.94	0.02	0.08	ninjurin1
0.88	0.02	0.10	microtubule-associated protein 6 mtap6; stop
0.63	0.03	0.09	ferritin subunit h fth1; heavy chain; ferritin-h
1.06	0.04	0.07	ATPase, H+ transporting, lysosomal V1 subunit D
0.94	0.03	0.15	3-hydroxy-3-methylglutaryl-coa synthase precursor ec 4.1.3.5
0.77	0.05	0.29	tropomyosin 5 slow-twitch alpha tm/htmnm homolog; nonmuscle tpm5

0.86	0.03	0.16	branched-chain alpha-ketoacid dehydrogenase kinase bckdhkin
0.65	0.03	0.11	solute carrier family 8 sodium/calcium exchanger, member 2 slc8a2
1.12	0.00	0.02	pkc-zeta-interacting protein zip
0.82	0.03	0.17	dynamin 2 dnm2; dyn2; iiaa
0.74	0.03	0.11	protein arginine n-methyltransferase prmt1; 1 hrmt1l2
0.86	0.00	0.03	rhodanese
0.66	0.03	0.13	jun b proto-oncogene junb; protein pjunb
0.91	0.04	0.25	similar to Myeloid/lymphoid or mixed-lineage leukemia protein 3 homolog
0.95	0.02	0.12	eukaryotic translation initiation factor 4 gamma, 3
0.72	0.04	0.20	EST
0.64	0.03	0.16	2-alpha globin 2-alpha-1 globin; major alpha-hemoglobin
0.63	0.02	0.08	zinc finger protein 53
0.72	0.02	0.05	EST
0.74	0.02	0.03	cytochrome p450 4f5 cyp4f5
0.95	0.02	0.12	tropic 1808 af078811; tropic1808
0.79	0.03	0.17	rat polymeric immunoglobulin receptor aa -18 to 751
0.68	0.05	0.06	EST
0.69	0.02	0.03	EST
0.61	0.03	0.14	aldolase a, fructose-bisphosphate aldoa; a ec 4.1.2.13
0.90	0.03	0.17	preprocathepsin d
0.79	0.03	0.16	chaperone, ABC1 activity of bc1 complex like
0.61	0.03	0.09	EST
0.68	0.02	0.08	EST
0.61	0.03	0.13	EST
2.23	0.02	0.14	trihydroxycoprostanoyl-coa oxidase thcox
0.63	0.05	0.27	fatty acid elongase 1 relo1
0.65	0.04	0.21	ovalbumin upstream promoter gamma nuclear receptor rcoupg
1.14	0.03	0.18	ARP2 actin-related protein 2 homolog
0.92	0.02	0.11	EST
0.79	0.03	0.12	cytoplasmic beta-actin actx; beta actin
0.91	0.02	0.10	androgen-inducible aldehyde reductase aiar
0.73	0.03	0.17	cdc5 cell division cycle 5, s. pombe, homolog -like cdc5l; cdc5-like protein
0.60	0.03	0.10	atp-stimulated glucocorticoid-receptor translocaton promoter gyk
0.79	0.03	0.17	nedd4

0.74	0.03	0.12	poly adp-ribose glycohydrolase parg
0.68	0.05	0.29	kangai 1 suppression of tumorigenicity 6; metastasis suppressor homolog
0.81	0.02	0.09	glycine methyltransferase gnmt; aa 1-293
0.71	0.02	0.09	EST
0.87	0.04	0.07	cell division cycle 2-like 2
0.66	0.03	0.14	g-protein beta-2 subunit; g protein beta 2
0.61	0.02	0.04	zinc finger protein 103 zfp103; kf-1 adgr34
0.74	0.02	0.05	transmembrane emp24 protein transport domain containing 9
1.02	0.03	0.19	acidic calcium-independent phospholipase a2 aipla2
0.90	0.03	0.17	strain fischer nuclear receptor car nr1i3
0.90	0.03	0.13	branched chain alpha-ketoacid dehydrogenase precursor
0.75	0.03	0.15	prosaposin sulfated glycoprotein, sphingolipid hydrolase activator psap
0.68	0.03	0.14	cathepsin h pre-pro-peptide; rch11; ctsh
0.80	0.03	0.03	beta-galactoside-binding lectin lgals1; protein
1.14	0.03	0.17	epidermal growth factor receptor egfr
1.16	0.04	0.07	5'-amp-activated protein kinase catalytic alpha-2 subunit; amp-activated prkaa2
1.32	0.02	0.17	ARP2 actin-related protein 2 homolog
0.73	0.04	0.20	interferon-inducible protein 16 loc80875
0.77	0.03	0.12	putative n-acetyltransferase camello 2 cml 2; cml5; cml2
0.69	0.02	0.05	triosephosphate isomerase 1 tpi1
0.87	0.03	0.14	atpase, na+k+ transporting, alpha 1 polypeptide atp1a1
0.71	0.02	0.09	riken cdna 2510006j11 txn2-pending; trx2
0.63	0.04	0.18	malic enzyme 3, NADP(+)-dependent, mitochondrial
0.71	0.02	0.07	EST
0.61	0.03	0.02	EST
1.01	0.02	0.06	striated muscle alpha tropomyosin aa 81-284
0.89	0.00	0.02	3-hydroxyanthranilate 3,4-dioxygenase haao
1.00	0.02	0.09	alpha-2u protein aa 1-198
0.61	0.04	0.19	RNA polymerase 1-3
0.68	0.04	0.19	complement component factor h-like 1
1.75	0.04	0.47	EST
0.80	0.02	0.11	sarcosine dehydrogenase sardh
0.75	0.02	0.06	pyruvate carboxylase pc
0.64	0.02	0.04	EST

2.28	0.05	0.22	similar to Ctps protein
0.65	0.02	0.07	cytochrome p450 iid3 protein p450 iid3; cyp2d3; p-450
0.71	0.03	0.13	EST
1.00	0.02	0.14	cAMP responsive element binding protein 3-like 3
0.68	0.05	0.28	dipeptidyl peptidase ii dpp ii; dpp2
0.62	0.03	0.12	ribosomal protein s16 aa 1-146
0.71	0.02	0.06	EST
0.96	0.00	0.02	acidic calcium-independent phospholipase a2 aipla2
0.61	0.04	0.17	ceruloplasmin ferroxidase cp; gpi-anchored
0.65	0.03	0.16	rrna promoter binding protein
0.95	0.02	0.08	acidic calcium-independent phospholipase a2 aipla2
0.96	0.02	0.06	acidic calcium-independent phospholipase a2 aipla2
0.93	0.00	0.03	acidic calcium-independent phospholipase a2 aipla2
0.81	0.05	0.30	lmarrlc1a; marrlc2a
0.86	0.03	0.15	acidic calcium-independent phospholipase a2 aipla2
0.71	0.03	0.11	catalase ec 1.11.1.6; cat
1.03	0.00	0.03	acidic calcium-independent phospholipase a2 aipla2
1.06	0.02	0.08	acidic calcium-independent phospholipase a2 aipla2
0.97	0.02	0.11	acidic calcium-independent phospholipase a2 aipla2
1.01	0.02	0.08	bmal1b
0.65	0.02	0.05	upstream transcription factor 1 usf1; usf-1
0.68	0.00	0.02	apolipoprotein a-v apoa-v; apoa5
0.61	0.03	0.11	stress-inducible chaperone mt-grpe#1 grpe#1; precursor
0.67	0.04	0.21	solute carrier family 14, member 1 slc14a1; urea transporter ut3; ut11
0.78	0.03	0.12	acidic calcium-independent phospholipase a2 aipla2
0.62	0.03	0.16	EST
0.86	0.02	0.11	EST
0.66	0.02	0.04	coagulation factor x f10
0.77	0.03	0.03	gtpase rab8b rab8b
0.71	0.03	0.10	protein-tyrosine kinase csk
0.67	0.03	0.11	delta subunit of f1f0 atpase
0.90	0.04	0.07	plakophilin 4 (predicted)
0.96	0.03	0.16	EST
0.64	0.02	0.08	hepatic nuclear factor 4 alpha transcription factor 4 hnf4a; hepatocyte; hnf-4

0.79	0.02	0.05	cdc37 cell division cycle 37, s. cerevisiae, homolog cdc37
0.68	0.03	0.16	gaba transporter
0.84	0.03	0.12	endothelial p22-phox protein p22-phox; cytochrome b558 alpha-subunit cyba
0.62	0.00	0.01	rat ribosomal protein l13a
0.65	0.02	0.05	cd14 antigen cd14
1.26	0.03	0.06	spliceosomal protein sap155
0.68	0.02	0.05	phosphatidylethanolamine binding protein pbp; phosphatidylethanolamine-binding
0.62	0.02	0.07	enoyl-coa hydratase, short chain 1, mitochondrial echs1
0.69	0.02	0.05	EST
0.97	0.02	0.04	guanine aminohydrolase gah; deaminase gda
0.66	0.03	0.11	paired-like homeodomain transcription factor 3 pitx3; ptx3 protein
0.73	0.02	0.08	asialoglycoprotein receptor 1 hepatic lectin asgr1
0.68	0.02	0.05	lis1-interacting protein nude2
0.63	0.03	0.11	pituitary tumor-transforming 1 interacting protein
0.78	0.05	0.36	HypB, AroK, ADK and rho factor domain containing protein RGD1303144
0.65	0.05	0.29	EST
0.60	0.03	0.14	organic cation transporter slc22a1
0.74	0.05	0.30	thiopurine s-methyltransferase tpmt
0.63	0.03	0.10	putative glycogen storage disease type 1b protein; glucose-6-phosphatase
1.05	0.02	0.14	amino acid system n transporter
0.63	0.05	0.27	EST
0.94	0.02	0.12	liver activating protein lap, also nf-il6, nuclear factor-il6,
0.89	0.04	0.24	guanine nucleotide binding protein g protein alpha 12 gna12
0.85	0.03	0.17	tweety homolog 1 (Drosophila)
0.76	0.02	0.07	EST
0.94	0.02	0.05	EST
0.75	0.03	0.17	p21 (CDKN1A)-activated kinase 4
4.86	0.05	1.95	cytosolic peroxisome proliferator-induced acyl-coa thioesterase
0.65	0.02	0.03	contrapsin-like protease inhibitor related protein cpi-23; serine 1
0.87	0.02	0.04	glucose regulated protein, 58 kda grp58; er-60 protease; er60; pi-plc i aa 1-504
0.72	0.02	0.00	thymidine phosphorylase
0.67	0.03	0.10	4-hydroxyphenylpyruvic acid dioxygenase hpd; 4-hydroxyphenylpyruvate hppd
0.66	0.05	0.29	evectin-1 evt1; kpl1
0.63	0.02	0.09	fructose-1,6-bisphosphatase fru-1,6-p2-ase

0.80	0.03	0.16	adp-ribosylation factor 5 arf5
0.80	0.02	0.07	prostaglandin i2 prostacyclin synthase ptgis; ratpgis
1.20	0.03	0.31	nadh dehydrogenase subunit 5 nd5
0.71	0.03	0.13	EST

entified in the spleen of rats fed rBV-VP60 in comparison with wtBV fed animals.

change log_2)	**q-value**	**SD**	**Description**
1.42	0.04	0.12	lsc protein lsc
0.68	0.03	0.04	putative rna helicase bat1; liver nuclear protein p47
0.61	0.03	0.04	coagulation factor iii thromboplastin, tissue factor f3; protein
0.83	0.00	0.02	EST
0.94	0.04	0.12	12/15-lipoxygenase 12/15-lipoxygenase, 12/15-lo, tk15lo
0.73	0.04	0.06	rt1.b-1 beta-chain
0.95	0.04	0.09	invariant polpypeptide of major histocompatibility class ii antigen-associated cd74
1.02	0.05	0.16	interleukin 1 receptor, type i il1r1; receptor; soluble il-1
0.80	0.04	0.10	a regulatory subunit of protein phosphatase 2a pp2a ara
1.11	0.05	0.15	phospholipase a2, group ib, pancreas pla2g1b; pancreatic a-2
1.60	0.03	0.07	uncoupling protein 2, mitochondrial ucp2; 2; protein-2
1.44	0.03	0.07	rst transporter homolog
0.73	0.04	0.07	lung kruppel-like factor lklf
0.63	0.04	0.06	pv-1 pv1
0.69	0.00	0.00	ankyrin repeat domain 12
0.65	0.05	0.12	epithelin 1 & 2; granulin; grn
0.70	0.05	0.10	ubiquitin-like 5
0.68	0.00	0.02	5-oxoprolinase atp-hydrolysing oplah; 5-oxo-l-prolinase
1.00	0.03	0.06	invariant chain aa 1-280
1.25	0.04	0.12	stress activated protein kinase beta serk2
0.63	0.04	0.07	EST
0.78	0.03	0.04	beta-1 adducin add1
0.95	0.05	0.17	neural visinin-like protein 1 vsnl1; nvp
0.77	0.05	0.13	pan-2; pan-1
0.70	0.05	0.11	carboxypeptidase a1 pancreatic cpa1; a precursor; preprocarboxypeptidase
0.68	0.03	0.03	immunoglobulin kappa-chain igkv

1.12	0.04	0.11	carboxypeptidase cpa2
0.83	0.04	0.08	reading frame preproalbumin
0.68	0.00	0.03	EST
1.37	0.04	0.15	ribosomal protein S2
0.67	0.04	0.07	talin 1
0.66	0.05	0.09	t-cell receptor alpha-chain c-region precursor
0.61	0.04	0.06	EST
0.60	0.03	0.03	coronin, actin binding protein 1b coro1b; coronin-like
0.63	0.04	0.05	dna polymerase gamma mip1; polg
0.66	0.05	0.13	yb2
0.65	0.05	0.11	preproprolactin-releasing peptide loc63850; prolactin-releasing prrp
1.45	0.00	0.05	cyclase-associated protein homologue cap1; mch1
0.61	0.05	0.09	EST
0.71	0.05	0.11	glutamate receptor, ionotropic, ampa4 alpha 4 gria4
1.55	0.00	0.05	d-beta-hydroxybutyrate dehydogenase; calreticulin; calr
1.17	0.03	0.07	annexin vi anxa6
0.63	0.05	0.13	eukaryotic translation elongation factor 2 eef2; ef-2; aa 1-858
0.64	0.04	0.06	janus kinase 3, protein-tyrosine kinase jak3
1.15	0.03	0.05	preprocaldecrin; elastase iv
1.14	0.04	0.12	alpha-actin aa 1-377
0.65	0.05	0.09	sushi domain containing 2 (predicted)
0.67	0.04	0.05	EST
0.71	0.05	0.10	s2 kallikrein; tonin; ton
0.98	0.05	0.19	nadh dehydrogenase subunit 5 nd5
0.77	0.03	0.04	
0.61	0.05	0.10	type 2 angiotensin ii receptor at2r; agtr2; type-2
1.07	0.03	0.06	cathepsin ec 3.4.22.1; b ctsb
1.16	0.05	0.25	similar to exosome component 10
1.59	0.05	0.24	atpase, na+k+ transporting, beta polypeptide 2 atp1b2
0.95	0.03	0.05	proline rich protein
0.65	0.05	0.13	EST
1.32	0.00	0.03	light chain 3 subunit of microtubule-associated proteins 1a and 1b; 1a/1b mpl3
1.64	0.00	0.05	selenoprotein w muscle 1 sepw1; selw
0.63	0.04	0.08	pctaire-1a protein kinase; pctaire-1b

0.67	0.03	0.03	ubiquitin-conjugating enzyme E2M (UBC12 homolog, yeast)
1.05	0.05	0.19	lutheran blood group auberger b antigen included lu
1.66	0.04	0.12	cofilin 1, non-muscle cfl1
0.73	0.05	0.15	oligodendrocyte-myelin glycoprotein
0.64	0.03	0.03	rab11b, member ras oncogene family rab11b
1.21	0.03	0.06	related to drosophila groucho gene aes; r-esp1
0.77	0.00	0.01	zyxin
1.60	0.00	0.04	plasma glutathione peroxidase precursor gpxp
1.65	0.04	0.14	chymotrypsin-like ctrl; chymopasin
0.86	0.05	0.17	hemicentin 1
0.85	0.05	0.13	transient axonal glycoprotein 1 tag-1 tax; precursor
0.69	0.04	0.06	EST
0.92	0.04	0.10	sepiapterin reductase ec 1.1.1.153
1.02	0.04	0.13	fission 1 (mitochondrial outer membrane) homolog (yeast)
0.61	0.05	0.10	avian myelocytomatosis viral v-myc oncogene homolog myc; c-myc protein
0.62	0.04	0.05	lyric
0.71	0.05	0.11	cytoplasmic beta-actin actx; beta actin
0.79	0.05	0.13	adrenergic receptor, alpha 1c adra1c; alpha1c receptor; alpha1c-adrenergic
0.77	0.04	0.06	small inducible cytokine a5 rantes scya5
1.13	0.05	0.17	ovalbumin upstream promoter beta nuclear receptor rcoupb
0.61	0.05	0.09	immunoglobulin kappa light chain variable region; kappa-chain igkv
1.20	0.03	0.07	myosin, light polypeptide 2, alkali ventricular, skeletal
1.30	0.04	0.13	carboxypeptidase b cpb
0.63	0.04	0.07	mad mothers against decapentaplegic, drosophila homolog 3 madh3; dpp mad3
1.31	0.05	0.21	adaptor-related protein complex 3, beta 2 subunit (predicted)
0.61	0.04	0.07	EST
0.74	0.05	0.11	nadh dehydrogenase subunit 2 nd2
0.90	0.05	0.18	ribosomal RNA processing 1 homolog (S. cerevisiae)
1.10	0.03	0.07	pancreatic triglyceride lipase; pnlip
1.04	0.04	0.10	pancreatic lipase-related protein 2 pnliprp2; lipase
0.97	0.05	0.13	sperm membrane protein
0.64	0.05	0.14	anti-idiotype immunoglobulin m light chain; lambda-2 igl
0.82	0.04	0.10	fatty acid binding protein 1, liver fabp1; fabp; sterol carrier
0.75	0.05	0.02	tektin 1 tekt1

1.13	0.04	0.09	pancreatic secretory trypsin inhibitor type ii precursor
0.80	0.05	0.12	putative alpha 1b-glycoprotein
1.33	0.04	0.14	protein kinase c, zeta subspecies; c; pkcz
0.62	0.04	0.06	colipase pancreatic clps
0.68	0.03	0.03	EST
0.68	0.04	0.07	malic enzyme 3, NADP(+)-dependent, mitochondrial (predicted)
0.94	0.05	0.14	platelete glycoprotein 5 gp5; platelet v
0.94	0.04	0.10	zinc finger transcription factor gli3 gli3
0.69	0.04	0.08	retinoic acid receptor beta2
2.17	0.05	0.31	protein kinase c-binding protein nell1 nell1
1.13	0.05	0.18	pancreatic lipase related protein 1 pnliprp1; triacylglycerol
0.68	0.05	0.09	coagulation factor XI
1.32	0.00	0.04	mhc a-beta rt1.b-b-beta cell surface glycoprotein
1.33	0.03	0.07	non-muscle alpha-actinin 1 actn1
0.70	0.05	0.13	gaba-b r2 receptor gaba-b r2; gb2; 2 gaba-br2; gpr51
0.82	0.04	0.08	immunoglobulin 4g6 light chain variable region
0.62	0.05	0.09	corticotropin-releasing factor binding protein; crh-binding
0.63	0.05	0.11	aryl hydrocarbon receptor ahr; ah
0.72	0.05	0.15	sarcomeric mitochondrial creatine kinase
0.86	0.05	0.13	cell membrane glycoprotein 110000mr surface antigen homolog
0.88	0.00	0.01	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activatioprotein
0.73	0.00	0.03	breakpoint cluster region protein, uterine leiomyoma,
0.62	0.05	0.11	sperm tail protein spag5
0.86	0.04	0.09	cardiac potassium channel subunit kv6.2 kv6.2
1.22	0.05	0.23	immunoglobulin mu-chain igm; mu-immunoglobulin
0.75	0.04	0.08	j domain protein 1 jdp1
1.10	0.05	0.23	ribosomal protein l38
1.22	0.05	0.21	rt1.b-1 beta-chain
0.68	0.05	0.12	EST
0.86	0.04	0.11	betacellulin btc
1.06	0.04	0.10	killer cell lectin-like receptor subfamily c, member 2 klrc2
0.68	0.05	0.11	nmda receptor glutamate-binding subunit
1.42	0.05	0.25	transforming growth factor beta-1 gene tgfb1; prepro-tgf-beta 1 aa -23 to 367
0.83	0.03	0.05	ubiquitin-conjugating enzyme e2n homologous to yeast ubc13 ube2n

1.23	0.04	0.11	proto-oncogene fyn p59fyn
1.03	0.04	0.11	cd37 antigen cd37
0.72	0.04	0.05	pyrimidine binding protein 2 pybp2
0.74	0.04	0.06	unconventional myosin myr2 i heavy chain myr2; myr 2
0.79	0.04	0.06	guanylate cyclase, soluble, beta 2 gtp pyrophosphate - lyase gucy1b2
0.93	0.05	0.18	profilin pfn1
0.74	0.00	0.01	lyn protein non-receptor kinase lyn; a tyrosine lyna; b lynb
0.72	0.04	0.07	unknown
0.67	0.05	0.12	cbl-b
1.10	0.05	0.03	calpastatin probably multiple splicing products cast
0.86	0.05	0.12	adh water channel; complete cds; aquaporin 2 aqp2
0.74	0.05	0.11	activity and neurotransmitter-induced early gene protein 4 ania-4 ania4
0.77	0.05	0.15	sequence-specific single-stranded-dna-binding protein ssdp
1.15	0.05	0.16	carboxypeptidase h precursor ec 3.4.17.10; e cpe; aa 1-476
1.37	0.04	0.09	reading frame preproelastase ii; elastase precursor; 2, pancreatic ela2
0.76	0.05	0.15	EST
0.84	0.04	0.07	pius protein pius
0.65	0.05	0.10	thymosin beta-4 precursor; peptide; protein; tmsb4x
0.62	0.04	0.05	vascular cell adhesion molecule 1 vcam-1; molecule-1; vcam1
1.32	0.00	0.05	EST
0.80	0.05	0.15	EST
0.70	0.03	0.04	WD repeat domain 24
1.25	0.05	0.17	solute carrier family 4, member 2, anion exchange protein 2 slc4a2
0.99	0.03	0.06	myosin regulatory light chain 17 is 2nd base in codon; mrlcb
0.67	0.05	0.10	chymotrypsin b chyb; ctrb
0.81	0.03	0.03	EST
1.07	0.00	0.03	preproelastase i; elastase; 1 ela1
0.72	0.04	0.06	ephrin a3
0.83	0.04	0.10	nadh dehydrogenase subunit 5 nd5

entified in the liver of rats fed rBV-VP60 in comparison with wtBV fed animals.

change (log_2)	q-value	SD	Description
0.82	0.05	0.32	heat shock protein 90, alpha (cytosolic), class A member 1
0.76	0.04	0.13	endothelin-converting enzyme 1 ece1
0.66	0.04	0.18	fk506 binding protein 1b 12.6 kda fkbp1b; fk506-binding
1.08	0.02	0.07	lipopolysaccharide binding protein lbp
0.86	0.04	0.17	EST
1.31	0.03	0.17	androsterone udp-glucuronosyltransferase ugt2b2
1.28	0.04	0.26	interleukin 1 receptor, type i il1r1; receptor; soluble il-1
0.62	0.03	0.05	angiotensinogen pat
0.99	0.03	0.08	peroxisomal enoyl-coa: hydrotase-3-hydroxyacyl-coa
0.88	0.00	0.03	carnitine o-octanoyltransferase crot; octanoyltransferase
0.63	0.04	0.11	2,4-dienoyl coa reductase 1, mitochondrial decr1; 2,4-dienoyl-coa precursor
0.65	0.03	0.08	EST
1.51	0.02	0.07	serine dehydratase aa 1 - 327; sdh2; sds
1.83	0.04	0.35	rst transporter homolog
1.51	0.05	0.11	ca2+/calmodulin-dependent protein kinase neuronal isoform delta a ca2
0.67	0.03	0.09	EST
0.69	0.03	0.08	betaine-homocysteine methyltransferase 2
0.60	0.04	0.16	EST
1.43	0.04	0.37	stress activated protein kinase beta serk2
0.83	0.04	0.21	synaptic vesicle glycoprotein 2 a sv2a; protein sv2
0.80	0.03	0.07	betaine-homocysteine methyltransferase 2
0.82	0.03	0.08	coiled-coil alpha-helical rod protein 1
0.64	0.04	0.09	transcription factor 1, hepatic lf-b1, hepatic nuclear factor hnf1 tcf1 tcf1
0.92	0.04	0.20	prostacyclin receptor
0.65	0.04	0.11	carnitine octanoyltransferase
0.87	0.04	0.13	zinc finger protein; kidney 1 kid1
0.82	0.04	0.22	dermatan sulfate proteoglycan-ii decorin; dcn
0.79	0.05	0.26	ets domain transcription factor pet-1
0.92	0.04	0.13	neural visinin-like protein 1 vsnl1; nvp
0.92	0.05	0.26	receptor tyrosine kinase ret
0.84	0.04	0.13	retinol dehydrogenase type ii

0.82	0.03	0.08	glutathione synthetase
0.98	0.00	0.02	putative zinc finger protein serz-1
0.64	0.03	0.07	EST
0.81	0.05	0.25	EST
0.71	0.05	0.26	EST
1.37	0.04	0.18	ribosomal protein S2
0.86	0.04	0.22	atp synthase
0.60	0.03	0.06	m2 pyruvate kinase pk
0.82	0.05	0.23	prion protein, structural prnp; protein prp; raprp; prion-related; prn
0.94	0.05	0.08	tafiib taf2b
0.61	0.04	0.12	cytochrome p450, subfamily ivb, polypeptide 1 see also d5m4rp1 cyp4b1
0.64	0.04	0.08	electron transfer flavoprotein alpha-subunit etf
0.62	0.04	0.12	neurotensin-degrading neutral metalloendopeptidase
0.64	0.04	0.10	kruppel-like factor 15 kidney klf15; transcription
0.90	0.04	0.13	type iii multi-pass transmembrane protein
0.88	0.05	0.27	protein tyrosine phosphatase epsilon ptpepsilon; m
0.64	0.04	0.14	cyclase-associated protein homologue cap1; mch1
0.80	0.03	0.09	d-beta-hydroxybutyrate dehydogenase; calreticulin; calr
0.61	0.04	0.09	sushi domain containing 2 (predicted)
0.95	0.05	0.27	aminocarboxymuconate semialdehyde decarboxylase
0.78	0.05	0.22	r8f dna-binding protein loc84382
0.95	0.05	0.30	activity and neurotransmitter-induced early gene protein 4 ania-4 ania4
0.66	0.02	0.04	transcriptional repressor crem transcriptional repressor crem, icer
0.70	0.03	0.08	paired-like homeodomain transcription factor drg11
0.85	0.03	0.10	phosphatidate phosphohydrolase type 2 ppap2
0.81	0.04	0.11	proline rich protein
1.48	0.05	0.49	unidentified reading frame
0.62	0.04	0.10	light chain 3 subunit of microtubule-associated proteins 1a and 1b; 1a/1b mpl3
0.62	0.03	0.08	cofilin 1, non-muscle cfl1
0.66	0.04	0.08	beta-carotene 15, 15'-dioxygenase bcdo
0.67	0.04	0.16	nuclear factor, interleukin 3, regulated nfil3; nfil3/e4bp4 transcription factor
0.67	0.04	0.18	SAR1 gene homolog A (S. cerevisiae)
0.74	0.00	0.01	oligodendrocyte-myelin glycoprotein
0.77	0.05	0.29	neuronal calcium sensor-1 freq; sensor ncs-1; frequenin-like protein

0.65	0.05	0.19	N-myc downstream regulated gene 1
0.61	0.04	0.01	lipid-binding protein; c-fabp; da11
0.99	0.05	0.39	adenylyl cyclase type iv; 4 adcy4
0.61	0.03	0.04	p85
0.89	0.05	0.35	EST
0.76	0.04	0.18	antigen identified by monoclonal antibodies 4f2 mdu1
1.33	0.04	0.18	laminin chain
1.04	0.05	0.32	platelet-derived growth factor receptor beta pdgfrb
1.00	0.04	0.14	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10
0.80	0.04	0.17	pdk2.1 pyruvate dehydrogenase kinase 2 subunit variant p45; pdk2
0.65	0.03	0.05	atp-stimulated glucocorticoid-receptor translocaton promoter gyk
0.84	0.03	0.09	adrenergic receptor, alpha 1c adra1c; alpha1c receptor; alpha1c-adrenergic
0.60	0.05	0.17	kidney androgen-regulated protein kap
0.76	0.02	0.04	nedd4
1.25	0.02	0.05	myosin, light polypeptide 2, alkali ventricular, skeletal, slow myl2
0.60	0.02	0.03	glycine receptor alpha 2 subunit glycine receptor, neonatal glra2
0.64	0.02	0.03	hepatocyte nuclear factor 6 alpha hnf-6; hnf6; beta
0.73	0.04	0.15	nuclear gtpase pike pike
0.66	0.05	0.22	Ras association (RalGDS/AF-6) domain family 1
0.65	0.04	0.12	n-acetylated alpha-linked acidic dipeptidase-like ileal peptidase i100 naaladasel
0.90	0.05	0.27	ca2+/calmodulin-dependent protein kinase ii alpha camk2a
0.65	0.02	0.03	sperm membrane protein
0.71	0.05	0.27	EST
0.61	0.03	0.05	solute carrier family 28 (sodium-coupled nucleoside transporter), member 2
0.77	0.04	0.18	adamts-4
0.84	0.05	0.24	prestin pres
0.76	0.05	0.22	putative alpha 1b-glycoprotein
0.93	0.05	0.29	protein kinase c, zeta subspecies; c; pkcz
0.62	0.03	0.06	arrestin, beta 1 arrb1; beta-arrestin
0.63	0.05	0.23	EST
0.78	0.05	0.05	platelete glycoprotein 5 gp5; platelet v
0.62	0.04	0.10	rap2a-like protein rap2a; rap2b
0.81	0.05	0.26	EST
0.99	0.05	0.29	zinc finger transcription factor gli3 gli3

0.61	0.05	0.18	EST
1.04	0.04	0.26	microtubule-associated protein aa 1-1830; 2 map2; microtubule associated
0.73	0.04	0.17	EST
1.25	0.05	0.35	solute carrier family 12,
0.73	0.05	0.27	brevican brain specific proteoglycan in the aggrecan family bcan;
0.80	0.02	0.03	utrophin homologous to dystrophin utrn; unnamed protein product
0.68	0.05	0.07	wingless-type mmtv integration site family, member 4 wnt4; wnt-4
0.66	0.05	0.24	inositol polyphosphate-5-phosphatase B
1.03	0.05	0.34	sperm tail protein spag5
0.68	0.04	0.09	cardiac potassium channel subunit kv6.2 kv6.2
0.80	0.02	0.05	udp-glucuronosyltransferase
0.61	0.02	0.04	sorting nexin 3
1.14	0.04	0.18	ribosomal protein l38
0.62	0.04	0.10	somatostatin transactivating factor-1 somatostatin transactivating factor-1,
1.16	0.04	0.25	killer cell lectin-like receptor subfamily c, member 2 klrc2
0.75	0.04	0.11	nude
0.90	0.04	0.19	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27
0.72	0.04	0.13	apoptosis inhibitor protein 3 api3; riap3; of
0.67	0.05	0.25	rat cyclin e
0.67	0.03	0.01	trp1 beta variant
0.87	0.05	0.29	adh water channel; complete cds; aquaporin 2 aqp2
1.27	0.02	0.07	integrin beta-7 subunit
0.64	0.04	0.18	glutamate receptor, metabotropic 5 grm5; receptor mglur5
1.75	0.05	0.50	nuclear core glycoprotein np62; p62; protein aa 1-525
0.66	0.03	0.06	apical endosomal glycoprotein
0.80	0.05	0.26	cgrp-receptor component protein
0.89	0.04	0.16	activating transcription factor atf-4 atf4
1.08	0.04	0.15	EST
0.67	0.04	0.16	SH3 and cysteine rich domain 3 (predicted)
0.63	0.05	0.21	mature T-cell proliferation 1
0.61	0.05	0.23	glutathione s-transferase yc2 subunit gsta5; transferase gst
0.73	0.03	0.08	liver activating protein lap, also nf-il6, nuclear factor-il6
0.71	0.04	0.11	retinoic acid receptor alpha1
0.74	0.05	0.30	EST

0.94	0.04	0.25	EST
0.64	0.05	0.18	kidney-derived aspartic protease-like protein kdap; napsin nap
0.70	0.03	0.05	asialoglycoprotein receptor 2 asgr2; rhl2; hepatic lectin aa 1-301
0.84	0.05	0.28	homeobox protein
0.76	0.05	0.23	somatostatin receptor subtype 1 sstr1
0.77	0.04	0.18	WD repeat domain 24
0.62	0.04	0.14	solute carrier family 4, member 2, anion exchange protein 2 slc4a2
0.68	0.03	0.07	dolichol-phosphate (beta-D) mannosyltransferase 1 (predicted)
0.62	0.00	0.02	d-beta-hydroxybutyrate dehydogenase; calreticulin; calr
0.78	0.05	0.27	ephrin a3
1.00	0.04	0.02	odorant receptor
1.09	0.04	0.16	protease, serine, 23

entified in small intestine epithelium of rats fed rBV-VP60 in comparison with wtBV fed animals.

change (og_2)	**q-value**	**SD**	**Description**
-1.30	0.00	0.05	calcium-binding protein, intestinal, vitamin d-dependent 9-kda cabp calb3
0.76	0.00	0.01	retinoic acid hydroxylase

9.2 Equipment

Centrifuges:	
Centrifuge 5417 C	(Eppendorf/Gilson, Hamburg)
Centrifuge CL GS-6R	(Beckmann, München)
Heraeus Labofuge 400	(Heraeus Instruments, Düsseldorf)
Electrophoresis chambers:	
Horizontal gel electrophoresis chamber D3, OWL	(Angewandte Gentechnische Systeme GmbH, Heidelberg)
RNA gel electrophoresis chamber	(Biometra, Göttingen)
Electrophoresis Power Supply:	
High Voltage Power Pack P30	(Biometra, Göttingen)
Vacuum concentrator:	
Univac D08	(Progen Scientific Ltd, Mexborough, South Yorkshire, UK)
Thermostat:	
Dri-Block® DB-3D	(Techne, Staffordshire, UK)
Homogenizator:	
Ultra Turrax T25 basic	(IKALabortechnik, IKA-Werke GMBH & CO.KG, Staufen)
Microwave:	
R3 V10	(Sharp Corporation, Thailand)
Vortexer:	
Vortex Genie 2TM G 560 E	(Scientific Industries, Bohemia, USA)
pH-meter:	
HI 221 Calibration-Check pH meter	(HANNA Instruments, Kehl am Rhein)

Laser scanner:	
428 Array Scanner	(Affymetrix Inc., Santa Clara, USA)
PCR instruments:	
PCR express thermal cycler	(Hybaid, Ashford, UK)
TRIO-thermal block TB 1	(Biometra, Göttingen)
Real-time PCR instrument:	
LightCycler 3	(Roche Diagnostics, Mannheim)
Spectrophotometer:	
Nano Drop® ND-100 Spectrophotometer	(NanoDrop Technologies, Wilmington, Delaware USA)
Waterbath:	
Medingen W6	(Prüfwerk Medingen, Medingen)
Magnetic stirrer:	
IKAMAG® RET	(Janke & Kunkel, Staufen)
UV Transilluminator:	
UV Transilluminator TFM 20	(Ultra-Violet Products Ltd, Cambridge, UK)
Gel documentation:	
Kaiser Repro RS1+RA1 5510	(Ultra-Violet Products Ltd, Cambridge, UK)
QImaging 32-0030C-104	(Ultra-Violet Products Ltd, Cambridge, UK)
QIGAM Mono 10-bit	(Ultra-Violet Products Ltd, Cambridge, UK)
Printer P91 D	(Mitsubishi Electric Europe, Ratingen)
Water Purification Systems:	
Milli-Q	(Millipore, Indien)
Sterile filtration:	
ME 24	(Schleicher & Schuell GmbH, Dassel)

9.3 Software and databases

Base SAS® (Version 9.1.3)	SAS Institute Inc., Cary, NC, USA
DAVID 2007	http://david.abcc.ncifcrf.gov/
GAZER (Gene Set Analyzer)	http://expressome.kobic.re.kr/ GAzer/index.faces
GeneSight (Version 3.5)	BioDiscovery Inc., Los Angeles, USA
ImaGene™ (Version 5)	BioDiscovery Inc., Los Angeles, USA
Lab Works (Version 4.5.)	Ultra-Violet Products Ltd, Cambridge, UK
LightCycler (Version 3.5)	Roche Diagnostics, Mannheim
MAVI Pro (Version 2.6.0)	BioDiscovery Inc., Los Angeles, USA
NCBI database	http://www.ncbi.nlm.nih.gov/
Primer3	http://frodo.wi.mit.edu/
QCapture (Version 1.66)	Ultra-Violet Products Ltd, Cambridge, UK
QVALUE	http://faculty.washington.edu/~jstorey/qvalue/
R version 2.5.2 (2007-06-27)	http://www.bioconductor.org/
TIGR MultiExperimentViewer	http://www.tigr.org/software/
”R” software	http://www.bioconductor.org
Rat genome database	http://rgd.mcw.edu/genes/?100

9.4 Chemicals, kits and solutions

9.4.1 Chemicals

10 x buffer - taq DNA Polymerase	(Promega, Madison USA)
2-ß-Mercaptoethanole	(Sigma Aldrich Chemicals, Steinheim)
3-(N-morpholino)propanesulfonic acid, MOPS	(Sigma-Aldrich, Steinheim)
5 x first strand buffer	(Invitrogen, Karlsruhe)
Acetic acid	(Roche, Mannheim)
Aqua bidestillata	(Qiagen, Hilden)
Bovine serum albumin, Cohn Fraction V, BSA	(Sigma-Aldrich, Steinheim)
Bromphenole blue, 0.1 %	(Serva, Heidelberg)
Desoxynucleoside triphosphates (dATP, dTTP, dCTP, dGTP), 10 mM	(Promega, Mannheim)
Diethylpyrocarbonate 0.1 %, DEPC	(Serva, Heidelberg)
Di-Natriumhydrogenphosphate, Na_2HPO_4	(Merck KGaA, Darmstadt)
Dithiotreitol, DTT, 100 mM	(Invitrogen, Karlsruhe)
DNase I (10 U/µl) + RDD buffer	(Qiagen, Hilden)

Ethanol, 96 - 100 %	(Applichem, Darmstadt)
Ethidiumbromide, 10 mg/µl	(Serva, Heidelberg)
Ethylene-diamine-tetra-acetic acid, EDTA	(Applichem, Darmstadt)
Ficoll Typ 400	(Amersham Pharmacia Biotech, Freiburg)
FluoroLink™ Cy3-UTP, 5 mM	(Amersham, Buckinghamshire, UK)
FluoroLink™ Cy5-UTP, 5 mM	(Amersham, Buckinghamshire, UK)
Formaldehyde, 37 %	(Roth, Karlsruhe)
Formamide, 99.5 %	(Sigma Aldrich Chemicals, Steinheim)
Magnesium acetate, Mg-ac	(Roth, Karlsruhe)
Magnesium chloride, $MgCl_2$	(Roth, Karlsruhe)
Magnesium sulfate, $MgSO_4$	(Roth, Karlsruhe)
M-MLV Reverse Transcriptase	(Invitrogen, Karlsruhe)
Potassium acetate	(Roth, Karlsruhe)
pBR322 DNA/AluI Marker 20	(Fermentas, St. Leon-Rot)
peq Gold Universal Agarose	(peqLab, Erlangen)
Poly T12 Primer, 50 pmol/µl	(Sigma Aldrich Chemicals, Steinheim)
Polyvinylpyrrolidone, PVP	(Sigma-Aldrich, Steinheim)
Proteinase K, 20 mg/ml	(Roche Diagnostics, Mannheim)
QIAzol® lysis reagent	(Qiagen, Hilden)
Rnase-free water	(Qiagen, Hilden)
RNasin, Ribonuclease Inhibitor, 40U/µl	(Promega, Madison USA)
Sodium acetate	(Merck KGaA, Darmstadt)
Sodium chloride	(Roche, Mannheim)
Sodium citrate	(Roth, Karlsruhe)
Sodium dihydrogen phosphate	(Merck KGaA, Darmstadt)
Sodium hydroxide, NaOH	(Roth, Karlsruhe)
Sodium-Dodecyl-Sulfate, SDS ultra pure	(Roth, Karlsruhe)
Taq DNA Polymerase, 5 U/µl	(Promega, Madison USA)
TRIS, 99.3 %	(Roth, Karlsruhe)
TRIS-Acetate, TRIS-ac	(Roth, Karlsruhe)

9.4.2 Kits

cDNA Synthesis System	(Roche, Mannheim)
High Pure RNA Tissue Kit	(Roche, Mannheim)

LightCycler® FastStart DNA (Master SYBR Green I)	(Roche, Mannheim)
MEGAscript® T7 Kit	(Ambion, Cambridgeshire, UK)
QIAquick® Gel Extraction Kit	(Qiagen, Hilden)
QIAquick® PCR Purification Kit	(Qiagen, Hilden)
RNeasy® Mini Kit	(Qiagen, Hilden)

9.4.3 Solutions

TRIS buffer	5 ml	1 M TRIS/HCl (pH 8,0)
	20 ml	0.5 M EDTA
	5 ml	10 % SDS
	70 ml	Aqua bidestillata
TE-buffer	2.5 ml	1 M TRIS/HCl (pH 8.0)
	0.5 ml	0.5 M EDTA
	247 ml	Aqua bidestillata
10 x TAE-buffer (pH 8.0)	48.46 g	TRIS-ac
	3.72 g	EDTA
	1 l	Aqua bidestillata
Loading buffer DNA	50 ml	Glycerin, 99 %
	1 ml	Bromphenole blue, 10 %
	24 ml	0.5 M EDTA
	1 ml	1 M TRIS/HCl (pH 8.0)
Agarose gel (0.1 M)	0.75 g	Agarose
	75 ml	TAE-buffer
	3 µl	Ethidiumbromide (10mg/µl)
Formaldehyde gel (2.2 M)	30 mg	Agarose
	21.70 ml	DEPC-H20
	5.37 ml	Formaldehyde, 37 %

Loading mix RNA	5 µl	Formamide
	1.75 µl	Formaldehyde, 37 %
	5 µl	10 x MOPS
	1.0 µl	Loading buffer RNA
	0.25 µl	Ethidiumbromide
Loading buffer RNA	30 ml	Glycerin, 99 %
	0.25 g	Bromphenole blue
	200 µl	10 mM EDTA
	0.25 g	Xylencyanole
10 x MOPS, pH 7.5	83.72 g	MOPS
	13.61 g	Na-ac
	3.72 g	EDTA
	1 l	Aqua bidestillata
5 x Fragmentation buffer	0.50 ml	1 M TRIS/HCl (pH 8.1)
	0.12 g	Potassium acetate
	0.08 g	Mg-ac
	24.50 ml	RNase-free water
Sodium Phosphate	50 mM	Di-Natriumhydrogenphosphate
(pH 8.0)	50 mM	Sodium dihydrogen phosphate
20 x SSC stock solution	175.3 g	Sodium Phosphate
(pH 7.0)	88.2 g	Sodium citrate
50 x Denhardt's solution	5 g	Ficoll Typ 400
	5 g	PVP
	5 g	BSA
	500 ml	Aqua destillata
Hybridization buffer	50 µl	1 M Sodium phosphate (pH8.0)
	500 µl	100 % Formamide
	300 µl	20 x SSC
	100 µl	50 x Denthardt´s solution
	50 µl	10 % SDS

Washing solution 1	100 ml	20 x SSC
	10 ml	10 % SDS
	890 ml	Aqua destillata
Washing solution 2	50 ml	20 x SSC
	950 ml	Aqua destillata
Washing solution 3	25 ml	20 x SSC
	975 ml	Aqua destillata

9.4.4 Analytical consumables

Centri Sep spin column	(Princeton Seperations, Inc.; NY, USA)
Cover slip (24 x 50 mm)	(Roth, Karlsruhe)
Falcon tube (15 ml, 50 ml)	(Roth, Karlsruhe)
LightCycler® short capillary (5 - 47 cm x 50 µm)	(Roth, Karlsruhe)
Microcon YM-10 concentrators	(Millipore, Indien)
Servisol® Aero Duster 100	(Ambersil Ltd., Somerset, UK)
Biosphere pipette filter tips	(Sarstedt AG & Co., Nümbrecht)
RNase-free pipette tips, sterile	(Eppendorf/Gilson, Hamburg)
Sterile filter (0,22 µm)	(Millipore, Indien)

Reaction tubes:

0.2 ml; 0.5 ml; 1.5 ml; 2 ml	(Roth, Karlsruhe)
Rubber cement	(REMA Tip Top, Poing)

9.5 Composition of ALTROMIN® Standard Diet 1310

Crude Nutrients [%]	
Dry Matter	89.0
Crude Protein	22.5
Crude Fat	5.0
Crude Fibre	4.5
Crude Ash	6.5
Nitrogen Free Extracts	50.5

Minerals [%]	
Calcium	0.9
Phosphorus	0.7
Magnesium	0.2
Sodium	0.2
Potassium	0.9

Amino Acids [%]	
Alanine	1.0
Arginine	1.5
Aspartic Acid	2.3
Glutamic Acid	4.3
Glycine	0.9
Histidine	0.5
Isoleucine	1.0
Leucine	1.8
Lysine	1.2
Met + Cyst	0.7
Phenylalanine	1.1
Phe + Tyr	1.9
Proline	1.4
Serine	1.1
Threonine	0.8
Tryptophan	0.3
Valine	1.1

Vitamins per kg	**Standard**
Vitamin A	15.000 IE | IU
Vitamin D3	600 IE | IU
Vitamin B1	18 mg
Vitamin B2	12 mg
Vitamin B6	9 mg
Vitamin B12	24 mcg
Vitamin C	36 mg
Vitamin K3	3 mg
Vitamin E	75 mg
Folic Acid	2 mg
Biotin	60 mcg
Nicotinic Acid	36 mg
Pantothenic Acid	21 mg
Choline Chloride	600 mg

Trace Elements [ppm]	
Iron	150
Manganese	50
Zinc	60
Copper	13
Iodine	1.4
Selenium	0.6
Cobalt	0.3

Metabolizable Energy		
Calories from protein*	12.5 MJ/kg	(2988 kcal/kg)
Calories from fat*	27 %	
Calories from carbohydrates*	13 %	
(*calculated using Atwater factors)	60 %	

DECLARATION

I do hereby declare that the present thesis is my original work and that no sources and utilities have been used other than those indicated in the thesis itself. In addition, I declare that this work and any part thereof have not been submitted elsewhere for the award of any other degree.

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die von mir angegebenen Hilfsmittel und Quellen benutzt habe. Zudem erkläre ich, dass diese Dissertation weder in gleicher noch in anderer Form bereits in einem Prüfungsverfahren vorgelegt wurde.

Rostock, den 1. Juli 2008

Anja Hartmann

THESES

"The application of gene expression profiling in the characterization of physiological effects of genetically modified feed components in rats"

Submitted by Dipl.-Ing. agr. Anja Hartmann

Background and aims

1. Genetic modification of plants has great potential to improve crop productivity as well as health or nutritional benefits for consumers. However, unexpected alterations may occur as a result of the modification process (e.g. changed plant composition). Before introducing such plants into the food chain, the physiological impacts of unintended compositional changes on the consumer have to be evaluated.

2. At present, mainly target-based experimental approaches are applied. Though these have been criticized as they focus generally on the analysis of single molecules/parameters or specific cellular processes. Therefore, the use of non-target-based profiling tools (transcriptome, proteome or metabolome analyses) was discussed lately, as they provide an open-ended broad view of the complex cellular processes in the food recipient.

3. In this study the adequacy of gene expression profiling for the characterization of unintended physiological effects caused by an intake of genetically modified dietary components was assessed. Possible gene expression changes and thereby alterations in biological processes due to the diet were investigated in rats.

4. Specific genetically modified potatoes (VP60-potato or nptII-potato) were fed in order to characterize physiological effects in response to alterations in plant composition and/or to the "foreign" genes. To specify effects induced solely by the VP60 molecule, an additional experiment with a VP60-baculovirus additive was conducted. Expression profiles were compared to the corresponding control group (non-GM potato or wildtype-baculovirus).

5. Thresholds (e.g. False Discovery Rate, Fold change) are commonly used in data analysis to define significant expression changes. As a precondition for the subsequent investigations, several threshold-settings were tested regarding their appropriateness for the data analyzed within this work.

Results and conclusions

6. Threshold stringency had a great impact on the number of differentially expressed genes and its associated biological processes. Consequently, threshold choice should always be taken in the context of the experiment and adapted within each study.

7. A False Discovery Rate threshold of ≤ 5 % was most appropriate for this work since a high reliability of the results was aimed. Additionally a moderate fold change filter of ≥ 1.5 was chosen.

8. In the spleen of rats the feeding of VP60-potatoes caused primarily a significant down-regulation of lipolysis-, proteolysis- and digestion-related genes and a prior up-regulated of genes associated with developmental processes. In small intestine epithelium a down-regulation of few immune related genes was detected, whereas in the liver expression remained unaffected.

9. Feeding of a VP60-baculovirus additive also induced a down-regulation of lipolysis- and proteolysis-associated genes in the spleen. These consistent results indicate that the VP60 molecule is probably at least partially involved in the observed expression changes. However, an impact of changes in potato composition due to transgenesis and/or somaclonal variation cannot be excluded.

10. In the liver a feeding of nptII-potatoes led to significant up-regulation of genes that are associated with electron transport, alcohol/monosaccharide and lipid metabolism. Since the nptII molecule has no known bioactivity these changes are probably attributed to alterations in the potato composition. In the spleen and in small intestine epithelium no expression differences were observed in response to the transgenic feed.

Outlook

11. Gene expression profiling allows a holistic approach to screen for unintended physiological side effects caused by an intake of feeds or feed additives as for instance genetically modified plants.

12. Transcriptomics and other “omics” technologies (proteomics, metabolomics) provide a great sensitivity in monitoring even slight changes in the complex physiological processes which might not be detected with conventional methods. Therefore, they could be regarded as a valuable completion of such traditional analyses.

13. Profiling methods could also be useful to characterize alterations in the composition of for instance genetically modified plants which are used in feeding trials in that this knowledge is important for a thorough interpretation of the results from these feeding studies.

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