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The Identification and Interpretation of Differences in the Transcriptomes of Organically and Conventionally Grown Potato Tubers

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Supporting Information

ABSTRACT: In the European integrated research project SAFEFOODS, one of the aims was to further establish the potential of transcriptomics for the assessment of differences between plant varieties grown under different environmental conditions. Making use of the knowledge of cellular processes and interactions is one of the ways to obtain a better understanding of the differences found with transcriptomics. For the present study the potato genotype Santé was grown under both organic and conventional fertilizer, and each combined with either organic or conventional crop protection, giving four different treatments. Samples were derived from the European project QualityLowInputFood (QLIF). Microarray data were analyzed using different statistical tools (multivariate, principal components analysis (PCA); univariate, analysis of variance (ANOVA)) and with pathway analysis (hypergeometric distribution (HGD) and gene set enrichment analysis (GSEA)). Several biological processes were implicated as a result of the different treatments of the plants. Most obvious were the lipoxygenase pathway, with higher expression in organic fertilizer and lower expression in organic crop protection; the starch synthase pathway, with higher expression in both organic crop protection and fertilizer; and the biotic stress pathway, with higher expression in organic fertilizer. This study confirmed that gene expression profiling in combination with pathway analysis can identify and characterize differences between plants grown under different environmental conditions.

KEYWORDS: *transcriptomics, microarrays, organic farming, pathway analysis, potato*

INTRODUCTION

In recent years a number of scientific publications have assessed the potential of transcriptomics to assess differences between plant varieties or similar plant varieties grown under different environmental conditions. Transcript profiling, mostly through microarrays, has been performed for investigations into drought-stress in wheat[1,2] and comparisons of genetically modified plants with (1) wild-type plants in maize and soybean,[3,4] (2) conventional breeding in wheat,[5] and (3) mutagenic breeding in rice.[6] Transcriptome profiling was also done for different stages of plant part development[7,8] and different forms of organic farming in potato.[9] Also other omics technologies, i.e. proteomics and metabolomics, have been applied for similar assessments.[10−12] The advantage of transcriptomics is that for an increasing number of crop species so-called "whole transcriptome" arrays are available that comprise the complete or the larger part of the transcriptome of individual crop species. Another benefit is that for each variable, i.e. gene or gene fragment, the exact sequence is known. However, it is clear that, as with the other omics techniques, knowing the variable itself is not always linked to knowledge of the biological function of that particular gene, protein or metabolite.

In the European integrated research project SAFEFOODS (http://www.safefoods.nl/), one of the aims was to develop comparative safety assessment methods for foods, in this case potato, produced by different breeding approaches and production practices and under different environmental conditions using modern profiling techniques.[13−15] Important for the establishment of such methods is the use of very well-defined plant material. In one of the studies, the samples were derived from another European project, QLIF (http://www.qlif.org/), which focused on the identification of differences between organically and conventionally grown crops.[16]

Organic farming has been claimed to be beneficial compared to conventional farming in a number of different areas. A beneficial effect of organic farming over conventional farming on the environment and its biodiversity has been shown.[17,18] Claims of greater disease resistance and a relatively enhanced food quality are still being debated, in particular the latter as it

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is complex, involving many different aspects.[19−26] Until now, few papers on profiling studies have focused on differences between organic and conventional farming. A recent proteomics study of organic and conventionally driven potato production[10] reported few (and minor) differences; strongest for organic vs conventional fertilizer, and less difference for organic vs conventional crop protection. Many of the identified proteins with increased expression in the organic fertilizer group of samples were linked with abiotic stress responses. However, no conclusion on nutritional impact was made. Conversely, a metabolomics study of organically or conventionally grown mature wheat ears found no differences between agricultural practices, although the study was based on only 62 compounds.[12]

Study of omics data on the level of cellular processes and metabolic and signaling pathways is one of the ways to obtain a better biological understanding of the differences found with transcriptomics. Several tools are available to fit omics data into known biological pathways and identify differentially regulated pathways rather than individual genes. In this study two such tools were used, HGD[27] and GSEA.[28,29] HGD compares the proportion of genes significantly affected within a given pathway to the proportion of significantly differentially expressed genes of all genes on the microarray. Significant differences can be identified using statistical models such as Student's *t* test or ANOVA. GSEA has the advantage that no initial filtering needs to be applied to the data set to select for significantly differentially expressed genes. GSEA first ranks all probe sets based on fold changes in expression (for example using the algorithm "signal-to-noise") between a treatment group and the control group. Subsequently, by using predefined sets of associated genes based on prior biological knowledge, GSEA calculates whether sets as a whole are enriched at the top or bottom of the fold change-based ranking list, or randomly distributed. This enables detection of significantly affected gene sets even when the fold change of expression of the individual genes can be relatively modest. Biological pathways can be used as gene sets, such as the MapMan gene ontology, as was done in the present study. The principle of the MapMan ontology is a hierarchical "BIN"-based structure. Each BIN comprises items of similar biological function, e.g. glycolysis, and can be further split into subBINs corresponding to submodes of the biological function. It was originally developed for *Arabidopsis*,[30] and adapted for use in *Solanaceae*.[31,32]

For the present study the potato genotype *Santé* was grown under organic or conventional crop protection as well as organic or conventional fertilizer in four different combinations. Samples were analyzed to determine if the differences in environmental conditions during growth were reflected in their transcriptome profiles at maturity, and whether these observed differences could be interpreted on the level of biological pathways. The data were analyzed with different statistical tools, multivariate (PCA) and univariate (ANOVA), and with pathway analysis tools (HGD and GSEA). These methods are discussed regarding their use for identification and interpretation of differences between plants grown under different environmental conditions.

MATERIALS AND METHODS

Field Experimental Design and Agronomy. The experiments presented were carried out within the Nafferton factorial systems comparison (NFSC) trial at the University of Newcastle's Nafferton Experimental Farm, Northumberland, U.K. (54:59:09 N; 1:43:56 W), which included 4 factorial experiments. Experiments were established in a field with a uniform sandy loam soil (alluvial deposit) of the Stagnogley type, with a mean organic matter content of 3.3% in 2001. Each experiment was a split−split plot design with rotation design, crop protection (CP) and fertility management (FM) as experimental factors (see ref 16 for a detailed description of the experimental design), but differed with respect to cropping sequence. The treatment plots were 6 × 24 m in size.

Potato samples used for analyses were grown in the NFSC trial in 2005 and were established after winter wheat. Potato seeds of the variety *Santé* were planted in ridges (distance between rows, 90 cm; distance between seeds within the row, 35 cm) using a semiautomatic two-row potato planter (Reekie, Forfar, Scotland, U.K.). After defoliation, tubers were left in the ground for 3 weeks to allow skin maturation and then harvested using a single row potato harvester (Ransomes, Ipswich, U.K.).

Potato crops for all treatments were planted on May 2, 2005, and harvested on September 13, 2005. The field trials were established as completely randomized block design with 4 replicate blocks, with all combinations of fertilization and crop protection being established on one plot in each block.

Fertilization Treatments. The two fertilization treatments were (a) composted cattle manure, equivalent to 170 kg N/ha (standard fertilization regime used for commercial organic potato crops) or (b) ammonium nitrate (Nitram), equivalent to 180 kg N/ha and superphosphate and KCl as a compound (0:20:300 fertilizer equivalent to 134 kg P/ha and 200 kg K/ha), which is the standard fertilization regime for commercial conventional crops.

Crop Protection and Defoliation. Two crop protection regimes typically used in organic and conventional production systems in the North East of England were compared. The organic crop protection regime consisted of ×2 mechanical rigging of potato rows (for weed control), 5 foliar sprays of copper oxychloride (product: Headland Copper) at a total rate of 6 kg Cu/ha (for late blight control) and mechanical removal of foliage by flailing to minimize tuber blight risk and encourage skin set in potato tubers. No soil treatments were used.

The conventional crop protection consisted of a preplanting soil application of aldicarp granules (67 kg/ha) (for nematode/slug and soil pest control), 1 spray of linuron (3.5 L/ha) (for weed control), 4 foliar sprays of fluazinam (2.85 L/ha) (for late blight control) and 1 foliar spray of the herbicide diquat (4 L/ha) for desiccation/removal of foliage to minimize tuber blight risk and encourage skin set in potato tubers.

Yield and Disease Assessment. Potato yields were assessed by harvesting the four middle rows of each plot. Fresh weights were determined by weighing tubers harvested in each plot immediately after harvesting. Dry weights were determined by drying a subsample of harvested tubers at 80 °C for two days using a drying oven (Genlab Ltd., Widnes,U.K.). Plots were examined for visible symptoms of foliar blight at 2 weekly intervals and other foliar diseases, but in 2005 no symptoms of foliar blight and/or other diseases could be detected.

Tuber Sampling Strategy. For each treatment and replicate, one bulk sample, comprising the same tuber numbers (4−6 tubers) to a combined fresh weight of ca. 800 g, was prepared. For each set of tubers, and to minimize the impact of metabolite gradients within these large tubers, two opposite eighths were removed from each tuber,[33] combined, chopped into 1 cm cubes and frozen in liquid N_2 prior to freeze-drying for one week. The freeze-dried samples were milled using a Retsch mill (Tecator Udy) with a 1 mm sieve. Freeze-dried potato powders were stored in resealable bags at −80 °C (in the dark) until required for analysis.

Total RNA Isolation from Potato Tubers. RNA was isolated from 0.4 g of freeze-dried tuber material. The protocol for RNA extraction from polysaccharide rich tissues, based on using CTAB and consecutive chloroform/isoamylalcohol extractions with an overnight LiCl precipitation,[34] was adapted for potato tuber samples as previously described.[9]

RNA concentration and purity were assessed from the absorbance measurements by Nanodrop 1000 instrument (NanoDrop Technologies, USA). To assess RNA integrity 1 μg of RNA was electrophoresed on a

1% agarose gel with 5% formamide. Gel images were analyzed using Quantity One 1-D (BIO-RAD, USA). Samples with the ratio of the quantity of 18S rRNA to total rRNA above 40% and the ratio of non-ribosomal RNA to ribosomal RNA below 30% were considered of adequate quality for microarray analysis. Adequate RNA quality was confirmed for all 16 RNA samples.

Microarrays. The 4K cDNA food safety potato microarray (FSPM) was used as previously described[9] with a few adaptations, resulting in FSPM 2.0, deposited under number GPL13246 in NCBI's Gene Expression Omnibus.[35] After purification, PCR fragments were dissolved in 20 μL of 50% DMSO-MiliQ. Microarrays were spotted on UltraGAPS slides (Corning) in 64 blocks containing 16 × 16 spots (ServiceXS, Leiden, The Netherlands). Two complete microarrays were spotted per slide. As positive controls three different luciferase fragments corresponding to the 5′, middle and 3′ part of the luciferase cDNA, as well as the entire luciferase cDNA, were spotted across the array as well as a *Salmonella* gene fragment as a negative control. A total of 264 controls were included. Microarrays were dried and stored at room temperature until use. Prior to hybridization DNA was UV-cross-linked to the glass surface with 150 mJ. Spotting quality was assessed for two of the spotted arrays in the series by staining the slides with 0.2 μM cyanine nucleic acid dye POPO-3 (Molecular Probes, USA).

The 44K Potato Oligo Chip Initiative (POCI) array[7] was purchased as a custom array (product code nr 015425, Agilent); the microarray platform had been deposited under number A-MEXP-1117 in the ArrayExpress database of the European Bioinformatics Institute.[36]

Fluorescent Labeling of cDNA and Hybridizations of the Microarray. *FSPM 4K Array.* 25 μg of RNA was labeled by incorporation of Cy5-dCTP during a cDNA synthesis reaction using 21-mer oligo-dT primers.[37−39] 1 μL of Luciferase RNA (1 μg, Invitrogen) was added to each sample. Labeled cDNA was dissolved in 80 μL of MilliQ water, to which 20 μL of Cy3-labeled universal control cDNA was added as well as 4 μL of denatured herring sperm DNA and 4 μL of denatured blocker (Agilent, USA). This mix was combined with 100 μL of 2× hybridization buffer (Agilent, USA), and hybridization was performed for 17 h at 60 °C with the Agilent gasket/chamber system in an Agilent hybridization oven with rotor. After hybridization the slides were washed with wash buffers 1, 2, and 3 (Agilent, USA) according to the manufacturer's instructions.

POCI 44K Array. The "One-Color Microarray-Based Gene Expression Analysis (Quick Amp Labeling) Protocol for Use with Agilent Gene Expression Oligo Microarrays Version 5.7, March 2008" was used for both labeling and hybridization. For each sample, 1 μg total RNA was used.

Scanning and Image Analysis. *FSPM 4K Array.* Microarrays were scanned after excitation of Cy5 dye with 633 nm laser and Cy3 dye with 543 nm laser using scanner ScanArray Express HT (Perkin-Elmer, USA). The microarrays were scanned at constant laser power (90%) and 10 μm resolution settings. Tiff images were imported into the ArrayVision software (Imaging Research, Waalwijk, The Netherlands), and the fluorescent intensity, background and signal-to-noise ratio (S/N) were determined for each spot. The background signal was defined as the average signal in the four corners surrounding each spot. The S/N was defined as the spot signal minus the background signal, divided by the standard deviation of the background signal.

POCI 44K Array. The "One-Color Microarray-Based Gene Expression Analysis (Quick Amp Labeling) Protocol for Use with Agilent Gene Expression Oligo Microarrays Version 5.7, March 2008. Protocol GE1-v5_95_Feb07" was used in combination with grid 015425 D F 20061105.

Microarray Data Analysis. *FSPM Array.* Control and empty spots were removed. The Cy5/Cy3 ratio was calculated and log2 transformed. For each array, normalization was performed by subtraction from all 3725 remaining spots of the median value of the log2(Cy3/Cy5) signal of 1080 spots with a target/background ratio of more than 2 on all 16 arrays. R statistical software and Genemaths XT software (Applied Maths, St-Martens-Latem, Belgium) were used for analysis. The data have been deposited in NCBI's Gene Expression Omnibus[35] and are accessible through GEO Series accession number GSE27662.

Table 1. Experimental Design

	crop protection	
fertilizer	organic	conventional
organic	OF-OCP, 4 reps	OF-CCP, 4 reps
conventional	CF-OCP, 4 reps	CF-OCP, 4 reps

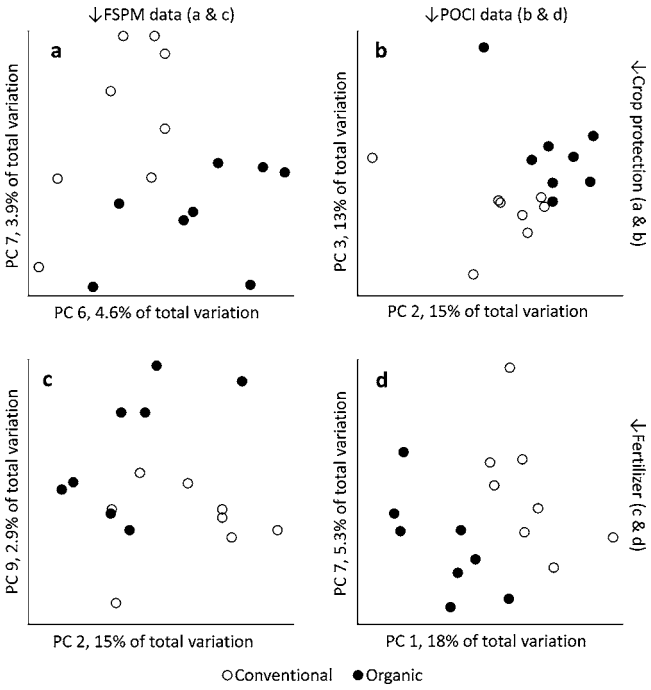

Figure 1. Samples produced using either conventional or organic treatments separate well in PCA. PCA scores are plotted for the two most discriminating principal components (PC). For each PC, the percentage of the variation that is explained is given. Axes were scaled to equal sizes such that the score values of each PC occupy the maximal space.

POCI Array. Control spots were removed; the gMeanSignal and gBGUsed columns from the raw scan files were used for selecting those spots that showed a signal higher than 2× the background on all 16 arrays (21142 spots). Per array data were normalized by dividing every value by the median value of that array. The data have been deposited under number E-MTAB-605 in the ArrayExpress database of the European Bioinformatics Institute.[36]

For further analysis also the expression values were median normalized for both platforms, based on the median gene expression per gene for all arrays in the data set.

PCA and ANOVA. PCA was performed in Genemaths XT software (Applied Maths, St-Martens-Latem, Belgium) to determine if the treatments influence the potato transcriptome, and/or distinct differences can be seen among various treatment groups.[40] Second, ANOVA was performed in the R statistical software with crop protection and fertilizer as factors plus the interaction between these factors.[41] False discovery rates (FDRs) were determined using the Benjamini and Hochberg correction.[42]

HGD. HGDs were calculated in Excel. The number of significant spots was based on the p-value for the ANOVA <0.01. This yielded 1622 spots for the factor fertilizer and 1288 spots for the factor crop protection.

GSEA. GSEA was performed to discover the differential expression of biologically relevant sets of genes that share common biological

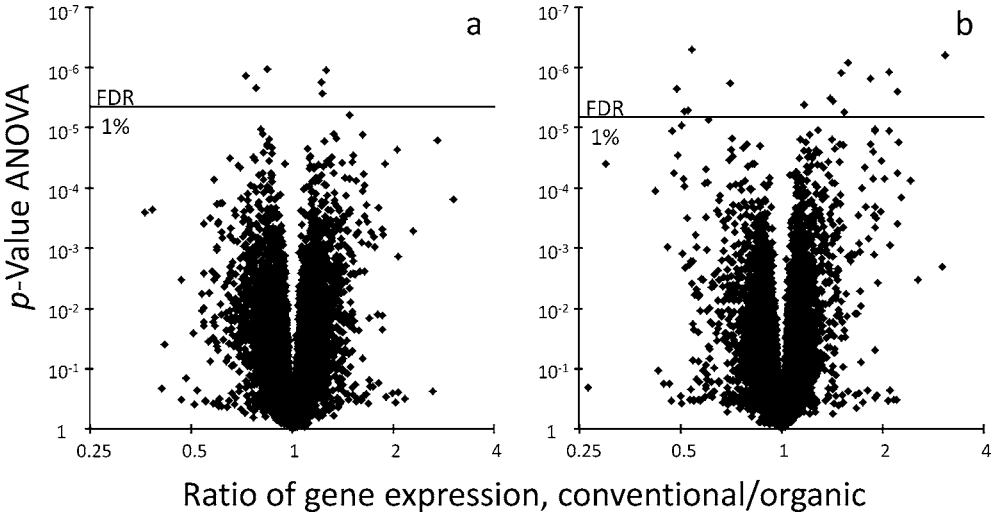

Figure 2. Volcano plots for fertilizer (a) and crop protection (b) for the POCI array data. Plotted are the *p*-values of the two-way ANOVA on the *y*-axes in $\log_2$ scale against the ratio of gene expression on the *x*-axes in $\log_{10}$ scale. The FDR threshold of 1% is indicated.

function or regulation.[28] GSEA was performed for pathways in which at least 5 spots showed significant data. If a gene was represented by more than one spot on the array, the spot with the most differential gene expression was used for the analysis in a particular comparison. Gene sets with a *p*-value <0.05 and a FDR-value <0.25 were considered as significant.

Reverse Transcription Quantitative PCR (RT-qPCR). RT-qPCR was used for the confirmation of the microarray results for lipoxygenase gene expression. Primers were designed in Beacon Designer software v. 5.10 (PREMIER Biosoft International). The starch phosphorylase gene was selected as a housekeeping/reference gene.[9] Primer sequences for the lipoxygenase gene were as follows: forward, 5′-TTACTGATCTTGCTAGTTCTTTGACTGG-3′; reverse, 5′-CACTACCTGCTGTTAATGGTGTTAGAG-3′, with 100% identity to the Stu.965 unigene for lipoxygenase and less for other lipoxygenase unigenes.

Total RNA (10 μg) was treated with RQ-1 RNase-free DNase (Promega, USA). 1 μg of RNA was reverse transcribed using random hexamers and iScript cDNA Synthesis Kit (Bio-Rad, USA), and 1 μL of cDNA (equivalent to 10 ng of reverse transcribed RNA) was added to a 25 μL real-time PCR reaction containing 2× iQ SYBR Green Supermix (Bio-Rad, USA) and amplified on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, USA). The thermal cycling conditions were as follows: 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 45 s at 60 °C followed by a melting curve analysis. Real-time PCR products for each assay were visualized on an agarose gel, and only single bands of expected size were observed (data not shown).

PCRs had previously been screened on DNase treated RNA; no traces of genomic DNA were detected. Transcript quantity was determined by interpolation of cycle threshold values in a standard regression curve and normalized for the quantity of the starch phosphorylase as a reference gene. The data was log transformed, and the ratio *lipoxygenase/starch phosphorylase* expression was calculated to enable a direct comparison to microarray data.

RESULTS

Overview of Microarray Results. Microarray analysis was performed using a two-factorial plot design comparing organic and conventional fertilization and crop protection of potato tubers. Plants were grown under either organic or conventional fertilizer (OF, CF), and each was combined with either organic or conventional crop protection (OCP, CCP), giving four different treatments. Four biological replicates were grown per treatment group (Table 1). Mature tubers were sampled for analysis at the same time postharvest to minimize differences in maturation status to influence the results other than those possibly induced by the difference in treatments. A more extensive developmental time course would be informative to address this but was beyond the practical scope of this study. Two microarray platforms were used, the FSPM 4K cDNA potato microarray and the POCI 44K oligo potato microarray. Selected and normalized gene expression values were analyzed with PCA to gain insight in the variation in the data sets. ANOVA analysis was applied to detect genes whose expressions were significantly correlated either to (1) crop protection, (2) fertilizer, or (3) the interaction crop protection × fertilizer.

PCA showed separation of the treatment groups for both fertilizer and crop protection with both the FSPM and POCI derived data sets. The separations showed up in combinations of components rather than individual components (Figure 1). The variation explained by the combination of principal components that best separated the samples ranged from 8.5% for the FSPM data to 28% for the POCI data, in both cases for crop protection (Figure 1a,b). Samples that differed in fertilizer were separated very well in the POCI array results in the combination of the first and the seventh component (Figure 1d), together explaining 23.3% of the variation in the data set.

For the FSPM data, ANOVA showed significant differential gene expression for both factors but none for the interaction (p = 0.01). Differential gene expression was observed for 15 spots for crop protection and for 31 spots for fertilizer. The FDRs for the two most significant spots for fertilizer were 21 and 36%, both representing *lipoxygenase1* (*lox1*); all other spots had FDRs higher than 67%. For the factor crop protection the FDRs were 70% for the two most significant spots, *lox1* and *ferritin 1*; all others were 100%.

For the POCI data, differential gene expression was shown for 1288 spots for crop protection and for 1622 spots for fertilizer. For fertilizer, FDRs ranged from 0.1 to 13% with 6 spots below 1% (Figure 2a). The FDRs for crop protection ranged from 0.5 to 16%, with 15 spots below 1% (Figure 2b). All spots with an FDR <1% are listed in Table 2. The differential gene expression of *ferritin 1* for crop protection found with the FSPM array was confirmed by the POCI array

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Table 2. *p*-Values and Ratios of Expression of the Most Significant Spots from the ANOVA Analyses for the Crop Protection and Fertilizer Factors for the POCI Array Data[a]

systematic name	*p*-value CP	ratio CCP/OCP	nucleotide blast hit *mapman pathway*
MICRO.15472.C1	5.1×10^{-7}	0.54	No Hits Found *not assigned*
bf_lbchxxxx_0059f05.t3m.scf	6.3×10^{-7}	3.06	No Hits Found *not assigned*
MICRO.6933.C1	8.6×10^{-7}	1.58	ferritin 1 [*Solanum tuberosum*] *metal handling.binding, chelation and storage*
MICRO.34.C2	1.2×10^{-6}	2.08	Superoxide dismutase [Mn], mitochondrial precursor—curled-leaved tobacco *redox.dismutases and catalases*
MICRO.7729.C1	1.3×10^{-6}	1.51	bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase [*Gossypium hirsutum*] *amino acid metabolism.degradation.aspartate family.lysine*
MICRO.1006.C4	1.6×10^{-6}	1.84	ascorbate peroxidase [*Solanum tuberosum*] *redox.ascorbate and glutathione.ascorbate*
MICRO.3214.C1	1.9×10^{-6}	0.70	unknown [*Ricinus communis*] pir_T10174 hypothetical protein—castor bean *not assigned*
MICRO.16662.C2	2.3×10^{-6}	0.49	No Hits Found *not assigned*
MICRO.14210.C1	2.6×10^{-6}	2.20	hypothetical protein protein containing a von Willebrand factor type A domain [*Oryza sativa*] *protein.degradation.ubiquitin.E3.RING*
MICRO.485.C1	3.3×10^{-6}	1.39	nam-like protein 10 [Petunia × hybrida] *RNA.regulation of transcription.NAC domain transcription factor family*
102H09AF.esd	**5.7×10^{-6}**	**1.54**	***Solanum tuberosum* mRNA for lipoxygenase** ***hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase***
cSTB11P3TH	3.7×10^{-6}	1.42	NA *misc.cytochrome P450*
MICRO.15301.C1	5.3×10^{-6}	0.53	*Vitis vinifera*, whole genome shotgun sequence, contig VV78 × 166385.10, clone ENTAV 115 *hormone metabolism.gibberelin.induced-regulated-responsive-activated*
bf_mxlfxxxx_0045b11.t3m.scf	4.3×10^{-6}	1.17	NA *not assigned*
bf_mxflxxxx_0005c04.t3m.scf	5.5×10^{-6}	0.52	NA *protein.targeting.nucleus*
systematic name	*p*-value F	ratio CF/OF	nucleotide blast hit *mapman pathway*
MICRO.6802.C2	1.1×10^{-6}	0.84	putative protein [*Arabidopsis thaliana*] *not assigned*
bf_cswbxxxx_0004f02.t3m.scf	1.1×10^{-6}	1.26	No Hits Found *not assigned*
MICRO.5169.C1	1.4×10^{-6}	0.73	amidohydrolase family protein [*Arabidopsis thaliana*] *nucleotide metabolism.degradation*
098H08AF.esd	1.8×10^{-6}	1.22	unknown [*Arabidopsis thaliana*] *not assigned*
bf_lbchxxxx_0047h03_t3m.scf	2.2×10^{-6}	0.78	No Hits Found *not assigned*
STMCY15TV	2.8×10^{-6}	1.22	unknown protein [*Arabidopsis thaliana*] *not assigned*

[a]Bold font indicates also significant differences in gene expression for the entire pathway. CCP: conventional crop protection. OCP: organic CP. CF: conventional fertilizer. OF: organic fertilizer.

for both spots in the data set, with one of the spots ranking as third most significant spot. *Ferritin 2* did not show differential gene expression. Differential gene expression for the *lipoxygenase* gene was also confirmed in the POCI data set and by performing q-RT-PCR (Figure 3). The ANOVA *p*-values and FDRs for all selected data are given in the Supporting Information.

Pathway Analyses: HGD and GSEA. The microarray data derived from the 44K POCI array were also investigated for effects on expression of genes belonging to known pathways or cellular processes in order to gain better insight into the impact of the different treatments. MapMan potato gene ontologies were used for this purpose. Potato contigs from the potato gene index (StGI) were previously mapped to the MapMan ontology to enable the application of the MapMan tools for potato microarrays.[31,43] The mapping that was used for analysis is given in the Supporting Information.

HGD was performed on genes that were significantly different between treatments groups according to ANOVA analysis (*p*-values <0.01). A number of pathways were significantly affected by crop protection and/or fertilizer. Crop protection showed 16 pathways affected with $p < 0.05$ and five with $p < 0.01$. Fertilizer showed 11 pathways with $p < 0.05$ and five with $p < 0.01$, as listed in Table 3.

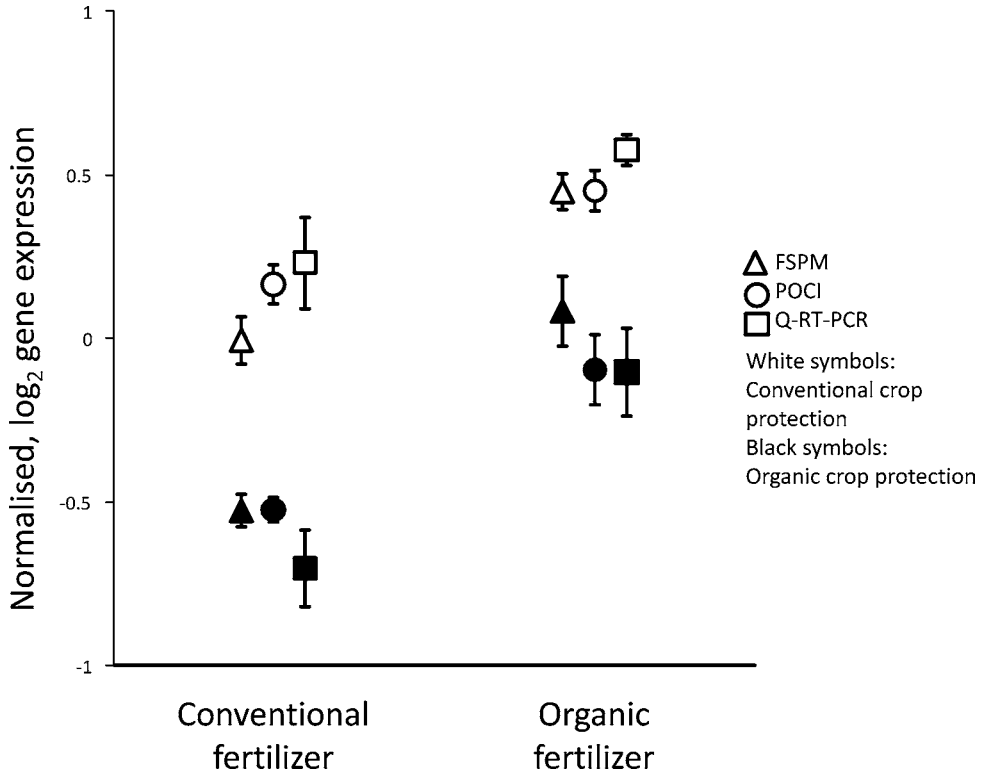

Figure 3. Concordance between the two microarray platforms and RT-qPCR for lipoxygenase gene expression. The $\log_2$ expression data were normalized separately for each technique so that the average gene expression of the four different treatments was zero. Error bars indicate the standard error of the mean of the four biological replicates.

The MapMan gene ontologies were also used for GSEA analysis. A total of six comparisons were made, resulting from the pairwise comparisons of all four different combinations of treatments in the study: OF-OCP vs OF-CCP; OF-OCP vs CF-CCP; OF-OCP vs CF-OCP; CF-CCP vsCF-OCP; CF-CCP vs OF-CCP; OF-CCP vs CF-OCP. A total of 168 pathways were found to be significantly affected by at least one of six treatment comparisons. We then focused on the most pronounced effects: pathways that showed a consistently differential expression for a type of fertilizer treatment in at least three out of the four comparisons, i.e. OF-OCP vs CF-OCP, OF-OCP vs CF-CCP, OF-CCP vs CF-OCP, and OF-CCP vs CF-CCP. The same criterion was used for consistent differences due to crop protection. Using these criteria, 13 gene sets were affected by the fertilizer treatment, and six gene sets by the crop protection treatment (Table 4). Heatmaps of all 19 gene sets are provided in the Supporting Information.

A number of pathways showed significance in both the HGD and GSEA analyses, such as the starch synthase pathway and the lipoxygenase pathway, designated "major CHO metabolism.synthesis.starch.starch synthase" and "hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase" in MapMan ontologies (Figures 4 and 5 and Tables 3 and 4).

For the starch synthase pathway 15 spots were included in GSEA, representing nine different *starch synthases*. A number of those showed high homology to well-annotated potato *starch synthase* unigenes, for instance for *soluble starch synthase II, IV* and *V*. Others showed highest homology to (putative) *starch synthases* of other plant species as *tomato* and *rice*. For those exhibiting higher expression in the organic crop protection group five of the 15 spots also exhibited differential gene expression in ANOVA ($p < 0.01$). These five spots represented four distinct starch synthase genes: *soluble starch synthase* (unigene Stu.202), *starch synthase V* (unigene Stu.15592), *tomato starch synthase VI* (unigene Les.8073) and *Solanum urticans granule-bound starch synthase I* (*GBSSI*, genbank accession code GU591152). For the higher expression in the organic fertilizer group, an overlapping set of four spots also showed differential gene expression in the individual ANOVAs. These represented the same *starch synthase V* and *Solanum urticans GBSSI*, a different *soluble starch synthase* (unigene Stu.198), and potato *GBSS* (genbank accession code X83220).

For the lipoxygenase pathway nine spots were included in GSEA, representing six different *lipoxygenases*. A number of those showed high homology to well-annotated potato lipoxygenase unigenes, for instance for *lipoxygenase*, *13-lipoxygenase* and *lipoxygenase 3*. Others showed highest homology to the *Arabidopsis lipoxygenase* family. For the higher expression in the conventional fertilizer group four of these nine spots showed also differential gene expression in ANOVA when analyzed individually. These represented four distinct lipoxygenases: *lipoxygenase* (unigene Stu.965), *lipoxygenase* (genbank accession code U24232) and two *Arabidopsis lipoxygenses* (genbank accession code AAL91142 and unigene At.27885). For the lower expression in the organic crop protection group an overlapping set of four spots also showed differential gene expression in ANOVA. These represented the same two *Arabidopsis*-like *lipoxygenases*, one of the same potato *lipoxygenases* (Stu.965) and potato *lipoxygenase 3* (unigene Stu.18586).

Glycoalkaloid Related Genes and Pathways. The glycoalkaloids solanine and chaconine are the major antinutrients in potato. Of the five genes implicated so far in glycoalkaloid synthesis,[44,45−47] only both oligos representing *β-solanine/β-chaconine rhamnosyl transferase* (*sgt3*, MICRO.11.C1,

Table 3. Pathways Significantly Differentially Expressed between Potatoes Grown under Conventional and Organic Fertilizer and Crop Protection According to HGD Analysis[a]

Crop Protection				
pathway	*p*-value	*S*	*T*	*S*/*T*
minor CHO metabolism.myo-inositol.InsP Synthases	8.0×10^{-5}	4	5	0.80
major CHO metabolism.degradation.sucrose.invertases.vacuolar	7.2×10^{-3}	3	7	0.43
minor CHO metabolism.raffinose family.raffinose synthases.putative	8.3×10^{-4}	5	13	0.38
hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	**5.0×10^{-4}**	**8**	**31**	**0.26**
major CHO metabolism.synthesis.starch.starch synthase	**2.0×10^{-3}**	**7**	**30**	**0.23**
RNA.regulation of transcription.ARR	**1.1×10^{-2}**	**4**	**15**	**0.27**
major CHO metabolism.degradation.starch.transporter	1.2×10^{-2}	2	3	0.67
minor CHO metabolism.others.Xylose isomerase	1.2×10^{-2}	2	3	0.67
hormone metabolism.jasmonate.synthesis-degradation.12-Oxo-PDA-reductase	1.7×10^{-2}	4	17	0.24
lipid metabolism.exotics (steroids, squalene etc).phosphatidylcholine.sterol O-acyltransferase	2.0×10^{-2}	3	10	0.30
hormone metabolism.auxin.synthesis-degradation	2.0×10^{-2}	3	10	0.30
hormone metabolism.cytokinin.signal transduction	**2.1×10^{-2}**	**6**	**37**	**0.16**
protein.aa activation.glutamate-tRNA ligase	2.2×10^{-2}	2	4	0.50
misc.gluco-, galacto- and mannosidases	3.3×10^{-2}	13	128	0.10
minor CHO metabolism.raffinose family.galactinol synthases.putative	3.4×10^{-2}	2	5	0.40
amino acid metabolism.degradation.glutamate family.arginine	4.7×10^{-2}	3	14	0.21
Fertilizer				
pathway	*p*-value	*S*	*T*	*S*/*T*
PS.lightreaction.photosystem II.LHC-II	**3.2×10^{-4}**	**18**	**94**	**0.19**
minor CHO metabolism.others.Xylose isomerase	5.3×10^{-4}	3	3	1.00
tetrapyrrole synthesis.chlorophyll b synthase	4.5×10^{-3}	3	5	0.60
polyamine metabolism.synthesis.agmatine deiminase	6.6×10^{-3}	2	2	1.00
hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	**7.9×10^{-3}**	**7**	**31**	**0.23**
nucleotide metabolism.phosphotransfer and pyrophosphatases.guanylate kinase	1.3×10^{-2}	3	7	0.43
lipid metabolism.FA synthesis and FA elongation.pyruvate kinase	1.4×10^{-2}	4	13	0.31
C1-metabolism	2.6×10^{-2}	5	23	0.22
glycolysis.PGM	2.7×10^{-2}	3	9	0.33
secondary metabolism.isoprenoids.mevalonate pathway.phosphomevalonate kinase	3.3×10^{-2}	2	4	0.50
major CHO metabolism.synthesis.starch.AGPase	4.5×10^{-2}	3	11	0.27

[a] *S*: number of spots with ANOVA < 0.01. *T*: total number of spots in pathway. Bold font indicates also a significantly enriched pathway as calculated with GSEA.

MICRO.11.C2) showed a 2-fold higher gene expression for organic crop protection, with *p*-values of 1.2×10^{-3} and 1.7×10^{-3} and FDRs of 6 and 7%. *UDPgalactose:solanidine galactosyltransferase* (*sgt1,* MICRO.2681.C1) did not show sufficient expression for analysis, while expression of *UDPglucose:solanidine glucosyltransferase (sgt2)* was either not present in all samples (MICRO.212.C5, MICRO.212.C7) or not significantly different between treatments (MICRO.212.C3, STMJM96TV). Also *3-Hydroxy-3-methylglutaryl coenzyme A reductase* (*hmgr,* MICRO.3134.C6, MICRO.6420.C1) and *squalene synthase* (*pss*, MICRO.2146.C1) did not show differential expression. Of these five genes, the *sgt 1, 2* and *3* were clustered in the same pathway according to MapMan: miscellaneous UDP glucosyl and glucoronyl transferases. The pathway did not show up as significantly differentially expressed in the HGD. However, the GSEA results for the four fertilizer comparisons of the *sgt3* containing pathway were all significant, with a higher expression in organic fertilizer. For the POCI array, this pathway contained 356 different genes. Further scrutiny of this pathway showed no significant homology after BLAST analysis between the three glycoalkaloid related glucosyltransferases and any of the oligo sequences of the other spots in this pathway, including those that dominated the differential pathway expression.

DISCUSSION

The goal of the present study was to further explore the potential of transcriptomics to identify and interpret differences between plants grown under different environmental conditions. A well-defined model study was used in which organic and conventional agricultural practices (fertilizer and crop protection) were applied to potato.

Separation of organic from conventionally grown potato plants was observed for both fertilizer and crop protection treatments with two microarray platforms using PCA as a data exploration tool. PCA is an unsupervised method which does not force samples into predefined groups as discriminant analysis does. Therefore, the segregations in the PCA clearly indicated that distinct gene expression profiles existed for both organic and conventional crop protection and fertilization in this study, providing a good basis for further exploration into the biological pathways being differentially expressed.

Biological interpretation of microarray data has improved with the development of pathway analysis tools. The first step is the correct annotation of genes according to their function in different biological pathways and networks. Much progress has been made in this area over the past few years, particularly for plants and crops, in the wake of human and other mammalian gene annotation initiatives. The MapMan gene annotation for

Table 4. Pathways Significantly Differentially Expressed between Potatoes Grown under Conventional and Organic Fertilizer and Crop Protection According to GSEA Analysis[a]

Higher Expression in Organic Crop Protection				
pathway	CCP-CF vs OCP-OF	CCP-OF vs OCP-CF	CCP-CF vs OCP-CF	CCP-OF vs OCP-OF
hormone metabolism.cytokinin.signal transduction	**0.0306**	**NS**	**0.0021**	**0.0022**
hormone metabolism.gibberelin.induced-regulated-responsive-activated	0.0158	NS	0.0220	0.0204
major CHO metabolism.synthesis.starch.starch synthase	**<0.0001**	**NS**	**<0.0001**	**0.0022**
secondary metabolism.phenylpropanoids.lignin biosynthesis.pal	0.0063	NS	0.0277	0.0060
RNA.regulation of transcription.arr	**0.0024**	**0.0019**	**0.0024**	**<0.0001**
Lower Expression in Organic Crop Protection				
pathway	CCP-CF vs OCP-OF	CCP-OF vs OCP-CF	CCP-CF vs OCP-CF	CCP-OF vs OCP-OF
hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	**NS**	**<0.0001**	**0.0320**	**<0.0001**
Higher Expression in Organic Fertilizer				
pathway	CF-CCP vs OF-CCP	CF-OCP vs OF-OCP	CF-CCP vs OF-OCP	CF-OCP vs OF-CCP
major CHO metabolism.synthesis.starch.starch synthase	**<0.0001**	**0.0210**	**<0.0001**	**NS**
major CHO metabolism.synthesis.starch.debranching	0.0044	0.0407	0.0044	NS
secondary metabolism.phenylpropanoids.lignin biosynthesis.4cl.	<0.0001	<0.0001	0.0231	0.0041
TCA _ org.transformation.other.organic acid transformations.ATP-citrate lyase	0.0423	0.0064	<0.0001	0.0062
stress.biotic	<0.0001	<0.0001	<0.0001	0.0063
transport.metabolite transporters at the envelope membrane	<0.0001	<0.0001	0.0049	<0.0001
hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	**<0.0001**	**<0.0001**	**NS**	**<0.0001**
misc.UDP glucosyl and glucoronyl transferases	<0.0001	<0.0001	0.0025	0.0043
RNA.regulation of transcription. HB,homeobox transcription factor family	<0.0001	0.0131	0.0048	0.0142
secondary metabolism.flavonoids.dihydroflavonols	0.0023	0.0080	0.0097	0.0373
development.storage proteins	<0.0001	0.0021	0.0023	NS
Lower Expression in Organic Fertilizer				
pathway	CF-CCP vs OF-CCP	CF-OCP vs OF-OCP	CF-CCP vs OF-OCP	CF-OCP vs OF-CCP
RNA.processing.RNA helicase	0.0051	<0.0001	<0.0001	0.0039
PS.lightreaction.photosystem II.LHC	**<0.0001**	**<0.0001**	**0.0037**	**<0.0001**

[a]Bold font indicates also a significantly enriched pathway as calculated with HGD. NS: not significant. CCP: conventional crop protection. OCP: organic CP. CF: conventional fertilizer. OF: organic fertilizer.

potato[31,43] was used to link microarray spot identifiers to pathways. Effects of treatments on pathways were then analyzed using (1) HGD on ANOVA significant spots and (2) GSEA on all spots.

One of the most consistent differences found was for the lipoxygenase pathway. Both pathway analysis approaches showed "lipoxygenase" to be significantly differentially expressed for both factors, though oppositely for crop protection and fertilizer. Organic fertilizer showed a higher expression while organic crop protection showed a lower expression. In the individual ANOVAs, one of the spots included in the lipoxygenase pathway showed up as one of the major differences and differential gene expression was confirmed with RT-qPCR.

Also, the starch synthase pathway showed differential gene expression in both pathway analyses. A consistent higher expression was observed for organic fertilizer treatment, with both analyses, as well as organic crop protection, but in the latter case only with GSEA. Interestingly, none of the individual spot signals showed a large difference, nor were the *p*-values of the individual ANOVAs among the 100 most significantly different spots. This indicated that the effect might be subtle and strengthened the notion that subtle effects can indeed be picked up with this technique, particularly where they may be pathway related.

Both the lipoxygenase and starch synthase pathways represent important processes for potato tubers, although for neither pathway is the complete interplay between the various enzymes and related metabolites known. This is underscored by the fact that, within the NCBI unigene assembly for *lipoxygenase*, transcripts are now grouped within the same assembly that were originally considered to be different, i.e. *lipoxygenase 1* and *lipoxygenase 2* in Stu.965. On the other hand, for the "*soluble starch synthase*", two distinct unigene assemblies exist: Stu.198 and Stu.202. Starch synthases belong to a group of isoenzymes involved in synthesis of starch, and relative expression of these different enzymes is likely to influence for instance the ratio of amylose to amylopectin.[48]

Lipoxygenases are enzymes that catalyze the conversion of (poly)unsaturated fatty acids to hydroperoxide derivatives which can in turn be further metabolized to elicitors of defense mechanisms in different stress situations, including herbivorous attack.[49−51] Another point to note regarding the changes in lipoxygenase expression is that these are key enzymes in determining the aroma and flavor characteristics of cooked potato. Palmitic, linoleic and linolenic acids when exposed to lipoxygenase can, during cooking, lead to the formation of many deleterious aroma aldehydes.[52−54] Therefore any lipoxygenase changes during development and at harvest can be important for the potato crop's utility and acceptability in the table and processed food market.

Apart from these two obviously interesting pathways another interesting gene that exhibited differential gene expression was

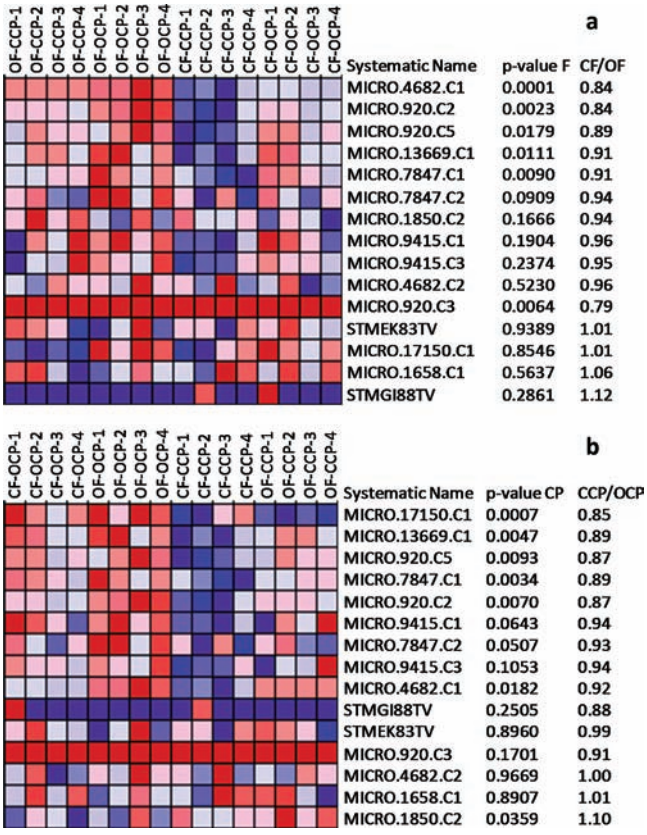

Figure 4. Starch synthase pathway genes have higher expression in potatoes grown under organic conditions. Shown are the heatmaps for gene expression after GSEA analysis for organic vs conventional fertilizer (a) and organic vs conventional crop protection (b). Dark red represents highest expression and darkest blue represents lowest expression per spot. For each spot also the *p*-value of the ANOVA is given and the numerical ratio of gene expression between the two treatments. CCP: conventional crop protection. OCP: organic CP. CF: conventional fertilizer. OF: organic fertilizer.

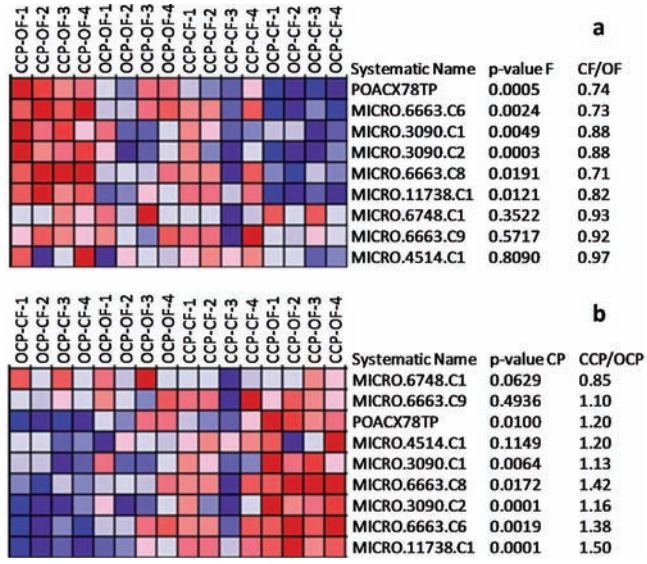

Figure 5. Significant difference of expression of the lipoxygenase pathway. Shown are the heatmaps for gene expression after GSEA analysis for organic vs conventional fertilizer (a) and organic vs conventional crop protection (b). Columns and rows were sorted according to the relevant comparison and the contribution to the enrichment respectively. Dark red represents highest expression and darkest blue represents lowest expression for each spot. For each spot also the *p*-value of the ANOVA is given and the ratio of gene expression between the two treatments. CCP: conventional crop protection. OCP: organic CP. CF: conventional fertilizer. OF: organic fertilizer.

that representing the *ferritin 1* gene, exhibiting a lower expression in organic crop protection, in both arrays for three independent spots. Ferritin 1 is an iron-binding protein, and *ferritin 1* gene expression has been shown to increase after pathogen attack, another form of biotic stress.[55] It is also a classic abiotic stress-response gene.[56] For *ferritin 1,* differential expression was found with both microarray platforms, indicating that the difference is real and unlikely to be a technical artifact. Indeed, for the FSPM microarray the two *ferritin 1* spots showed the lowest FDR. Nevertheless, the pathway including *ferritin 1*, "metal handling.binding. chelation and storage", did not show a general differential gene expression.

Genes and pathways involved in stress response seemed to be an important factor in this study: lower expression in organic crop protection for *ferritin 1* and the lipoxygenase pathway, and higher expression in organic fertilizer for the lipoxygenase pathway and also the biotic stress pathway. Also Leheseranta et al. identified stress response as an important factor in their 2D gel proteomics study.[10] In fact, Lehesranta et al. analyzed very similar samples, belonging to the same QLIF study, from an earlier year of harvest. One clear overlap was a Kunitz-type enzyme inhibitor, with the other proteins identified by Lehesranta et al. all generally involved in abiotic stress, such as maintenance of redox balance and detoxification mechanisms.

From the pathway analyses, the numerous changes to sugar metabolism may indicate changes in carbon flux in the potatoes. Likewise, from the ANOVA analysis (Table 2) many of the most significantly differentially expressed genes are known redox-response genes[57] or are susceptible to deactivation as a result of changes to iron's redox state (ferritin and lipoxygenase). These differences could be correlated with the use of diquat and linuron, which are redox antagonists.

Lu et al. reported on a transcriptomics study in winter wheat grown under different concentrations and sources of nitrogen, one of which was cattle manure as an organic source.[58] One of the most pronounced effects they observed was the higher gene expression for some 20 genes involved in nitrogen metabolism for the wheat with the organic nitrogen source. When cross-checked with the MapMan pathways used in our study, 18 potato homologues were found in several nitrogen related pathways such as amino acid transport and metabolism, both synthesis and degradation, and also transport and metabolism of nitrate and ammonia. None of the identified pathways showed a differential gene expression for the fertilizer treatment in our study. This could be due to different actual nitrogen levels or a different reflection of differential gene expression in diverse plant tissues; in Lu's study wheat grain endosperm was investigated while in our study mature potato tubers were used.

One of the most important pathways directly related to food safety of potato is that of glycoalkaloid synthesis. The metabolic routes and underlying genes for these natural potato toxins present in cultivated potato have not yet been entirely elucidated.[44] However, three *steroidal alkaloid glycosyltransferase* genes *sgt1, sgt2 and sgt3* have been identified that are involved

in the biosynthesis of the two major potato glycoalkaloids α-solanine and β-chaconine.[45−47] Also, for the genes *hmg1* and *pss1*, gene expression was correlated with high levels of steroidal glycoalkaloids.[44] In the MapMan gene annotation, these five genes were not grouped in a separate pathway. All three *sgt* genes were assigned to a group of glucosyltransferases: "misc. UDP glucosyl and glucoronyl transferases", i.e. a grouping based on type of protein function rather than a specific metabolic route. For the POCI array, this pathway contained 356 different genes, of which the vast majority are probably not involved in glycoalkaloid synthesis. Analysis of the five genes proven or suspected to be involved in glycoalkaloid synthesis showed that *sgt3* was the only gene to be differentially expressed.

The comparison of the two microarray platforms showed largely similar results. The PCA plots for the data sets of both platforms showed segregations for both crop protection and fertilizer. This indicated that a smaller array can distinguish different sample groups similarly as a larger array. This is important for species for which no full genome arrays are available or in studies where the distinction between different types of samples is more important than the elucidation of as many genes as possible playing a role in a particular type of samples. For fertilizer, the POCI PCA plots exhibited clearly a more distinct separation between organic and conventional treatment than the FSPM PCA plots. In this respect the full or nearly full genome POCI array performed better. Besides the larger amount of data, these arrays might have performed better also because of the type of probes (oligos vs cDNAs) and the manufacturing process (industrial vs small-scale in-house). With the larger coverage of the transcriptome, also pathway analyses are more comprehensive. The latest technique for transcriptome analysis provides a flexible coverage of genes. RNA-seq, the term used for next generation- or deep-sequencing when applied to transcriptomics, allows the user to choose the approximate number of genes investigated, dependent on the so-called sequencing depth.[59,60] In this way, exploratory studies into broad differences between groups of samples can be performed with less depth and cost per sample than a full investigation into a particular biological question with the goal to fully retrieve data on all genes potentially involved.

This study revealed the variation in gene expression due to specific agricultural practices in a well-defined study. As such, it contributed to the goals of the SAFEFOODS project to establish a natural bandwidth of expression profiles as a benchmark for safety evaluation of novel foods. This study also confirmed that pathway analysis can provide added value to the interpretation of microarray results beyond a mere list of differentially expressed genes. This is an important aspect of safety evaluation where identified differences are to be interpreted for their potential toxicological impact. It is also important in an academic setting where differences in gene expression between relevant situations need be not only identified but also incorporated in the existing biological understanding. In this study several biological processes were implicated as a result of the different treatments of the plants. As such, the data provide a basis for further research into understanding differences between different agricultural practices, in this particular case organic and conventional potato cultivation, even if no direct link could be made yet with the claimed or proven benefits of organic farming. Challenges for biological interpretation of microarray results are still the correct annotation of genes into pathways and networks. Various efforts are ongoing to fill in the blanks, like for potato.[61] Further elucidation of biological networks on gene level, but also of the interplay between genes, proteins and metabolites, will advance the biological interpretation of the differential gene expression found in the current as well as in future data sets.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The *p*-values for the ANOVA are presented in two separate worksheets for the two different platforms. The mapping that was used for analysis is presented. The heatmaps of all significant pathways after GSEA are presented in a pdf file. You may view this information in the following SI files: jf204696w_si_003.pdf; jf204696w_si_004.xls; jf204696w_si_005.xls. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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