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Commentary

The Effect of Replicate Number and Image Analysis Method on Sweetpotato [*Ipomoea batatas* (L.) Lam.] cDNA Microarray Results

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Abstract. Microarray analysis makes it possible to determine the relative expression of thousands of genes simultaneously. It has gained popularity at a rapid rate, but many caveats remain. In an effort to establish reliable microarray protocols for sweetpotato [*Ipomoea batatas* (L.) Lam.], we compared the effect of replication number and image analysis software with results obtained by quantitative real-time PCR (Q-RT-PCR). Sweetpotato storage root development is the most economically important process in sweetpotato. In order to identify genes that may play a role in this process, RNA for microarray analysis was extracted from sweetpotato fibrous and storage roots. Four data sets, Spot4, Spot6, Finder4 and Finder6, were created using 4 or 6 replications, and the image analysis software of UCSF Spot or TIGR Spotfinder were used for spot detection and quantification. The ability of these methods to identify significant differential expression between treatments was investigated. The data sets with 6 replications were better at identifying genes with significant differential expression than the ones of 4 replications. Furthermore when using 6 replicates, UCSF Spot was superior to TIGR Spotfinder in identifying genes differentially expressed (18 out of 19) based on Q-RT-PCR. Our study shows the importance of proper replication number and image analysis for microarray studies.

Key words: cDNA microarray, image analysis, replicates, sweetpotato

Abbreviations: AGPase, ADP-glucose pyrophosphorylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; limma, Linear Models for Microarray Data; Q-RT-PCR, quantitative real-time PCR; SuSy, Sucrose Synthase; TAIR, The Arabidopsis Information Resource; TIGR, The Institute for Genomic Research.

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Introduction

Microarray technology (Schena et al., 1995) is widely used today to monitor gene expression in various organisms (for review see Goldsmith and Dhanasekaran, 2004). It is a hybridization-based technique that makes it possible to determine the expression level of a large number of genes simultaneously. This is important from a biological point of view since genes and gene products do not function in isolation and a single gene's expression by itself is rarely very informative. Despite its frequent use, there are no universally agreed upon standard procedures for microarray experiments. This can make initial microarray experiments especially difficult.

There are numerous sources of variation associated with microarray analysis (Spruill et al., 2002; Churchill, 2002; Drăghici, 2003). Churchill (2002) divides these into 3 levels: biological variation, technical variation and measurement error. Since most studies are interested in detecting variation between treatment groups, replication at the first two levels is essential in good experimental design (Churchill, 2002).

Without replication, statistical analysis of microarray data is not possible (Lee et al., 2000; Churchill, 2002; Yang and Speed, 2002). Various factors can influence the sample size required for obtaining meaningful results. These include population variability, the p-value cutoff and the fold-change one wants to detect (Wei et al., 2004). Replication itself must be considered at two levels, biological and technical. Biological replicates represent samples from different individuals, while technical replicates represent multiple independent RNA extractions from the same individual. As always, the more replicates the better, but the cost of materials and labor can be limiting.

Spot detection and quantification is an important, but often-overlooked task of microarray analysis. It generally involves spot localization (addressing or gridding), image segmentation, quantification, and spot assessment (Yang et al., 2002a; Drăghici, 2003; Qin et al., 2005). Many of the sources of variation associated with measurement error, such as grid placement, spot segmentation, etc. (Drăghici, 2003) are dependent on the software package used for spot detection and quantification. Numerous systems are currently available to accomplish this aspect of microarray analysis. In a comparison of image analysis software, Yang et al. (2002a) found that the choice of background adjustment method can have a large effect on results. This study also indicated that image segmentation methods did not affect results to the same degree. TIGR Spotfinder (Saeed et al., 2003) is a freely available image processing software package that is part of the TM4 Microarray software suit. UCSF Spot (Jain et al., 2002) is a fully automated detection system that reduces the time needed for quantification and is reported to yield highly reproducible results. Both methods are currently reported in published research and it was our aim to determine which method gives superior results for our experiment. Neither of these methods was included in the Yang et al. (2002a) study. Korn et al. (2004) compared UCSF Spot with GenePix® (Axon Instruments, Union City, CA, USA), a popular commercial package. It is not our intention to carry out a comprehensive comparison of all available spot detection and quantification packages; rather we compare two freely available packages.

Sweetpotato [*Ipomoea batatas* (L.) Lam.] is recognized as the 4th most important crop in the tropics (FAO, 1993) and 7th in the world (Woolfe, 1992). Unfortunately, its great agronomic importance has not been matched with a comparable investment in scientific research. To develop procedures for sweetpotato microarray analysis we decided to investigate genes differentially expressed between storage and fibrous roots. Storage root formation is the most economically important process in sweetpotato. In early root development, colorless fibrous roots are formed. In some fibrous roots, elongation ceases and they start to rapidly thicken and develop into storage roots. This thickening is mainly due to cell division in the vascular cambium of the root xylem (Wilson and Lowe, 1973). Wilson and Lowe (1973) classified sweetpotato roots into the following three categories based on root thickness, fibrous (<2 mm), thick (2 to 5 mm) and storage (>5 mm).

The present experiments were undertaken to determine the appropriate replicate number, spot quantification method, and reliability of fold change data based on Q-RT-PCR in sweetpotato for a 3072 feature cDNA microarray. Our objectives were to: (1) compare changes in gene expression between storage and fibrous roots using four and six replicates, (2) compare two publicly available software programs, UCSF Spot and TIGR Spotfinder, for spot detection and quantification, and (3) validate changes in gene expression with Q-RT-PCR.

Materials and Methods

Plant Materials

Sweetpotato [*Ipomoea batatas* (L.) Lam. cv Jewel] plants were grown in a greenhouse from 3-leaf shoot cuttings originating from virus tested plant material. Six weeks after planting, fibrous roots (F) (<2 mm diameter) and storage roots (1 – 3 cm diameter) were harvested from 45 plants. Root material from 15 plants was pooled to represent a biological replicate and 3 biological replicates were used for each root type. Samples were pooled to limit the effects of biological variation. Kendziorski et al. (2005) showed that this can be useful as long as different biological samples are used to construct the pools, and the pools contain an appropriate large number of individuals.

RNA extractions

Two RNA extractions (technical replicates) were performed from each of the 3 biological replicates to yield 6 RNA samples from storage roots and 6 from fibrous roots. Roots were ground with a Waring blender (Model 33BL79, Dynamics Corporation of America, New Hartford, CT) cooled with dry ice, then ground with a mortar and pestle using liquid nitrogen. Total RNA was extracted using the RNeasy Maxi Kit (Qiagen, Valencia, CA) following the manufacturer's instructions, except that the RLC lysis buffer was replaced with RLT lysis buffer. RNA was then cleaned further and eluted in a smaller volume using the RNeasy Mini Kit (Qiagen). DNaseI digestion was carried out on the column as recommended by manufacturer.

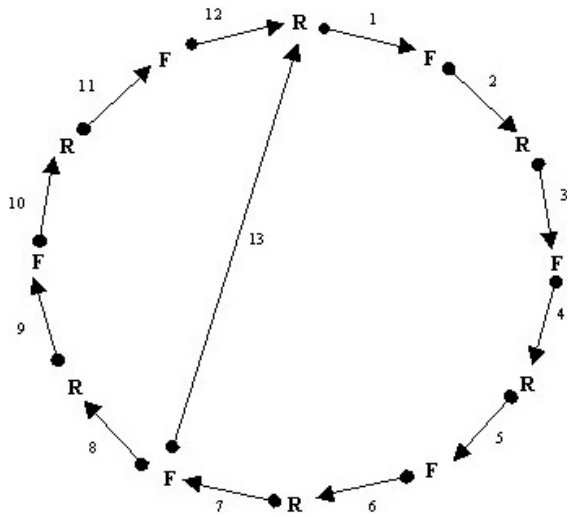

Figure 1. The connected loop design for hybridization of sweetpotato fibrous (F) and storage (R) root samples. Samples connected with arrows were hybridized on the same array and the filled circle end indicates labeling with Cy3 while the arrow point indicates labeling with Cy5. The numbers next to the arrows represent the 13 arrays used in this experiment.

RNA quality and concentration were determined by agarose gel electrophoresis and UV absorbance.

RNA labeling and hybridization

The TIGR protocol for aminoallyl labeling of RNA for microarrays (http://atarrays.tigr.org/PDF/Aminoallyl.pdf) was used to label 10 μg of RNA from each sample with Cy3 or Cy5 to yield 24 labeled samples. These samples were then hybridized in a connected loop design (Rosa et al., 2005) (Fig. 1) using the Pronto hybridization kit (Corning, NY) following the manufacturer's instructions. A 13th array was used to complete a smaller loop to investigate the effect of using 4 replications (8 arrays) vs. 6 replicates (12 arrays). Arrays 1-12 were used for the 6 replicate experiment and arrays 1-7 and 13 were used for the 4 replicate experiment.

cDNA library construction

At ARC Seibersdorf (ARCS), total RNA was extracted from young sweetpotato leaves using the CsCl gradient method (Glisin et al., 1974), and the cDNA library was constructed using the SMART PCR cDNA library construction kit (Clontech, Palo Alto, CA) according to the supplier's instructions. The quality and length of approximately 2000 fragments were checked on an agarose gel after PCR amplification, and only fragments between the 400 bp and 1200 bp range were chosen. The plasmid was extracted from 1104 clones using the Qiagen Plasmid extraction

kit and the forward and reverse sequencing was performed with a Big Dye Primer Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The cycle sequencing products were purified with Millipore MultiScreen HV filter plates (MAHVN4510) and sequenced on an ABI Prism 3100 capillary sequencer. Sequences were analyzed using Sequencer software. The 1104 clones represent 608 individual genes with an average insert size of about 800 bp. Sequences were deposited in GenBank and were assigned accession numbers CB329881 to CB330959.

At North Carolina State University (NCSU), a developing storage root library was constructed from RNA isolated from the sweetpotato cultivar 'Beauregard' selection B94-14. Primary storage roots were harvested once they had clearly differentiated (approximately 2-3 weeks). RNA extractions were performed by a modified CTAB protocol (Doyle and Doyle, 1987) with a lithium chloride precipitation substituted for the ethanol precipitation. mRNA was isolated using a Messenger RNA Isolation Kit (Stratagene, La Jolla, CA), and cDNA was synthesized using a cDNA Synthesis Kit (Stratagene). The cDNA was directionally cloned into the Lambda ZAP-CMV vector, and a mass excision of the primary library was performed using the manufacturer's protocol. Roughly 5000 clones were randomly sequenced using BigDye 3.1 and an Applied Biosystems 3700 capillary sequencer. Quality scores for the sequencing reads were made using the phred/phrap suite (Ewing and Green, 1998), and a unigene set was developed using CAP3 (Huang et al., 1999) with a cutoff value of 90. Sequences were deposited in GenBank.

The ARCS_Sp02 Array

cDNA inserts were amplified using the appropriate primers and purified with Montage PCR96 filter plates (Millipore, Billerica, MA). Samples were evaluated using agarose gel electrophoresis before being spotted in a final concentration of 3XSSC and 1.5M Betaine on Corning GAPSII slides (Corning, NY) using a Genemachines Omnigrid microarray printer (GeneMachines, San Carlos, CA).

The ARCS_Sp02 array contains a total of 9216 spotted features, consisting of 3072 features spotted in triplicate. These include 284 control features, 1060 features from the ARCS leaf library and 1728 features from the NCSU storage root library. In order to functionally classify genes on the array according to the Gene Ontology Consortium (Ashburner et al., 2000), all clone sequences were compared with *Arabidopsis thaliana* protein sequences (http://www.arabidopsis.org/) (BLASTX E-value < 1E-5). Four hundred and sixty clones showed no homology to known *Arabidopsis* protein sequences. The clones that did show homology were annotated by biological process using GO Slim terms developed by TAIR and are graphically represented in Figure 2. An explanation and description of the GO slim terms developed by TAIR can be found at http://www.arabidopsis.org/help/helppages/go_slim_help.jsp.

Array scanning and image quantification

All arrays were scanned using and AlphaArray™ Reader (Alpha Innotech, San Leandro, CA). Image data for all 13 arrays were quantified using UCSF Spot

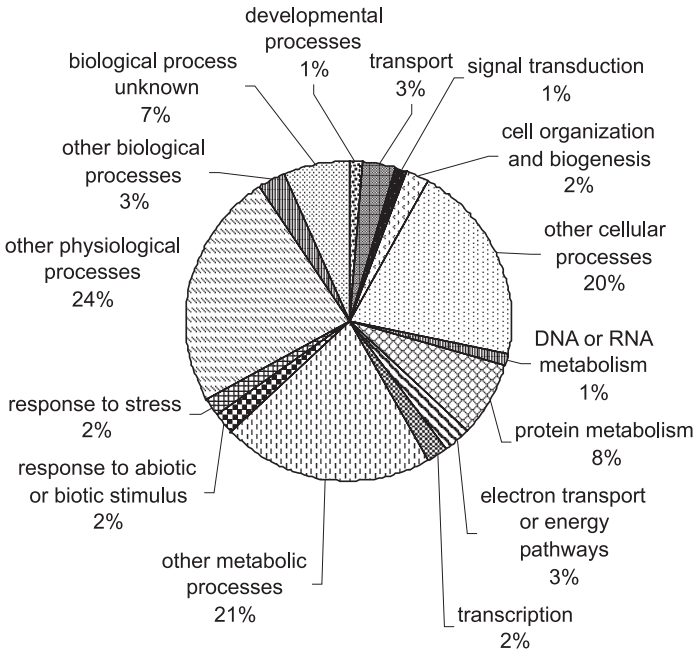

Figure 2. Functional classification of the sweetpotato genes, spotted on the ARCS_Sp02 array, classified according to GO slim terms developed by TAIR. Only genes with homology (BLASTX E-value < 1E-5) to Arabidopsis protein sequences are represented in this figure.

(Jain et al., 2002) and TIGR Spotfinder 2.2.3 (Saeed et al., 2003) to yield 4 data sets (Spot4, Spot6, Finder4 and Finder6). For UCSF Spot the composite test/reference images were used for segmentation, while default values were used for other settings. The histogram segmentation option was selected for TIGR Spotfinder analysis and default values for the quality control (QC) filter. For both packages grids were manually adjusted if necessary according to the instructions in the user's manuals. Spots flagged by the image analysis packages were treated as missing data in subsequent analysis.

Data transformation, normalization and analysis

Data were log (base 2) transformed, normalized and analyzed using the Linear Models for Microarray Data (limma) software package (Smyth, 2005). Background intensities were subtracted from foreground intensities for the UCSF Spot data sets (TIGR Spotfinder reports intensities after median, local background correction). Data were normalized within arrays using loess and between arrays using Aquantile normalization (Smyth and Speed, 2003). Triplicate spots were handled using the duplicateCorrelation function (Smyth et al., 2005), while the loop design and technical replications were handled as suggested in Smyth (2005). Empirical Bayes methods were used to determine differentially expressed genes (Smyth, 2004). Genes were considered to be differentially expressed if the p-value was smaller than 0.01 after Holm (1979) multiple testing correction.

Q-RT-PCR

Nineteen genes were selected for validation with Q-RT-PCR. The genes were selected to ensure that they represent as much of the scatterplot distribution as possible, while focusing on genes of interest to the authors (e.g., genes involved in sucrose and starch metabolism, and housekeeping genes) and genes with known, expected expression levels (e.g., Sporamin). Some genes were chosen specifically because they yielded different results in the 4 different data sets.

The same 12 RNA samples used for microarray analysis were used for Q-RT-PCR. First-strand cDNA synthesis for the two-step reaction was carried out using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA.) following protocols supplied by the manufacturer. The resulting reaction was diluted by adding 40 µl water, and 1 µl of the dilution was used for RT-PCR on the ABI PRISM® 7000 Sequence Detection System using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 600 nM of each primer in a final volume of 25 µl (Table 1). Primers were designed with Primer Express (version 2.0) (Applied Biosystems) using default parameters.

Standard curves were generated for all primer pairs using a dilution series that encompassed all sample concentrations. The efficiency of all reactions was between 90% and 110% (slopes between –3.1 and –3.6). Q-RT-PCR reactions were carried out in duplicate for every sample, and the average was taken for further analysis. First-step reactions without reverse transcriptase were used to verify that no DNA contamination was present. Dissociation curves were inspected to detect nonspecific amplification and data were normalized using 18S ribosomal RNA specific primers (Applied Biosystems). Significance was determined using a t-test (variances not assumed equal) of normalized values. Genes were considered to be differentially expressed if $p = 0.05$.

Results

All four data sets (Spot4, Spot6, Finder4 and Finder6) were normalized using loess normalization (Smyth and Speed, 2003). Control features on the array, expected to be non-differentially expressed, were examined to ensure that these had the expected approximate 1:1 ratio after normalization.

The number of detected features calculated to be differentially expressed for the 4 data sets varied from 792 for Spot4 to 1037 for Finder6 (Table 2). By and large the data sets with 6-replications identified the vast majority of features identified by the 4-replication data sets, and a number of additional features. The number of features common to different data sets is indicated in Table 2.

The scatter-plot distributions of the 4 data sets are similar, and generally the fold changes of specific features are comparable across data sets. In order to determine which data set yielded the best results, Q-RT-PCR was carried out on 19 genes and results were compared with microarray results (Table 3). In general all 4 data sets correctly identified the direction of regulation (up or down). The single exception was gene CB330845, but expression of this gene was not significant with Q-RT-PCR or any of the microarray data sets.

Table 1. Primers used for Q-RT-PCR analysis.

NCBI Accession		Primers (5′ → 3′)
DV037657	Fwd	GAATCCACCGTGGTCATGG
	Rev	GAGCTTGTTGGTGGCGATGT
DV037548	Fwd	CCCCCCTTTGTTGCTTTAGC
	Rev	TGACACGCACAAACTCCCAA
DV035666	Fwd	GCAACCCGCTTTCTATGAGG
	Rev	CAGTCCACAGGTCATGGCCT
CB330696	Fwd	CGCCTCATCCTCAATTATGGA
	Rev	GGGAGCTGGTGATTCCTTTGT
DV037575	Fwd	ATGCCCTGGATCTTGGTGG
	Rev	GCTTTCCCTCCCAACTGCA
DV037119	Fwd	AGAGTAGCGGCTGATGGAGC
	Rev	AGGGTTGTGGCTAGCAGTCAA
DV036464	Fwd	GCGGTAAATCCTCCGAGGTT
	Rev	TCGGAAACAGCCTTTGGAGA
DV035127	Fwd	AGTAGCAACGGCGGTCTGAT
	Rev	CGCAGAGTGCGTAGTTGGG
DV035220	Fwd	GTGCTCATGGATCTGGAGCC
	Rev	TCTGTCCGTAAGGGCCAGTC
DV036713	Fwd	GGTCAAGGCTGGGTTTGCT
	Rev	CGGCCAACTATGCTAGGGAAT
CB330166	Fwd	TGGTCGCTAGGGTTGCTCTG
	Rev	GAACGGGTCGTTGACTGCA
CB330823	Fwd	ATTGCACGACGAAGCTGATG
	Rev	TCAAAGGAGAACGGGACCG
CB330845	Fwd	AAGGCTACAGAGGGAGGACATG
	Rev	ACAGGTGCAGTTTGATCCACACT
CB330655	Fwd	CTGAGTCTCTCGTCATCGTCGA
	Rev	CAGTGCCCGGAAAGGTCTC
CB330120	Fwd	CGGGTGCAAGATGTACCCAG
	Rev	CGCCAAGAACAAGGGTCTCA
DV036043	Fwd	AGGCGAAGTGTCCCAGGTAA
	Rev	CCCTGACCTCACACTGTTCGT
DV037562	Fwd	CGTCGTCCGTTAAGGCTAAGA
	Rev	CCACCGAAAACCTTGTGCAC
DV035035	Fwd	GTGTTCCGACGCCGTTTCT
	Rev	CCGTCTTCGTTCCAGGAGATC
CB330724	Fwd	GTGCAACACGGTGTTCAACAG
	Rev	GCCGTTCATGACGTAGCTAAGG

The differences among the results from the 4 data sets were in their ability to identify whether regulation was significant or not. The results from Spot6 showed the best agreement with Q-RT-PCR results (18/19), while Spot4 had the least agreement (15/19). The results from 6-replicate experiments are closer to that obtained with Q-RT-PCR than the ones from 4-replicate experiments.

Discussion

Our first objective was to determine the appropriate replicate number. Our results

Table 2. Differentially expressed sweetpotato genes identified by the 4 data sets. Diagonal numbers indicate the total number of differentially expressed genes identified for each data set. Numbers in bold indicate the number of common genes for specific data sets. Numbers in parentheses indicate the number of genes not shared between 2 data sets. The first number is associated with data sets in rows and the second number with data sets in columns.

	Spot4	Spot6	Finder4	Finder6
Spot4	792	**787**	**752**	**783**
Spot6	(222)(5)	1009	**804**	**957**
Finder4	(82)(40)	(29)(205)	834	**826**
Finder6	(254)(9)	(80)(52)	(183)(7)	1037

Spot4 = 4 replicates using UCSF Spot; Spot6 = 6 replicates using UCSF Spot; Finder4 = 4 replicates using TIGR Spotfinder; Finder6 = 6 replicates using TIGR Spotfinder.

indicate that the 6 replicate data sets identify approximately 20% more differentially expressed genes than the 4 replicate data sets. It has previously been suggested that 3 would be an appropriate number of replicates for microarray analysis (Lee et al., 2000). Our results are more in line with Wei et al. (2004) and Pavlidis et al. (2003) who found that fewer than 5 replicates is rarely sufficient. Indeed both of these studies found that more than 8 replicates are often needed to yield reliable results. Since sweetpotatoes are clonally propagated, and the biological replicates in our study (and probably in most cases for studies on sweetpotatoes) are clones, one would expect that fewer replicates would be needed than for outbred species. Wei et al. (2004) showed that more replicates are needed for unrelated human subjects than for inbred mice to obtain the same statistical power.

It is clear that no "magic" number of replicates can be applied to all microarray experiments, and that the current apparent consensus on 3 replicates is probably not sufficient in most cases. Naturally the advantage of identifying an additional 20% differentially expressed genes should be weighed against the cost and effort involved in using more replicates. It should also be noted that the empirical Bayes method employed by the limma package used for statistical analysis in this study is robust even for a small number of replicates (Smyth, 2004). Other statistical analysis methods may yield even larger discrepancies between the results from 4 replicates and 6 replicates.

Our second objective was to compare spot quantification methods. UCSF Spot performed better when 6 replicates were used, while TIGR Spotfinder was superior for 4 replicates. Although the number of genes validated with Q-RT-PCR is small, the results from the comparison are in line with the results from Table 2. In the comparison with Q-RT-PCR, the only difference between Spot4 and Finder4 is that Spot4 wrongly identifies gene DV037119 as not significantly differentially expressed (Table 3). This would seem to indicate that, overall, Spot4 would identify a smaller number of differentially expressed genes. This is indeed true, and in a similar way Spot6 has one fewer false positive result (CB330823) than Finder6, and overall identifies fewer differentially expressed genes than Finder6 (Table 2).

Both UCSF Spot and TIGR Spotfinder use histogram segmentation and similar 'median value in local square region' background calculations (Jain et al., 2002; Saeed et al., 2003). UCSF Spot also replaces outliers in the background

Table 3. Comparison of results obtained for 19 genes with microarray analysis and Q-RT-PCR. Positive fold changes indicate up-regulation in sweetpotato storage roots, while negative fold changes indicate up-regulation in fibrous roots. Numbers in bold indicate fold changes that are statistically significant. Key genes are in italics.

	Fold change					Homology (BLASTX)		
GenBank ID	Spot4	Spot6	Finder4	Finder6	Q-RT-PCR	Description	Species	E-Value
DV037657	**7.66**	**6.51**	**10.47**	**8.92**	**320.19**	Sporamin precursor	*Ipomoea batatas*	4e-110
DV037548	**5.34**	**5.30**	**5.54**	**5.80**	**2.51**	Sucrose Synthase 1	*Craterostigma plantagineum*	4e-97
DV035666	**1.97**	**1.87**	**2.06**	**1.97**	**6.67**	Sucrose Synthase 2	*Coffea canephora*	6e-122
CB330696	**2.51**	**2.77**	**3.20**	**3.62**	**11.66**	Fructokinase	*Solanum tuberosum*	1e-23
DV037575	1.32	1.35	1.31	1.37	1.13	Hexokinase	*Nicotiana sylvestris*	1e-67
DV037119	1.46	**1.50**	**1.78**	**1.80**	**2.74**	Phosphoglucomutase (cytosolic)	*Solanum tuberosum*	1e-84
DV036464	**3.24**	**3.42**	**3.87**	**4.01**	**137.08**	ADP-glucose pyrophosphorylase	*Brassica napus*	2e-86
DV035127	**1.73**	**1.73**	**1.97**	**1.93**	**2.03**	Invertase inhibitor-like protein	*Ipomoea batatas*	7e-54
DV035220	1.57	**1.57**	1.56	**1.61**	**12.21**	Beta-tubulin	*Oryza sativa*	5e-47
DV036713	**1.95**	**1.90**	**1.94**	**1.92**	**3.57**	Actin	*Nicotiana tabacum*	7e-113
CB330166	**1.70**	**1.99**	**1.90**	**2.42**	**4.24**	GAPDH (cytosolic)	*Musa acuminata*	1e-160
CB330823	**-2.32**	-1.83	**-2.78**	**-2.24**	-1.16	Mitogen-activated protein kinase	*Lycopersicon esculentum*	2e-76
CB330845	-1.03	1.03	1.34	1.55	-1.33	Metallothionein-like type 1 protein	*Ipomoea batatas*	3e-16
CB330655	**-2.86**	**-2.83**	**-3.41**	**-3.36**	**-4.55**	Probable glutathione-*S*-transferase	*Capsicum annuum*	4e-36
CB330120	**2.29**	**2.33**	**2.42**	**2.47**	**5.04**	Metallothionein type 2	*Arachis hypogaea*	3e-06
DV036043	**2.67**	**2.46**	**3.16**	**2.97**	**2.22**	Unknown protein	*Arabidopsis thaliana*	2e-44
DV037562	**3.96**	**3.58**	**4.72**	**4.17**	1.60	Unknown protein	*Arabidopsis thaliana*	1e-43
DV035035	**2.13**	**3.77**	**2.54**	**2.23**	**1.69**	Heat shock factor RHSF2	*Oryza sativa*	2e-47
CB330724	**-2.58**	**-2.67**	**-2.98**	**-3.10**	**-5.26**	Lipid transfer protein precursor	*Davidia involucrata*	7e-29

Spot4 = 4 replicates using UCSF Spot; Spot6 = 6 replicates using UCSF Spot; Finder4 = 4 replicates using TIGR Spotfinder; Finder6 = 6 replicates using TIGR Spotfinder.

relative to the median with the median. It seems unlikely that the difference in performance of the two packages is due to background adjustment as in the Yang et al. (2002a) study, since both methods use similar background adjustments. It seems more plausible that the differences between the results from UCSF Spot and TIGR Spotfinder are due to differences in spot flagging. TIGR Spotfinder flags include flags assigned by the QC filter, spots with higher background than signal, and spots not detected (low intensity). In UCSF Spot, only spots deemed to be absent are flagged, leading to a very low number of flagged spots. Overall TIGR Spotfinder flagged approximately 8% of spots, while UCSF Spot only flagged ~0.01% of spots (data not shown). Korn et al. (2004) found that UCSF Spot flagged 0.04% of spots and noted that compared to GenePix, UCSF Spot gave superior results at low intensities. UCSF Spot is a particularly attractive option for spot detection and quantification because it is an automated system and a single array can be processes in less than 20 minutes. Our results indicate that UCSF Spot is certainly an appropriate, and even superior, analysis method, as long as a reasonable number of replicates is used. For a smaller number of replicates, TIGR Spotfinder may be a better option. We feel that the UCSF Spot software could benefit greatly from an interface that allows for manual flagging of spots with obvious defects.

Our third objective was to validate changes in gene expression. Fold changes calculated for all 4 data sets were very similar and showed good agreement with Q-RT-PCR results. Since the dynamic range of Q-RT-PCR is much larger than that of microarrays, some discrepancies are expected, especially at high fold changes, but our results are similar to what have been found in other studies (Yang et al., 2002b; Yuen et al., 2002; Brinker et al., 2004; Larkin et al., 2004). Interestingly, 3 genes commonly considered as housekeeping genes, β-tubulin (DV035220), actin (DV036713), and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) (CB330166), were shown to be significantly up-regulated in storage roots. This is not surprising considering the expected increase in cell division during the formation of storage roots, and the fact that actin (Gilliland et al., 2002; Ringli et al., 2002) and tubulin (Hussey et al., 1990; Joyce et al., 1992) have been implicated in playing roles in root development. Differential expression of these housekeeping genes has been reported previously in plants (Kim et al., 2003; Volkov et al., 2003; Iskandar et al., 2004) and other systems (Ullmannová and Haškovec, 2003; Bas et al., 2004; Wong and Medrano, 2005). Our study confirms that they cannot be universally classified as housekeeping genes. Previous studies (Kim et al., 2003; Ullmannová and Haškovec, 2003; Bas et al., 2004) have found that 18S rRNA is often an appropriate reference gene for Q-RT-PCR. Indeed we found that when equal amounts of total RNA were used for Q-RT-PCR, no statistically significant difference could be found between 18S rRNA of fibrous and storage roots of sweetpotato (data not shown). The underlying assumption here is that the proportion of 18S rRNA in the total RNA is constant between treatments.

Differential expression of several genes identified by Q-RT-PCR is confirmed by previous studies. Sporamin (DV037657) is the main storage protein found in sweetpotato storage roots (Shewry 2003) and therefore its massive up-regulation is not unexpected. Both Sucrose Synthase (SuSy) (DV037548 and DV035666)

and ADP-glucose pyrophosphorylase (AGPase) (DV036464) have been shown to be up-regulated during storage root formation in sweetpotato (Li and Zhang, 2003).

The differences among the results of the 4 data sets are in the significance of the regulation. Comparison of results from Spot4 (15/19) and Finder4 (16/19) with Q-RT-PCR uncovered false positive as well as false negative errors. The results from Spot6 and Finder6 contained 1 and 2 false positives respectively and no false negatives. It is certainly expected that more replications will lead to the identification of more differentially expressed genes (false negatives in the 4 replication data sets are expected). The Holm (1979) multiple testing correction used in this study is considered very stringent, and an even more stringent method (Bonferroni correction) did not eliminate the false positives (data not shown). On the other hand, the less stringent, false-discovery rate (FDR) (Benjamini and Hochberg, 1995) method led to even more false positives, as expected (data not shown).

The gain in accuracy and number of differentially expressed genes identified due to the use of more replicates have to be weighed against the additional cost and effort involved. Our results also indicate that the spot detection and quantification system represent another variable one must consider in developing microarray protocols. In order to obtain the best results, all aspects of a specific experiment must be evaluated together to determine the most appropriate method.

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