Microarray analysis of gene expression profiles in ripening pineapple fruits

Jonnii H Koia, Richard L Moyle and Jose R Botella

Abstract

Background: Pineapple (Ananas comosus) is a tropical fruit crop of significant commercial importance. Although the physiological changes that occur during pineapple fruit development have been well characterized, little is known about the molecular events that occur during the fruit ripening process. Understanding the molecular basis of pineapple fruit ripening will aid the development of new varieties via molecular breeding or genetic modification. In this study we developed a 9277 element pineapple microarray and used it to profile gene expression changes that occur during pineapple fruit ripening.

Results: Microarray analyses identified 271 unique cDNAs differentially expressed at least 1.5-fold between the mature green and mature yellow stages of pineapple fruit ripening. Among these 271 sequences, 184 share significant homology with genes encoding proteins of known function, 53 share homology with genes encoding proteins of unknown function and 34 share no significant homology with any database accession. Of the 237 pineapple sequences with homologs, 160 were up-regulated and 77 were down-regulated during pineapple fruit ripening. DAVID Functional Annotation Cluster (FAC) analysis of all 237 sequences with homologs revealed confident enrichment scores for redox activity, organic acid metabolism, metalloenzyme activity, glycolysis, vitamin C biosynthesis, antioxidant activity and cysteine peptidase activity, indicating the functional significance and importance of these processes and pathways during pineapple fruit development. Quantitative real-time PCR analysis validated the microarray expression results for nine out of ten genes tested.

Conclusions: This is the first report of a microarray based gene expression study undertaken in pineapple. Our bioinformatic analyses of the transcript profiles have identified a number of genes, processes and pathways with putative involvement in the pineapple fruit ripening process. This study extends our knowledge of the molecular basis of pineapple fruit ripening and non-climacteric fruit ripening in general.

Keywords: Pineapple, Non-climacteric, Fruit ripening, Microarray

Background

Pineapple (Ananas comosus) is a tropical fruit crop of significant commercial value and is the most important economic plant in the Bromeliaceae family. However, surprisingly little research has been undertaken to understand the molecular basis of pineapple fruit development - an essential prerequisite for future improvement by molecular breeding or genetic modification.

In terms of fruit ripening, fleshy fruits are classified as either climacteric or non-climacteric. In climacteric fruits, such as apples and bananas, a burst of ethylene biosynthesis and an increase in respiration is observed at the onset of ripening. Conversely, non-climacteric fruits such as pineapple lack the autocatalytic ethylene burst and the increase in respiration. Although the physiological changes that occur during pineapple fruit development are well known, little is known about the molecular events that govern pineapple fruit ripening.

The advent of high throughput sequencing and microarray technologies has facilitated large-scale studies on gene expression changes during fruit development in a number of species, including climacteric fruits tomato and apple, and the non-climacteric fruit strawberry [1-3]. In a previous study, we reported the construction of subtracted and normalized EST cDNA libraries, including the green mature and yellow mature ripening stages of pineapple fruits [4]. More recently, short read next...
generation sequencing technology was applied to a ripe pineapple fruit gene discovery project [5]. In this study, we report the development of an EST-based pineapple microarray and its use to identify differentially expressed genes during fruit ripening. Online bioinformatics tools were used to assign putative identity and function to those pineapple genes displaying differential expression during fruit ripening. We also applied visual mapping tools such as MapMan (v3.1.1), the Kyoto Encyclopedia of Genes and Genomes (KEGG) online resource and Heat Maps generated through DAVID to visualize biological processes and pathways of significance during pineapple fruit ripening. Quantitative real-time PCR analysis was used to validate a subset of the differentially expressed genes. This study contributes to our understanding of the molecular basis of pineapple fruit ripening and non-climacteric ripening in general.

Results and discussion

Microarray analysis of pineapple fruit ripening identifies gene expression changes associated with important metabolic pathways and processes

9277 cDNAs isolated from several pineapple tissues including roots, green mature and yellow mature fruits were spotted in duplicate onto microarray slides (Australian Genome Facility, University of Queensland). Hybridization was carried out using probes derived from mature green fruits and fully ripened yellow fruits. In total, three replicate microarray hybridizations were performed and analysis of the results identified 271 differentially expressed ESTs (> 1.5 fold). Of these, 160 were up-regulated and 77 down-regulated during pineapple fruit ripening. Among the 271 EST sequences, 184 shared significant homology with known gene products, 53 shared homology with products of unknown function, and 34 had no significant homology with any known plant gene sequence in the GenBank database. EST sequences with homologs were subjected to functional classification analysis and assigned an Arabidopsis thaliana homolog ID tag (e.g. At2g43640). The microarray data generated in this study has been deposited in NCBI’s Gene Expression Omnibus [6] and are accessible through GEO series accession number GSE38521 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38521).

The expression data was analyzed using the Functional Annotation Cluster (FAC) tool contained in the Database for Annotation, Visualization and Integrated Discovery (DAVID) [7]. DAVID FAC analysis of the 160 up-regulated genes (> 1.5-fold) produced a total of 37 enriched functional clusters under high stringency conditions and protein catabolism was the most significant biological process in green mature fruit (Figure 1b).

Redox activity

Reduction-oxidation (redox) activity showed the highest enrichment score in the FAC analysis of genes up-regulated in mature yellow fruits (Figure 1a). DAVID heat map analyses identified 26 genes with an expression range of 1.51 to 7.56 that functionally clustered into common GO terms related to oxidation-reduction and oxidoreductase activity (Figure 2A). A number of genes identified in the heat map relate to dismutase, peroxiredoxin, glutaredoxin, ascorbate-glutathione and thioredoxin activities.

The state of oxidation for a given organism is influenced by stress response-related enzymes such as dismutases, peroxidase, glutaredoxin, ascorbate-glutathione and thioredoxin [8]. The state of oxidation may influence the rate at which ethylene is produced at the onset of ripening in climacteric fruits [9]. Fruit maturation may be thought of as a catabolic process involving an array of redox enzymes causing degradation of fruit tissue and emanation of ethylene as a catabolic by-product that accelerates ripening. Possible differences between the oxidation state and modes of catabolism stress responses ought to be investigated between climacteric and non-climacteric fruit species. The observed redox activity in pineapple might also be related to the large size of the fruit and the low partial pressure of oxygen.

Organic acid metabolism

FAC analysis identified organic acid metabolism as an important biological process during pineapple fruit ripening (Figure 1a). DAVID heat map analyses identified 18 genes with an expression range of 1.50 to 8.52 that functionally clustered into common GO terms related to organic acid metabolism and other associated processes such as cellular ketone metabolism, oxoacid metabolism and carboxylic acid metabolism (Figure 2b).

Organic acid content has been reported to increase during ripening of climacteric and non-climacteric fruits [10-13]. In pineapple, acidity increases during development and starts declining once the fruit approaches maturity and ripens [14]. Citric and malic acid are the two main organic acids that contribute to the sourness and acidity of most climacteric and non-climacteric fruits such as peach [11,15,16], apple [17], kiwifruit [18], grape [19], orange [20], cherry [21], strawberry [12] and pineapple [22]. Furthermore, citric acid was shown to undergo the greatest increase in developing pineapple, reaching a peak prior to
fruit ripening, whereas malic acid showed little change during development [22].

**Metalloenzyme activity**

FAC analysis identified responses to inorganic compounds, metal ion cofactors and cadmium as significant biological processes during pineapple fruit ripening (Figure 1a). A total of 16 genes ranging in expression from 1.51 to 4.09 fold, were identified in the DAVID heat map (Figure 2c).

A number of metalloenzymes activated in response to inorganic substances, metal ion cofactors and cadmium were identified in the heat map analysis (Figure 2c). These include superoxide dismutase (SOD), metallothionein (MET) and phytochrome A (PHYTA) homologs. Metalloenzymes are known to regulate the redox state of many plant crops and are involved in many important biological processes such as oxidative stress, metal ion homeostasis, pathogenicity, metal-mediated catalysis and cell death. Pineapple waste (over-ripened fruit) has been used in the bioremediation removal of toxic heavy metals from wastewater and sewage plants [23,24]. This is possibly due to an abundance of metalloenzymes such as MET, SOD and PHYT in ripe fruit, where these enzymes have the ability to act as ligands and chelate excess metal ions in wastewater treatment procedures. Indeed, MET has been implicated in cadmium detoxification and metal ion homeostasis [25]. Previous studies confirm that yellow pineapple fruit contains a small transcriptome that is dominated by high levels of metallothionein transcript [4].

**Glycolysis and sugar catabolism**

FAC and DAVID heat map analysis identified genes involved in glycolysis and sugar catabolism (Figure 1a,
| Arab ID     | Expn | Contig Gene Name                  |
|-------------|------|-----------------------------------|
| At2G35120   | 1.75 | 12-oxophytodienoic acid reductase |
| At5G40370   | 1.62 | 12-oxophytodienoic acid reductase |
| At1G23740   | 2.68 | 12-oxophytodienoic acid reductase |
| At2G26080   | 1.57 | 12-oxophytodienoic acid reductase |
| At3G04120   | 4.09 | 12-oxophytodienoic acid reductase |
| At1G16300   | 7.56 | 12-oxophytodienoic acid reductase |
| At1G13440   | 2.70 | 12-oxophytodienoic acid reductase |
| At5G23300   | 1.45 | 12-oxophytodienoic acid reductase |
| At4G19710   | 1.52 | 12-oxophytodienoic acid reductase |
| At2G19800   | 2.02 | 12-oxophytodienoic acid reductase |
| At3G48280   | 1.55 | 12-oxophytodienoic acid reductase |
| At5G39950   | 1.53 | 12-oxophytodienoic acid reductase |
| At1G79750   | 1.83 | 12-oxophytodienoic acid reductase |
| At2G28190   | 2.69 | 12-oxophytodienoic acid reductase |
| At1G77120   | 2.09 | 12-oxophytodienoic acid reductase |
| At5G05580   | 1.60 | 12-oxophytodienoic acid reductase |
| At1G76690   | 2.00 | 12-oxophytodienoic acid reductase |
| At4G14710   | 1.73 | 12-oxophytodienoic acid reductase |
| At1G08830   | 2.31 | 12-oxophytodienoic acid reductase |
| At1G75280   | 3.66 | 12-oxophytodienoic acid reductase |
| At4G26260   | 2.56 | 12-oxophytodienoic acid reductase |
| At5G05340   | 1.51 | 12-oxophytodienoic acid reductase |
| At5G53560   | 1.67 | 12-oxophytodienoic acid reductase |
| At3g15353   | 1.95 | 12-oxophytodienoic acid reductase |
| At2g36530   | 2.28 | 12-oxophytodienoic acid reductase |
| At4g14880   | 2.32 | 12-oxophytodienoic acid reductase |
| At3G22890   | 2.11 | 12-oxophytodienoic acid reductase |
| At5g26220   | 1.95 | 12-oxophytodienoic acid reductase |
| At1G77120   | 2.09 | 12-oxophytodienoic acid reductase |
| At1g09780   | 1.56 | 12-oxophytodienoic acid reductase |
| At1G13440   | 2.70 | 12-oxophytodienoic acid reductase |
| At3G55440   | 2.08 | 12-oxophytodienoic acid reductase |
| At1G08830   | 2.31 | 12-oxophytodienoic acid reductase |
| At2G28190   | 2.69 | 12-oxophytodienoic acid reductase |
| At4G35000   | 2.55 | 12-oxophytodienoic acid reductase |
| At1g09570   | 2.13 | 12-oxophytodienoic acid reductase |

**GO Terms:**
- GO:0006020 –inositolmetabolic process
- GO:0019751 –polyolmetabolic process
- GO:0042364 –water-soluble vitamin biosyn. Process
- GO:0006096 –glycolysis
- GO:0019752 –carboxylic acid metabolic process
- GO:0006007 –glucose catabolic process
- GO:0043436 –oxoacid metabolic process
- GO:0019320 –hexose catabolic process
- GO:0006800 –oxygen and ROS metabolic process
- GO:0042180 –cellular ketone metabolic process
- GO:0044275 –cellular carbohydrate catabolic process
- GO:0016491 –organic acid metabolic process
- GO:0046365 –monosaccharide catabolic process
- GO:0016209 –antioxidant activity
- GO:0019560 –oxoacid metabolic process
- GO:0046164 –alcohol catabolic process

**See legend on next page.**
Figure 2d). KEGG pathway visual analysis identified six genes in the glycolysis pathway map that were up-regulated in yellow ripe fruits by 1.56 to 7.56 fold (Figure 3a, Figure 2d, Table 1). These findings indicate the importance of glycolysis and sugar catabolism during pineapple fruit development. Most of the genes identified on the KEGG map were also identified in the corresponding heat map matrix.

Interestingly, gene expression profiling of non-climacteric grape, also identified a significant cluster of up-regulated genes involved in glycolysis and sugar catabolic activity [26]. Taken together, the results in grape and pineapple underlay the significance of glycolytic activity during non-climacteric ripening. It is possible that ripening fruits generate increased ATP energy reserves and reducing power by increasing glycolysis that could be later used by enzymes involved in important fruit metabolic processes. The up-regulation of glycolysis related genes might also be related to phloem unloading of sugars and their storage in the vacuole.

Anti-oxidant activity and reactive oxygen species (ROS) metabolism

FAC analysis shows a significant enrichment of L-ascorbate (vitamin C) biosynthesis genes, anti-oxidant activity and ROS metabolism during pineapple fruit ripening (Figure 1a). DAVID heat map analysis revealed a total of five pineapple genes that functionally cluster with GO terms related to vitamin biosynthesis, polyol metabolism, inositol metabolism and L-ascorbic acid biosynthesis (Figure 2e). To name a few, myo-inositol oxygenase 4 (MIOX4), myo-inositol oxygenase 2 (MIOX2) and L-galactose-1-phosphate phosphatase (GPP), were identified with expression levels ranging from 1.55 to 2.56-fold increase. Radiotracer evidence showed that grape GPP (VTC4) is a key enzyme involved in L-ascorbate biosynthesis as non-climacteric grape fruits develop [27]. Our pineapple GPP expression profile also correlates with the peach [28] and tomato [29] GPP orthologs during fruit ripening. The pineapple MIOX4 and MIOX2 genes correlate with MIOX orthologous genes found in grape [30] and tomato [29].
and are involved in the conversion of myo-inositol to D-glucoronic acid during L-ascorbic acid biosynthesis.

DAVID heat map analyses also identified a cluster of six pineapple genes found to be involved in anti-oxidant activity, response to ROS, oxygen and ROS metabolism, cellular response to oxidative stress and cellular response to ROS activity (Figure 2f). L-ascorbate [27,31] and glutathione [32,33] have been linked to plant antioxidant activity and fruit ripening. In fact, fruit ripening is considered to be an oxidative process, where L-ascorbate and glutathione are linked by a cycle of enzymes whose biological role is to detoxify hydrogen peroxide [34].

KEGG pathway mapping (Figure 3b, Table 1) identified a cluster of genes involved in L-cysteine biosynthesis, an important precursor for glutathione. Aside from ripening, L-ascorbate production is influenced by various environmental cues such as light [35], temperature [29,36] and ambient ozone concentrations [37]. These stresses induce the formation of ROS, which are removed by the plant's antioxidant system involving catalase, superoxide dismutase, peroxidases and enzymes involved in the ascorbate-glutathione cycle [27]. Non-climacteric grape orthologs of pineapple GPP, APOX3, PXDN-B1, SOD-119 and SOD-3200 were also reported to be involved in antioxidant activity [30]. Hydrogen peroxide (H2O2) is the most stable ROS species and plays a crucial role as a signaling molecule in various physiological processes [38]. H2O2 is also involved in cell membrane and wall degradation of fleshy tomato fruit tissue, thereby causing fruit softening [39]. This may also be the case in developing pineapple fruit tissue.

### Table 1: List of pineapple ESTs from KEGG pathway maps with contig ID numbers (www.pgel.com.au), Arabidopsis ID tag, normalized expression value and annotated gene description

| Schematic Name | Arabidopsis homologue | CloneID | Normalized Expression Levels | Description | KEGGID |
|----------------|-----------------------|---------|------------------------------|-------------|--------|
| **Glycolysis - KEGG PATHWAY** | | | | | |
| contig 763 | At3g04120 | 4.1 | Glyceraldehyde 3-phosphate dehydrogenase, cytosolic | Ac763 |
| contig 251 | At4g33070 | 3.4 | Pyruvate decarboxylase 1 | Ac251 |
| contig 30 | At2g36530 | 2.3 | Enolase | Ac30 |
| contig 1110 | At1g77120 | 2.1 | Alcohol dehydrogenase | Ac1110 |
| contig 3191 | At3g55440 | 2.1 | Triose-phosphate isomerase | Ac3191 |
| contig 441 | At1g09780 | 1.6 | Phosphoglycerate mutase | Ac441 |
| **Cysteine and Methionine Metabolism** | | | | | |
| contig 3189 | At4g14880 | 2.3 | Cysteine synthase | Ac3189 |
| contig 117 | At4g14710 | 1.7 | ATARD2 acireductone dioxygenase2 | Ac117 |
| contig 113 | At3g25570 | 1.6 | S-adenosylmethionine decarboxylase | Ac113 |
| contig 1009 | At5g11520 | 1.5 | Aspartate aminotransferase | Ac1009 |
| contig 3350 | At4g19710 | 1.5 | Bifunctional aspartokinase/homoserine dehydrogenase | Ac3350 |
| **Ubiquitin-Mediated Proteolysis - KEGG Pathway** | | | | | |
| contig 1299 | At2g02760 | 3.1 | Ubiquitin conjugating protein | UBE2A |
| contig 154 | At1g16890 | 1.6 | Ubiquitin carrier protein E2 36 | UBE2N |
| contig 861 | At5g22920 | 1.6 | Putative PGPD14 protein (pollen germination related protein) | PIRH2 |
| contig 1793 | At5g42190 | 1.5 | SKP1-like protein 1B | SLP1 |
| contig 1433 | At1g64230 | –2.0 | Ubiquitin carrier protein E2 28 | UBE2D_E |
| contig 153 | At3g57870 | –2.2 | Ubiquitin carrier protein | UBE2I |

Proteolysis and Ubiquitin-type housekeeping processes

DAVID FAC analysis indicated that proteolysis and ubiquitin protein catabolism are active in both green and yellow pineapple fruit (Figure 1a, 1b). The enrichment score for proteolysis and ubiquitin protein catabolism was comparable between both FAC analyses (Figure 1a, 1b), however it was significantly lower than other up-regulated biological processes (Figure 1a). DAVID heat map analysis identified six down-regulated genes (Figure 4a) and 14 up-regulated genes (not shown) involved in proteolysis and ubiquitin-type activity during pineapple fruit ripening.
Proteolysis is essential for many aspects of plant development. It is responsible for cellular housekeeping and stress responses by removing abnormal and miss-folded proteins, supplying amino acids needed to make new proteins, controlling homeostasis by reducing the abundance of key enzymes and regulatory proteins, and controlling gene function and programmed cell death of specific plant organs or cells [40-42]. Our bioinformatics analysis indicates that protein degradation during pineapple fruit development is a complex process involving a multitude of proteolytic pathways. Selective proteolysis of damaged proteins is mainly controlled through ubiquitin-mediated processes involving ubiquitin and several ubiquitin conjugating enzymes [43].

Pineapple fruit bromelain
DAVID FAC analysis of the 77 down-regulated genes (> 1.5-fold), suggested that cysteine type peptidase activity was the most significant process in green mature fruit (Figure 1b). This is mainly due to two pineapple fruit bromelains, Ac-122 (−17.76-fold) and Ac-3033 (−31.75-fold) that were identified on a DAVID heat map (Figure 4A) and found to be strongly down-regulated as pineapple ripens from mature green to mature yellow fruit. Bromelain is a cysteine-type protease unique to pineapple. Cysteine-type proteases are also found in other fruits, such as kiwifruit [44], papaya and fig [45]. Pineapple EST library analyses identified an abundance of a bromelain inhibitor (Ac124) and a fruit bromelain (Ac122) from green mature fruit [4]. The bromelain inhibitor Ac124 was the most abundant EST in the green fruit library with 52 clones isolated. Northern analyses further confirmed decreased expression of Ac122 and Ac124 as pineapple fruit develops [4]. The Ac124 bromelain inhibitor corresponds to a non-cystatin bromelain inhibitor precursor homolog in pineapple [46,47], that does not have an inhibitory effect on pineapple fruit bromelain. It is possible that Ac-122 and Ac-3033 are more abundant in green mature pineapple fruit due to the role that cysteine-type proteases play in plant defense mechanisms [45], particularly before fruit maturation.

Validation of microarray data by real-time RT-PCR
In order to validate our microarray results we performed quantitative real time PCR (qRT-PCR) to determine the expression levels of ten pineapple genes randomly selected from the list of genes differentially expressed across mature green and mature yellow fruits. To avoid bias, the tested genes were chosen from an array of different processes including redox activity, organic acid
Figure 5 KEGG pathway map analyses of differentially expressed genes (> 1.5-fold). (a) Glycolysis. (b) Cysteine and methionine metabolism. (c) Ubiquitin mediated proteolysis. Yellow and red boxes (filled and bordered) indicate up-regulated and down-regulated homologs, respectively. All pineapple homologs identified on the KEGG maps are shown in Table 1.
metabolism, metalloenzyme activity, vitamin C biosynthesis, antioxidant activity, cysteine-type peptidase activity and ubiquitin proteolysis. The qRT-PCR expression results correlated with the microarray expression data for 9 out of the 10 genes tested (Figure 6, Table 3), qRT-PCR determination of MGS and G3PDH mRNA levels showed a 2.6-fold and 5.0-fold increase respectively in mature yellow fruits over mature green fruits and these results compare favorably to the 2.6-fold and 8.5-fold increase respectively in mature green to mature yellow stages of fruit ripening. Of these, 160 ESTs were up-regulated and 77 ESTs down-regulated during pineapple fruit ripening. Of these 271 ESTs, 237 displayed significant homology to plant sequences encoding proteins of known or unknown function. Of these, 160 ESTs were up-regulated and 77 ESTs down-regulated during pineapple fruit ripening. Furthermore, quantitative real-time PCR analysis validated the microarray results for nine out of ten genes randomly chosen from different cellular processes.

Bioinformatics analyses of the microarray results suggest that redox activity is a prominent biological process during pineapple fruit ripening. Organic acid metabolism of citric and malic acid and metalloenzyme activity were also up-regulated during pineapple fruit ripening. The significant increases observed in metalloenzyme encoding genes, may contribute to the bioremediation properties of pineapple waste [23]. Glycolysis related genes were also significantly up-regulated during pineapple fruit ripening, possibly to generate ATP energy reserves for important ripening processes or in response to the influx of sugars and their storage in the vacuole.

Furthermore, L-ascorbate biosynthesis, antioxidant activity and ROS metabolism were determined to be
up-regulated during pineapple fruit development. The antioxidant properties of L-ascorbate and glutathione may be involved in detoxifying \( \text{H}_2\text{O}_2 \) as pineapple fruit ripens. \( \text{H}_2\text{O}_2 \) may have a biological role activating an up-regulated response of vitamin C accumulation and antioxidant activity during pineapple fruit development.

Our studies also identified two pineapple bromelains, Ac-122 and Ac-3033, which were highly abundant in green mature pineapple tissues and may possibly be involved in plant defense mechanisms against pathogenic attack prior to fruit maturation.

Applying a microarray based approach to studying large scale gene expression changes during fruit development has contributed to our understanding of the molecular basis of pineapple fruit ripening and non-climacteric fruit ripening in general. The development of the pineapple microarray will also enable the future large scale analysis

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### Table 3 qRT PCR validation of microarray results

| Schematic Name                               | Microarray Result | qRT-PCR-Result | Target Gene                                                                 |
|---------------------------------------------|-------------------|----------------|-----------------------------------------------------------------------------|
| Redox Activity and Metalloenzyme Activity    |                   |                |                                                                             |
| contig 816                                  | 2.7 increase      | 2.6 increase   | GSPD-816 (glyceraldehyde 3-phosphate dehydrogenase, cytosolic)              |
| contig 3096                                 | 2.3 increase      | 2.6 increase   | ASP (peroxisome type ascorbate peroxidase)                                  |
| contig 119                                  | 2.7 increase      | 2.3 increase   | CSD2 (copper/zinc dismutase 2)                                              |
| Metalloenzyme Activity                       |                   |                |                                                                             |
| contig 180                                  | 1.95 increase     | 3.5 increase   | MET (metallothionein)                                                       |
| Vitamin C Biosynthesis and Antioxidant Activity |             |                |                                                                             |
| contig 3096                                 | 2.3 increase      | 2.6 increase   | ASP (ascorbate peroxidase, peroxisome type)                                 |
| contig 119                                  | 2.7 increase      | 2.3 increase   | CSD2 (copper/zinc dismutase 2)                                              |
| contig 3227                                 | 1.5 increase      | 2.1 increase   | GPP (L-galactose-1-phosphate phosphatase)                                   |
| Organic Acid Metabolism                      |                   |                |                                                                             |
| contig 816                                  | 2.7 increase      | 2.6 increase   | GSPD-816 (glyceraldehyde 3-phosphate dehydrogenase, cytosolic)              |
| contig 604                                  | 8.5 increase      | 5.0 increase   | MGS (monogalactosylacylglycerol synthase)                                   |
| Stress Related                              |                   |                |                                                                             |
| contig 35                                   | 6.3 decrease      | 4.5 decrease   | GAST (GAST-like protein)                                                    |
| Cysteine-Type Peptidase and Ubiquitin Proteolysis |             |                |                                                                             |
| contig 122                                  | 31.7 decrease     | 20.6 decrease  | Ac-122 (Fruit Bromelain Ac-122)                                            |
| contig 756                                  | 4.5 decrease      | 2.0 increase   | OTU (OTU-like cysteine protease family protein)                             |
| contig 1299                                 | 3.1 increase      | 2.1 increase   | UBE2A (Ubiquitin conjugating protein)                                       |

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Figure 6 Validation of microarray data. A total of ten pineapple genes showing differential expression in our microarray experiments were randomly selected and their relative expression determined using qRT-PCR. See Table 3 for more detail.
of gene expression changes in other pineapple developmental processes, conditions and treatments.

Methods
Plant material and RNA extraction
Commercial field-grown pineapples (A. comosus L. Smooth Cayenne, Clone C10) were harvested early in the morning and graded according to ripening appearance [4]. The skin and pith tissues were removed and the middle third of the fruit flesh was snap frozen in liquid nitrogen, pulverized, and stored at –80°C. RNA was extracted using TRIzol reagent (Invitrogen).

Microarray design and transcript profiling
PCR amplified cDNA elements derived from pineapple fruit, root tip, root and nematode infected roots and gall cDNA libraries were printed in duplicate onto Corning UltraGAPS slides by the Microarray Facility at the ARC Special Research Centre for Functional and Applied Genomics, University of Queensland [49]. The array contains 5533 previously sequenced cDNA elements [50] and 3744 unsequenced elements.

Fruit flesh from the middle third of unripe mature green pineapple and ripe mature yellow pineapple was used for RNA extraction, as previously described [4]. The RNA was labeled with Cy3/Cy5 fluorescent dyes using the LabelStar Array Kit (Qiagen) according to the manufacturer instructions. Labeled cDNA was mixed with 20xSSC (20 μl), liquid block (12 μl), 2%SDS (4 μl) and dH2O (up to 200 μl total volume). Denatured labeled target was hybridized to a pineapple microarray slide in submersible hybridization cassette chambers (Ambion) at 55°C for 12 hours. The arrays were then sequentially washed in 2x SSC/0.5% SDS, 1x SSC, 0.5x SSC and 0.05x SSC for 5 minutes at room temperature. Each slide was scanned using an ArrayWorx scanner (Applied Precision) and spot-edited using SoftWorx tracker software (Applied Biosystems). Each array dataset was then imported into GeneSpring software for analysis.

For each replicate slide, the mean and background intensity values from each channel (W595, W685) were log2 transformed and normalized using the LOWESS algorithm to remove intensity dependent effects within the calculated values. Normalized values were used to calculate the Cy3/Cy5 fluorescence ratios from experimental and biological repeats before all replicates were combined. Normalized data with a p-value of < 0.1 that passed a 2-fold standard deviation (2xSD) test from each replicate slide was individually tested. All normalized data possessing a differential expression value of 1.5-fold or more was further considered (Additional file 1: Table S1). Due to redundancy features, unsequenced clones that passed the p-value and 2xSD test, and found to be differentially expressed by 1.5-fold or more were sequenced and updated in the raw data file of each slide. Data was compiled from three hybridizations, including one dye-swap replicate. The microarray data has been entered into the Gene Expression Omnibus at NCBI and the project has been assigned the accession code GSE38521.

Real-time PCR validation
A total of ten differentially expressed genes were selected based on their function and involvement in pathways and processes important to pineapple fruit development. Biological processes such as redox activity, organic acid metabolism, metalloenzyme activity, L-ascorbate (vitamin C) biosynthesis, antioxidant activity, cysteine-type peptidase activity and ubiquitin proteolysis were considered. A total of 18 green mature pineapple fruits and 18 yellow mature pineapple fruits, were harvested in the morning and processed as previously described [4]. Three biological replicate samples were produced for both green mature and yellow mature pineapple fruit types based on six fruit per replicate. All fruit were cored, sliced from the middle third and diced prior to liquid nitrogen treatment. Frozen diced fruit samples were pooled together according to each biological replicate and tissue type prior to being ground to a fine powder under liquid nitrogen conditions. All ground tissue from each biological replicate was homogenized and thoroughly mixed prior to RNA extraction procedures. A total of 2.5ug RNA was extracted for each biological triplicate tissue type according to methods previously described [51]. The total RNA was used for RT-cDNA synthesis using SuperScript® III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and the resulting cDNA template was diluted five-fold in molecular grade water (Promega, Madison, WI). Real-time assays were conducted for all ten target genes by using the 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) and ActinB was used as the reference gene. Real-time assays were based on three biological and technical replicates of green mature and yellow mature pineapple fruit tissue types. Gene expression and statistical analysis was performed using SDS version 2.2.2 software (Applied Biosystems, Foster City, CA).

Bioinformatics analysis of normalized microarray data
The National Centre Bioinformatics Information (NCBI) BlastX tool was used to putatively annotate all normalized genes (1.5-fold) of which Arabidopsis thaliana homolog tags were assigned to each clone using The Arabidopsis Information Resource (TAIR) online database. Gene Ontology (GO) classification systems was used to assign putative function to each clone by way of biological process, molecular function and cellular components. The Database for Annotation, Visualization and
Integrated Discovery (DAVID) v6.7b [7] was used to determine pathways and processes of major biological significance and importance through the Functional Annotation Cluster (FAC) tool based on the GO annotation function.

**DAVID functional annotation cluster analysis**

DAVID FAC analysis was conducted on two independent normalized gene lists containing the 1.5-fold up-regulated normalized genes and 1.5-fold down-regulated normalized genes. High stringency ease score parameters were selected, to indicate confident enrichment scores of functional significance and importance of the given pathways and processes investigated. The Gene Ontology (GO) system in DAVID was utilized to identify enriched biological themes in both gene lists.

**Mapping and visual pathway analysis**

MapMan (v3.1.1) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway tools were used to visually map cluster of pineapple genes involved in common pathways and processes for both pathway-specific and molecular overview purposes. KEGG pathway tools were utilized through DAVID online tools. Since visual mapping was the primary objective, all normalized genes that were differentially expressed by 1.5-fold were considered for the MapMan and KEGG pathway analysis. Heat map analyses were also conducted through DAVID to produce a matrix of enriched GO terms with common pineapple genes that were 1.5-fold or more up- and down-regulated. The green and black shading on the heat map matrix indicates a positive and negative correlation between the enriched GO term and given pineapple gene, respectively.

**Enzyme abbreviations**

superoxide dismutase (SOD-119, SOD-3200), metallothionein (MET), phytochrome A (PHYTA), myo-inositol oxygenase 4 (MIOX4), myo-inositol oxygenase 2 (MIOX2), L-galactose-1-phosphate phosphatase (GPP), 2-cys peroxiredoxin BAS1 (PXDN-B1), superoxide dismutase (SOD-119, SOD-3200) and L-ascorbate peroxidase 3 (APOX3), fruit bromelains (Ac-122, Ac-3033), MGS (mono-galactosyl-diacyl-glycerol synthase), glyceraldehyde 3-phosphate dehydrogenase (G3PD-816), ascorbate peroxidase (AP), copper/zinc superoxide dismutase 2 (CS2D2), O2U-like cysteine protease family protein (OTU) ubiquitin conjugating protein (UBE2A), GAST-like protein (GAST).

note: where an enzyme abbreviation appears with an italicized number separated with a hyphen, it indicates an internal identifier corresponding to the related contig number for that enzyme (e.g. G3PD-763, SOD-119, Ac-122).

**Additional file**

**Additional file 1: Table S1.** Full list of microarray elements displaying at least 1.5x change in expression between mature green and mature yellow stages of pineapple (Pine3) and mature yellow (Pine5) stages of fruit ripening. Systematic ID refers to the contig number assigned during EST sequence assembly. Normalized integer refers to the fold difference in expression between mature green (Pine3) and mature yellow (Pine5) stages of fruit ripening. A positive normalized integer indicates up-regulation in mature yellow fruit (or down-regulation in mature green fruit) while a negative normalized integer indicates up-regulation in mature green fruit (or down-regulation in mature yellow fruit).

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

JHK undertook bioinformatics analysis of normalized microarray data, DAVID functional annotation cluster analysis, mapping and visual pathway analysis and real-time PCR validation analyses and contributed to manuscript preparation. RLM contributed to experimental design, undertook cDNA library construction, target labeling and microarray hybridizations, spot editing and normalization procedures. JRB conceived, designed and directed the research and contributed to manuscript preparation. All authors read and approved the final manuscript.

**Acknowledgements**

The authors would like to thank Gavin Scarr, Managing Director of Piñata Farms for kindly donating pineapple material used for this study.

Received: 21 August 2012 Accepted: 30 November 2012
Published: 18 December 2012

**References**

1. Alba R, Payton P, Fei Z, McQuinn R, Debbie P, Martin GB, Tanksley SD, Giovannoni JJ. Transcriptional and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. *Plant Cell* 2005, 17:2994–2965.
2. Aharoni A, O’Connell AP. Gene expression analysis of strawberry acheine and receptacle maturation using DNA microarrays. *J Exp Bot* 2002, 53:2073–2087.
3. Janssen BJ, Thodey K, Schaffer RJ, Alba R, Balakrishnan L, Bishop R, Bowen JH, Crowhurst RN, Gleave AP, Ledger S, et al. Global gene expression analysis of apple fruit development from the floral bud to ripe fruit. *BMC Plant Biol* 2008, 8:16.
4. Moyle R, Fairbairn DJ, Rijp J, Crowe M, Botella JR. Developing pineapple fruit has a small transcriptome dominated by metallothionein. *J Exp Bot* 2005, 56:101–112.
5. Ong WD, Voo LY, Kumar VS. De novo assembly characterization and functional annotation of pineapple fruit transcriptome through massively parallel sequencing. *PLoS One* 2012, 7:e46937.
6. Edgar R, Domrachev M, Lash AE. Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* 2002, 30:207–210.
7. Huang DW, Sherman BT, Lempicki RA: Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Prot* 2008, 4:44–57.
8. Davletova S. Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of arabidopsis. *Plant Cell Online* 2005, 17:268–281.
9. Burg S, Burg E. Biosynthesis of ethylene. *Nature* 1964, 203:869–870.
10. Yin Y-G, Tomimura T, Iijima Y, Aoki K, Shibata D, Aihara H, Nishimura S, Ezura H, Matsuura C. Metabolic alterations in organic acids and y-aminobutyric acid in developing tomato (solanum lycopersicum L.) fruits. *Plant Cell Physiol* 2010, 51:1300–1314.
11. Borsani J, Dudke CO, Pizzini L, Lauermann MA, Lombardo VA, Murray R, Andreo CS, Drinovcicz MF, Lara MV. Carbon metabolism of peach fruit after harvest: changes in enzymes involved in organic acid and sugar level modifications. *J Exp Bot* 2009, 60:1823–1837.
12. Moing A, Renaud C, Gauldierre M, Raymond P, Roudebecq P, Denoue-Rothan B: Biochemical changes during fruit development of four strawberry cultivars. J Am Soc Hortic Sci 2001, 126:394–403.

13. Famiani F, Casilli V, Proietti P, Walker RP, Battistelli A: Organic acid metabolism in grape: role of phosphoenolpyruvate carboxykinase. Acta Hortic 2007, 754:539–543.

14. Singleton VL, Garnier WA: Chemical and physical development of the pineapple fruit II. Carbohydrate and acid constituents. J Food Sci 1965, 30:19–23.

15. Moing A, Rothan C, Svanella L, Just D, Diakov P, Rolin D, Gauldierre JP, Monet R: Organic acid metabolism during the development of two peach cultivars. Acta Hortic 1998, 465:425–432.

16. Wu BH, Qiu JB, Gerard M, Kevella JLI: SH changes in sugar and organic acid concentrations during fruit maturation in peaches, P. Davidiana and hybrids as analyzed by principal component analysis. Sci Hortic 2005, 103:409–439.

17. Beruter J: Carbohydrate metabolism in two apple genotypes that differ in malate accumulation. J Plant Physiol 2004, 161:1011–1029.

18. Walton ET, De Jong TM: Growth and compositional changes in kiwifruit berries from three californian locations. Ann Bot 1990, 66:285–298.

19. Kiefer WW, Howorth L, Dorr M: Concentrations of tartaric acid and malic acids and their salts in vitis vinifera grapes. Am J Enol Vitil 1967, 18:82–94.

20. Albertini MV, Carcoudet E, Pailly O, Gambotti C, Luro F, Berti L: Changes in organic acids and sugars during early stages of development of acidic and acidless citrus fruit. J Agric Food Chem 2008, 56:8335–8339.

21. Usienk V, Fabijc J, Stamparc F, Sugars, organic acids. Phenolic composition and antimicrobial activity of sweet cherry (prunus avium L). Food Chem 2008, 107:185–190.

22. Saradhiulh P, Paul R: Pineapple organic acid metabolism and accumulation during fruit development. Sci Hortic 2007, 112:297–303.

23. Dacera-Ddel M, Iabel S: Heavy metals removal from contaminated sewage sludge by naturally fermented raw liquid from pineapple wastes. Water Sci Technol 2007, 56:145–152.

24. Tilay A, Bul M, Kishenikumar J, Amnrapuer U: Preparation of ferulic acid from agricultural wastes: its improved extraction and purification. J Agric Food Chem 2008, 56:7644–7648.

25. Leszczynski OI, Blandauer CA: Zinc transfer from the embryo-specific lipoxygenase in tomato fruit. J Plant Physiol 2001, 159:815–819.

26. Ophir R, Pang X, Halaly T, Venkateswari J, Lavee S, Galbraith D, Or E: Characterization of growth-regulating hormone responsive genes during tomato fruit development and ripening in response to stress conditions. J Exp Bot 2009, 60:663–678.

27. Cruz-Rus F, Botella MA, Valpuesta V, Gomez-Jimenez MC: Analysis of genes involved in ascorbic acid biosynthesis in growing and ripening of grape berries. J Plant Physiol 2010, 167:739–748.

28. Debolt S, Melino V, Ford CM: Ascorbate as a biosynthetic precursor in plants. Ann Bot 2007, 99:3–8.

29. Giovannozzo D, Amico L, Parodi IA, Bottini R, Sparvoli F, DeGara L: Antioxidant metabolite profiles in tomato fruit constitutively expressing the grapevine stilbene synthase gene. Plant Biotech J 2004, 2:57–69.

30. Jimenez A, Creissen G, Kular B, Firmin J, Robinson S, Verhoeven M, Mullineaux P: Changes in oxidative processes and components of the antioxidant system during tomato fruit ripening. Plant Physiol 2002, 129:1511–1518.

31. Considine M: Ascorbate and glutathione in fruit ripening: is cycling important for fruit health? In Advances in postharvest technologies for horticultural crops. Edited by Noureddine B, Norio S. 2006:275–296.

32. Li M, Ma F, Zhang M, Qu F: Distribution and metabolism of ascorbic acid in apple fruits (malus domestica borkh cv. Gala). Plant Sci 2008, 174:606–612.

33. Ma Y, Ma F, Zhang J, Ming L, Wang Y, Liang D: Effects of high temperature on activities and gene expression of enzymes involved in ascorbate-glutathione cycle in apple leaves. Plant Sci 2008, 175:761–766.

34. Luwe M: Antioxidants in the apoplast and symplast of beechn (fagus sylatica L) leaves: seasonal variations and responses to changing ozone concentrations in air. Plant Cell Environ 1996, 19:321–328.

35. Sapek O, Libk S, Karpinka B, Karpinski S, Miziański Z: The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. Acta Biochim Pol 2007, 54:39–50.

36. Todd JF, Pahlath G, Thompson JE: Characteristics of a membrane-associated lipoxigenase in tomato fruit. Plant Physiol 1990, 94:1225–1232.

37. Vienstra RD: Proteolysis in plants: mechanisms and functions. Plant Mol Biol 1996, 32:275–302.

38. Callis J: Regulation of protein degradation. Plant Cell 1995, 7:845–857.

39. Cook W, Jeffrey L, Sullivan M, Vienstra R: Three dimensional structural of a ubiquitin conjugating enzyme (E2). J Biol Chem 1992, 267:15116–15121.

40. Vienstra R: Protein degradation in plants. Annu Rev Plant Physiol Plant Mol Biol 1993, 44:385–410.

41. Balasubramanian C, Senapati A, Sitaramaiah VS, Prasad A, Tripathi R, Sahal S, Briere J, Morin NA, Prakash A, Mohan M, Bhattacharya S, Calvo P, Vamshi: A new proteinase inhibitor from pineapple fruit. Biochim Biophys Acta 2009, 1794:1–8.

42. Balasubramanian C, Senapati A, Sitaramaiah VS, Prasad A, Tripathi R, Sahal S, Briere J, Morin NA, Prakash A, Mohan M, Bhattacharya S, Calvo P, Vamshi: A new proteinase inhibitor from pineapple fruit. Biochim Biophys Acta 2009, 1794:1–8.

43. Balasubramanian C, Senapati A, Sitaramaiah VS, Prasad A, Tripathi R, Sahal S, Briere J, Morin NA, Prakash A, Mohan M, Bhattacharya S, Calvo P, Vamshi: A new proteinase inhibitor from pineapple fruit. Biochim Biophys Acta 2009, 1794:1–8.

44. Balasubramanian C, Senapati A, Sitaramaiah VS, Prasad A, Tripathi R, Sahal S, Briere J, Morin NA, Prakash A, Mohan M, Bhattacharya S, Calvo P, Vamshi: A new proteinase inhibitor from pineapple fruit. Biochim Biophys Acta 2009, 1794:1–8.

45. Balasubramanian C, Senapati A, Sitaramaiah VS, Prasad A, Tripathi R, Sahal S, Briere J, Morin NA, Prakash A, Mohan M, Bhattacharya S, Calvo P, Vamshi: A new proteinase inhibitor from pineapple fruit. Biochim Biophys Acta 2009, 1794:1–8.

46. Balasubramanian C, Senapati A, Sitaramaiah VS, Prasad A, Tripathi R, Sahal S, Briere J, Morin NA, Prakash A, Mohan M, Bhattacharya S, Calvo P, Vamshi: A new proteinase inhibitor from pineapple fruit. Biochim Biophys Acta 2009, 1794:1–8.

47. Balasubramanian C, Senapati A, Sitaramaiah VS, Prasad A, Tripathi R, Sahal S, Briere J, Morin NA, Prakash A, Mohan M, Bhattacharya S, Calvo P, Vamshi: A new proteinase inhibitor from pineapple fruit. Biochim Biophys Acta 2009, 1794:1–8.

48. Balasubramanian C, Senapati A, Sitaramaiah VS, Prasad A, Tripathi R, Sahal S, Briere J, Morin NA, Prakash A, Mohan M, Bhattacharya S, Calvo P, Vamshi: A new proteinase inhibitor from pineapple fruit. Biochim Biophys Acta 2009, 1794:1–8.

49. Balasubramanian C, Senapati A, Sitaramaiah VS, Prasad A, Tripathi R, Sahal S, Briere J, Morin NA, Prakash A, Mohan M, Bhattacharya S, Calvo P, Vamshi: A new proteinase inhibitor from pineapple fruit. Biochim Biophys Acta 2009, 1794:1–8.

50. Balasubramanian C, Senapati A, Sitaramaiah VS, Prasad A, Tripathi R, Sahal S, Briere J, Morin NA, Prakash A, Mohan M, Bhattacharya S, Calvo P, Vamshi: A new proteinase inhibitor from pineapple fruit. Biochim Biophys Acta 2009, 1794:1–8.

51. Balasubramanian C, Senapati A, Sitaramaiah VS, Prasad A, Tripathi R, Sahal S, Briere J, Morin NA, Prakash A, Mohan M, Bhattacharya S, Calvo P, Vamshi: A new proteinase inhibitor from pineapple fruit. Biochim Biophys Acta 2009, 1794:1–8.