Evaluation of automated cell disruptor methods for oomycetous and ascomycetous model organisms

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Two automated cell disruptor-based methods for RNA extraction, disruption of thawed cells submerged in TRIzol Reagent (method QP), and direct disruption of frozen cells on dry ice (method CP), were optimized for a model oomycete, *Phytophthora capsici*, and a model filamentous ascomycete, *Neurospora crassa*. The results were compared with more conventional methods of manual grinding in a mortar and pestle under liquid nitrogen (method M&P) and those using lyophilized samples. A chip-based electrophoresis system showed that methods CP and M&P yielded high integrity RNA from both *P. capsici* and *N. crassa*. In contrast, method QP and lyophilized sample-based methods resulted in inconsistent RNA integrity between the two organisms, indicating they are not safe alternatives for method M&P. Microarray mRNA profiling for *P. capsici* revealed alterations in global mRNA profiles in those samples that the chip-based electrophoresis detected slight decreases in RNA integrity. Despite this, RNA integrity of these samples could still be high enough to pass conventional stringent quality control measures. This demonstrated the necessity of global mRNA profiling for the evaluation of RNA extraction protocols.

Emerging high-throughput transcriptome analysis technologies such as microarray mRNA profiling (DeRisi *et al.*, 1997) and more recently RNA-Seq (Nagalakshmi *et al.*, 2008) enable us to monitor thousands of gene activities simultaneously and greatly facilitate the study of functional genomics. In responding to the ever-growing demand for transcriptomic analysis for large numbers of samples, automated cell disruptor-based methods for high-throughput RNA extraction are gaining popularity (Van der Vorst *et al.*, 2009). It should be emphasized that sensitivity and accuracy of high-throughput transcriptome analysis relies heavily upon the quality of input RNA samples. It is not clear if automated cell disruption is appropriate for high-throughput transcriptome analysis or if it is compatible with only certain tissue types.

Unlike genomic DNA, mRNA transcripts are extremely sensitive to growth conditions of the cell. Transcript levels can change on a minute scale due to both degradation and de novo transcription. Consequently, alterations in transcript profiles that are not representative for the condition of interest are easily introduced during sample harvesting (Fleige & Pfaffl, 2006; Pieterse *et al.*, 2006) and/or during sample preparation. Accordingly, in order to correctly interpret the mRNA profiling data, extent of such experimental noise needs to be evaluated and minimized. As a means to minimize such unwanted changes, biological samples are traditionally snap-frozen in liquid nitrogen, and kept at -80°C. RNA extraction is then carried out by means of a mortar and pestle with liquid nitrogen (Sambrook & Russel, 2006). Ground tissues are kept frozen and subsequently transferred to a tube containing extraction buffer and are homogenized immediately. A chaotropic reagent in the extraction buffer, such as guanidinium thiocyanate (Chomczynski & Sacchi, 1987), denatures and deactivates enzymatic activities, such as nuclease and mRNA transcription activities, thus protecting RNA integrity. In contrast, in a typical automated cell disruption method, beads such as small glass, ceramic or steel beads and extraction buffer are added to frozen cell cultures resting on an ice bath prior to cell disruption. Thus, unlike the traditional mortar and pestle-based method, samples are inevitably thawed on ice or in the extraction buffer prior to mechanical disruption of the cell. Judging from the inherent instability of mRNA transcripts, thawing can perturb global mRNA profiles, thus introducing experimental noise. To our surprise that reproducibility of global mRNA profiling data for cell disruptor-based methods has not been evaluated. Furthermore, no standardized protocols are available for cell disruptor-based methods for fungi or oomycetes.

In this study, a model oomycete *Phytophthora capsici* (Lamour *et al.*, 2007), and a model filamentous ascomycete, *Neurospora crassa* (Galagan *et al.*, 2003), were chosen as models, and frozen as well as lyophilized samples were subjected to cell disruption. There are two main methods for cell disruption; (1) disruption of cells submerged in extraction buffer, and (2) that under liquid nitrogen or dry ice without extraction buffer. Choice of beads for cell disruption is also crucial. Four distinctive beads with different sizes and materials; Lysing Matrix A (MP Biomedicals, Solon OH, USA, sold for lysis of hard samples such as cartilage, bone and seed), Lysing Matrix C (MP Biomedicals, for lysis of yeast, algae and fungi), Lysing Matrix D (MP Biomedicals, for lysis of plant and animal tissue) and Lysing Matrix R, which was a home made mixture of two different sized glass beads, were used in combination with the two aforementioned cell disruption methods, and evaluated for RNA yield. RNA integrity and quality was then evaluated by means of a chip-based electrophoresis system for the two model organisms, and microarray mRNA profiling for *P. capsici*. We found that global mRNA profiles were perturbed in response to thawing and lyophilization of samples. GO ontology enrichment analysis was then used to infer cellular activities which were responsible for the alteration of mRNA profiles. A means to minimize experimental noise associated with cell disruptor-based protocols are discussed.

Methods

Strain and culture conditions

Phytophthora capsici

P. capsici isolate LT1534, which was used for the genome sequencing project, was kindly given by Kurt Lamour, the University of Tennessee. The isolate was maintained on 20% clarified V8 solid medium with 1.5% agar at 23°C (Englander & Roth, 1980). A small mycelial plug was then transferred to each of the 60mm x 15mm Falcon Petri dishes (catalog no. 351007, BD Bioscience, Franklin Lakes, NJ, USA) containing 7 ml of clarified V8 solid medium overlaid with a polycarbonate membrane filter (catalog no. 28157-927; VWR, Brisbane, CA, USA), and grown for 48 hours in 23°C under constant light, as a precaution to avoid experimental noise due to circadian rhythm. The samples were cut into approximately four equal sizes, lifted from the polycarbonate membrane surface, each was transferred to a 2 ml screw-cap microcentrifuge tube (catalog no. 72.694.996 Sarstedt; Fisher Scientific, Santa Clara, CA, USA) and immediately snap-frozen in liquid nitrogen unless otherwise stated. Each of the quartered colony pieces weighed approximately 100 mg. The samples were kept at -80°C until further use. For the optimization of RNA extraction methods, samples originating from identical colonies were used for assessing methods with different beads and adaptor combinations. For microarray mRNA profiling all the samples, with the exception of one pair, were derived from different colonies.

Neurospora crassa

The wild type laboratory strain, FGSC2489, used for the *N. crassa* genome project, was grown in a 200 ml Vogel's liquid minimal medium (Vogel, 1956) in 500 ml Erlenmeyer flask at 25°C for 48 hours in a gyratory shaker at 150 rpm. The mycelium was blotted on paper towels, and yielded a wet weight of 9.3 g. A number of samples, each weighing approximately 100 mg, were excised from the mycelium mat, transferred to microcentrifuge tubes, snap-frozen in liquid nitrogen and kept in -80°C until further use.

Post-harvest treatments of samples

P. capsici samples were subjected to one of seven distinctive post-harvest treatments prior to RNA extraction (Table 2). For all samples but lyophilized ones, were homogenized by method QP with Lysing Matrix R (described in tissue homogenization section). In order to assess the effects of thawing on mRNA profiles, 1 ml of 4°C TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was added to each of the frozen samples, and incubated at 4°C for 10 min (Table 2, treatment 1). To assess thawing effects without TRIzol, frozen samples were incubated for 10 min at 4°C in a water bath prior to RNA extraction (treatment 2). For the RNALater ICE treatment (treatment 3, Ambion, Austin, TX, USA), c.a. 100 mg of fresh tissue was sliced into 5 mm strips prior to snap-freezing in liquid nitrogen. One ml of -80°C pre-chilled RNALater ICE solution was added to each frozen tissue and stored at -20°C for at least 24 hours. For the RNALater treatment (treatment 4, Ambion), 1 ml of RNALater RNA stabilization solution was added to c.a. 100 mg of sliced fresh tissue, and incubated at 4°C for 24 hours. Samples were then transferred to new tubes and advanced to RNA extraction. In addition, one quarter of a fresh mycelium was transferred directly to a microcentrifuge tube and incubated at 4°C for 10 min, and proceeded to RNA extraction (treatment 5). Tissue samples of *N. crassa* were also subjected to the post-harvest treatment as described above, except for the treatment 5.

Tissue homogenization and RNA extraction

The *P. capsici* and *N. crassa* tissues were either ground in liquid nitrogen with a mortar and pestle (method M&P), or homogenized using a FastPrep-24 Instrument (MP Biomedicals) as follows. For the FastPrep-24 cell disruptor, cells were disrupted either in a QuickPrep adaptor (method QP, MP Biomedicals) or CoolPrep adaptor (method CP, MP Biomedicals). For both adaptors, four unique sets of beads were tested: Lysing Matrix A (premix of 150 mg garnet sand and a 0.6 mm ceramic sphere), Lysing Matrix C (700 mg of 1 mm silica spheres), Lysing Matrix D (1 g of 1.4 mm ceramic spheres), all of which were purchased from MP Biomedicals, and Lysing Matrix R, which was a combination of two of 6.4 mm diameter glass beads (catalog number 11079635, Biospec Products, Bartlesville, OK, USA) and 50 mg of finer glass beads (106 um, catalog number G8893, Sigma-Aldrich, St. Louis, MO, USA).

For method QP, four 2ml screw-cap microcentrifuge tubes, each containing approximately 100 mg of a frozen mycelium were prepared in triplicate. Into each microcentrifuge tube 1ml of TRIzol Reagent, which is a guanidinium thiocyanate-based extraction buffer, and one of each of the four lysing matrices, chilled at -20°C, was added. Cells were then disrupted at a speed setting of 6 meters/second for 40 seconds. Total RNA was subsequently extracted according to the manufacturer's protocol for TRIzol Reagent.

As with method QP, for method CP, four 2 ml screw-cap microcentrifuge tubes, each containing approximately 100 mg of a frozen

mycelium were prepared in triplicate. However, for method CP, the microcentrifuge tubes were kept on a microcentrifuge tube rack (Subzero Blue IsoFreeze Flipper Rack, GeneMate; BioExpress, Kaysville, UT, USA) on dry ice. Into each microcentrifuge tube one of each of the four lysing matrices, chilled at -20°C, was added. The cells were then disrupted at a speed setting of 6 meters/second for 40 seconds, twice, in a CoolPrep adapter, which was filled with crushed dry ice. One ml of TRIzol Reagent was then added to the pulverized sample, and vigorously vortexed until the sample was completely thawed and homogenized. Total RNA was subsequently extracted according to the manufacturer's protocol for TRIzol.

For the method M&P, up to 200 mg of mycelium was ground with mortar and pestle in liquid nitrogen. Approximately 100 mg of ground cell tissue was then transferred to each of the 2 ml screwcap microcentrifuge tubes, which were kept on dry ice. 1 ml of TRIzol Reagent was then added to each of the tubes and immediately vortexed to homogenize the samples. RNA was then extracted according to the manufacturer's protocol for TRIzol Reagent.

In addition, two widely used methods for RNA extraction from lyophilized samples were adapted for an automated cell disrupter. Fresh cell cultures or those stored in -80°C, approximately 100 mg each, were snap-frozen in liquid nitrogen and subjected to lyophilization for 24 hours under 20 to 100 mTorr. For each model organism three separate lyophilizations were conducted for biological controls. Lyophilized samples were either pulverized with Lysing Matrices A at room temperature on a QuickPrep adapter (method LQ, treatment 7 in Table 2) or snap-frozen in liquid nitrogen and pulverized on a CoolPrep adapter with dry ice (method LC, treatment 6). For both methods, cells were disrupted at a speed setting of 6 meters/second for 40 seconds, twice. One ml of TRIzol Reagent was then added and RNA extraction was carried out as method CP described above.

Up to 100ug of total RNA was further cleaned using the RNeasy mini protocol for RNA cleanup (Qiagen, Valencia, CA, USA). RNA purity and concentration were determined by NanoDrop 3300 spectrophotometer (Thermo Scientific, Waltham, MA, USA) with RiboGreen fluorescent dye (Invitrogen Life Technologies). Integrity of RNA was determined by both agarose gel electrophoresis and Experion automated electrophoresis system (Bio-Rad, Hercules, CA, USA). Experion's RNA Quality Indicator (RQI), is independent of sample concentration, and is equivalent to RNA Integrity Number (RIN, Agilent Technologies). Although, different algorithms are employed, RQI and RIN are adjusted to give approximately the same values for identical RNA samples (Denisov *et al.*, 2008).

Microarray design

The *P. capsici* whole-genome expression oligonucleotide 60-mer arrays (4x72K multiplex format, Roche NimbleGen, Madison, WI, USA) were designed by the manufacturer on the basis of 17,383 gene models derived from the *P. capsici* database at DOE Joint Genome Institute (PhycaF7_best_transcripts.fasta.gz; http://genome.jgi-psf.org/PhycaF7/PhycaF7.home.html). On average four probes per open reading frame (ORF), a total of 69,421 probes for 17,363 gene models, were synthesized on each of the four arrays on the multiplex array slide.

cDNA synthesis, hybridization and image acquisition

cDNA synthesis, labeling, hybridization procedure, data acquisition and normalization were carried out according to the manufacturer's instructions (Roche NimbleGen). Briefly, 10 ug of total RNA and oligo dT primer were used to synthesize the first strand of cDNA, which was followed by the synthesis of the second strand of cDNA to yield double stranded cDNA. The Cy3 cyanine dye-labeled random 9-mers were then used to label the cDNA. The cDNA was then precipitated with isopropanol, vacuumed dried, and afterward used for hybridization. Hybridization was done at 42°C for 16 to 20 hours on a MAUI Hybridization System (BioMicro Systems, Salt Lake City, UT, USA). After three steps of washing, microarrays were scanned on an Axon GenePix 4000B (Molecular Devices, Sunnyvale, CA, USA). Quantile normalization and background correction across arrays were performed using Robust Multi-chip Average (RMA) algorithm (Irizarry *et al.*, 2003) implemented in NimbleScan Version 2.5 software. A MIAME-compliant microarray dataset (Brazma *et al.*, 2001) has been deposited in Filamentous Fungal Gene Expression Database at Yale University (http://bioinfo.townsend.yale.edu/) (Zhang & Townsend, 2010). Table S1 lists mRNA profiling results and functional annotations.

Data analysis

Normalized intensity data for 17,363 genes across 17 arrays were obtained by RMA. First, between pairwise samples, an intensity ratio of each gene was plotted against the average intensity of gene expression to visualize intensity-dependent ratio (MA plot, (Dudoit *et al.*, 2002)). Background noise for genes with normalized hybridization intensity of below 64 was disproportionately high. Therefore, genes with average hybridization intensity below 64 were removed from the dataset. The remaining 12,504 genes were used for further analysis. Pearson's correlation coefficient between global mRNA expression patterns was then use to cluster cDNA samples using hclust function with average linkage option in a statistical software R 2.7.1 (http://bioconductor.org). Between hierarchical clusters of cDNA samples, two-sample t-test was used to identify differentially expressed genes using the software R 2.7.1. In order to identify difference in cellular activities between the hierarchical clusters, gene ontology (GO)-based functional enrichment analysis was conducted. Gene ontology (GO) annotation (Harris *et al.*, 2008) for *P. capsici* genome was

obtained from the DOE Joint Genome Institute (JGI, PhycaF7_GO.tab.gz; http://genome.jgi-psf.org/PhycaF7/PhycaF7.download.ftp.html). Out of the 17,363 *P. capsici* gene models that were represented on the microarray, a total of 1,677 GO terms were assigned to 7,767 gene models according to the annotation scheme conducted by JGI. Over- or under- representation of GO terms in differentially expressed gene groups in relation to the genome was evaluated against an expected hypergeometric distribution using Fisher's exact test in the software R 2.7.1. A significant level of 0.05 was used with multiple testing corrections according to Benjamini and Hochberg (Benjamini & Hochberg, 1995).

Results

Representatives of oomycetes and filamentous ascomycetes, *Phytophthora capsici* and *Neurospora crassa*, respectively, were used for optimization of RNA isolation using a bench-top cell disruptor, FastPrep-24 Instrument (MP Biomedicals). Integrity and global mRNA profiles of obtained RNA specimens were then cross-examined with total RNA isolated by mortar and pestle under liquid nitrogen (M&P) and two lyophilization-based methods.

Optimization of RNA extraction using a cell disruptor

Cell disruptors are widely used for DNA and RNA extraction as high-throughput alternatives for mortar and pestle. Several different methods of cell disruption and various lysing matrix beads, with which tissues are pulverized, are available. First, we searched for a method that gives high quality RNA at high and consistent yields. Two distinctive cell disruption methods, each with a specialized adaptor, were tested. For the first cell disruptor method, 4°C TRIzol Reagent was added to frozen tissues, and homogenized immediately on a QuickPrep adaptor (method QP). For the second method, frozen tissues were pulverized at a frozen state on a dry ice-filled CoolPrep Cryogenic Adapter, and subsequently 4°C TRIzol Reagent was added and vortexed (method CP). For both methods, four distinctive lysing matrix beads were evaluated for RNA yield: i.e. Lysing Matrix A, Lysing Matrix C, Lysing Matrix D, and Lysing Matrix R (details in Introduction and Materials and methods). Additionally, two lyophilized sample-based methods, samples disrupted on a dry ice-filled CoolPrep adapter with Lysing Matrix A (method LC) and samples disrupted on a QuickPrep adapter at room temperature with Lysing Matrix A without extraction buffer (method LQ), were conducted.

Similar trends for performance of extraction methods for *P. capsici* and *N. crassa* were recognized. For method QP, the yield difference due to lysing matrix beads were small (Table 1). However, for *N. crassa* Matrix R significantly underperformed any other lysing matrices ($p<0.001$). For method CP, difference in performance of matrices was more prominent than that of method QP. Matrices A and R, which were comprised of fine and large beads, outperformed Matrices C and D, both of which were comprised of small uniform beads, for *P. capsici* (t test, $p<0.01$) and for *N. crassa* ($p<0.001$). RNA yields from lyophilized samples (methods LC and LQ) and those from a mortar and pestle under liquid nitrogen (method M&P) were comparable to the highest values for method CP. The quality of RNA obtained by the five methods, QP, CP, LC, LQ and M&P, judged by intensity of 28S and 18S ribosomal RNA bands on agarose gel, was indistinguishable (data not shown).

Table 1. Summary for total RNA yield due to different methods

Method[a]	Lysing matrix[b]	No. replicates	Average weight mg	Average total RNA yield µg	Yield (1SD) µg/mg-tissue
P. capsici					
QP	A	3	96.7	78.6	0.8 (0.5)
QP	C	3	86.7	87.3	1.0 (0.8)
QP	D	3	86.7	100.6	1.2 (0.2)
QP	R	3	100.0	82.0	0.8 (0.4)
CP	A	3	86.7	129.9	1.5 (0.4)
CP	C	3	93.3	14.1	0.2 (0.4)
CP	D	3	80.0	66.3	0.8 (0.6)
CP	R	3	83.3	119.3	1.4 (0.6)
LC	A	3	78.3	133.4	1.6 (0.6)
LQ	A	3	77.0	108.0	1.4 (0.2)
M&P		1	225.0	343.5	1.5 (0.0)
N. crassa					
QP	A	3	97.0	751.5	7.7 (1.0)
QP	C	3	98.3	513.1	5.2 (0.5)
QP	D	3	100.7	575.3	5.7 (0.3)
QP	R	3	100.3	302.2	3.0 (0.3)

CP	A	3	103.3	601.6	5.8 (0.9)
CP	C	3	99.0	94.3	1.0 (0.2)
CP	D	3	102.3	28.3	0.3 (0.4)
CP	R	3	106.7	902.0	8.5 (1.4)
LC	A	3	94.7	796.4	8.4 (1.1)
LQ	A	3	103.3	670.5	6.5 (2.3)
M&P		1	210.0	1326.3	6.3 (0.0)

[a] QP: samples submerged in TRIzol, homogenized on a QuickPrep adapter.
CP: frozen samples disrupted on a dry ice-filled CoolPrep adapter.
LC: lyophilized sample disrupted on a dry ice-filled CoolPrep adapter.
LQ: lyophilized samples disrupted on a QuickPrep adapter at room temperature.
M&P: a mortar and pestle under liquid nitrogen.
[b] various beads for cell disruption. Details in Introduction.

Experimental design for evaluation of cell disruptor protocols by means of a chip-based capillary electrophoresis and microarray mRNA profiling

With appropriate combinations of cell lysing matrices and adaptors, the high throughput cell disruptor was shown to yield a comparable quantity of RNA in comparison to method M&P. The next objective was to examine whether RNA samples derived from the cell disruptor, fresh or lyophilized samples, had high integrity and were able to reproduce global mRNA profiles of RNA samples derived from method M&P. Especially, unlike M&P, in method QP, TRIzol Reagent is needed to add to samples prior to cell disruption, which could potentially impact global mRNA profiles. Also, effect of lyophilization on transcriptome has not been investigated.
For each of the five RNA isolation methods, the number of biological replicates used for the measurement of RQI values and sample names of biological replicates used for microarray profiling were shown in Table 2. For the QP method, no more than four samples were handled simultaneously, and no longer than 2 minutes had elapsed between unscrewing caps for the addition of TRIzol Reagent to frozen samples and the initiation of mechanical disruption.

Table 2. Effects of post-harvest treatments on the integrity of RNA samples

Treatment No.	Post-harvest treatment[a]	Homogenization	Lysing Matrix	n	Average RQI	Samples used for microarray
P. capsici				-		
	no	M&P	-	3	9.8	MP1
	no	CP	A	5	9.5	CP1, CP2, CP3
	no	QP	R	2	9.5	QP1, QP2[b], QP3[b]
1	4°C TRIzol 10min	QP	R	3	9.6	TR1, TR2
2	4°C 10min	QP	R	3	9.3	FT2, FT3
3	RNALater ICE, -20°C, 1 day	QP	R	3	-	
4	RNALater, 4°C, 1 day	QP	R	1	9.7	RL2
5	Never frozen, 4C 10min	QP	R	2	9.5	NF1
6	lyophilization	CP (LC)	A	6	8.1	LC1, LC2
7	lyophilization	QP w/o buffer (LQ)	A	6	6.8	LQ1, LQ2
N. crassa						
	no	M&P	-	1	9.7	
	no	CP	A	1	9.7	
	no	QP	R	4	9.4	
1	4°C TRIzol 10min	QP	R	5	8.9	
2	4°C 10min	QP	R	2	9.2	
3	RNALater ICE, -20°C, 1 day	QP	R	2	-	
4	RNALater, 4°C, 1 day	QP	R	2	-	

6	lyophilization	CP (LC)	A	3	9.2
7	lyophilization	QP w/o buffer (LQ)	A	3	8.8

[a] All samples except for treatment 5 were snap-frozen in liquid nitrogen prior to post-harvest treatment.
[b] QP2 and QP3 are technical replicates; cDNAs were prepared from the same RNA sample with separate reverse transcription reactions.

Additionally, we simulated a situation where a large number of samples are handled together. Commercially available cell disruptor such as Mini-Beadbeater (Biospec, Bartlesville, OK) and Precellys Homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) are able to hold 45 and 24 microcentrifuge tubes, respectively. The maximum sample load for a QuickPrep adaptor on FastPrep 24 is 24 microcentrifuge tubes. When 24 samples are handled, 5 to 10 minutes is needed between unscrewing the first sample and initiation of a homogenizer. In such a scenario, if samples are kept in an ice bath, they are exposed to 0°C or a higher temperature, and thus there is an opportunity for thawing. Upon the addition of 4°C TRIzol Reagent, samples will inevitably be thawed in a short period of time. It is possible that prolonged duration in a thawed state could introduce noise to mRNA profiles. In order to account for high-throughput RNA extraction, two additional treatments were designed for method QP (Table 2). First, approximately 100 mg each of frozen *P. capsici* tissues were incubated with 4°C TRIzol Reagent in a 4°C water bath for 10 minutes to evaluate the effects of TRIzol and thawing (Table 2, treatment 1). Frozen samples of *P. capsici* were also incubated in microcentrifuge tubes in a 4°C water bath to assess the thawing effect alone (treatment 2). In addition, RNAlater (Ambion), an aqueous reagent widely used to preserve RNA in fresh animal tissues (treatment 4) (Florell *et al.*, 2001; Mutter *et al.*, 2004), and RNALater-ICE (treatment 3) used to preserve RNA in previously frozen animal tissues (Li *et al.*, 2004), were tested for the hope that they were capable of preserving mRNA profiles in *P. capsici* after harvest. In order to assess mRNA response to cold-shock alone, cultures were harvested and transferred to microcentrifuge tubes, without liquid nitrogen snap-freeze, and incubated at 4°C for 10 minutes (treatment 5). The two lyophilized sample-based methods (treatments 6 and 7), were also included for evaluation of their mRNA quality.

Effect of post-harvest treatments on mRNA profiles of *P. capsici*

Except for the RNAlater ICE-treated samples, all the *P. capsici* samples produced comparable quantities of total RNA. Several attempts were made to extract RNA from RNALater ICE-treated samples, however, the yielded RNA was always degraded. RNA quality was then assessed by RNA quality indicator (RQI) values presented by Experion microfluidic capillary electrophoresis system (Bio-Rad). For those samples without post-harvest treatment, average RQI values ranged from 9.5 to 9.8, indicating that RNA integrity was high and comparable among the three extraction methods (Table 2).

For those samples subjected to various post-harvest treatments, a diverse range of responses was observed. Thawed samples in the absence of TRIzol Reagent (method 2) showed slightly decreased RQI values (9.3). It was our surprise that lyophilized samples showed the lowest RQI values accompanied with elevated baseline signals. Note that method LQ yielded lower RQI (6.8) than method LC (RQI 8.1), We repeated lyophilization a total three times including six biological replicates for each method but decrease in RQI value was consistently detected. Remaining samples with post-harvest treatment showed RQI values comparable to samples without post-harvest treatment. Integrity measures of RNA for these thawed samples were in the range of "intact RNA" (Denisov *et al.*, 2008), and those for lyophilized samples with method LC were in the range of "less intact" but reliable for microarray mRNA profiling (Copois *et al.*, 2007). RQI values for lyophilized samples with method LQ were below the reliable range for microarray but in the reliable range for quantitative reverse transcription PCR (Fleige & Pfaffl, 2006; Fleige *et al.*, 2006). All the RNA samples except for that treated with RNAlater ICE were, thus, processed for microarray mRNA profiling.

In order to visualize effects of sample treatments on mRNA, seventeen *P. capsici* samples were hierarchically clustered according to pairwise distance (1 - correlation coefficients) between global mRNA profiles (Fig. 1). Three distinctive clusters, designated groups A, B and C, were observed. The group A includes all the samples prepared by methods M&P (MP1), QP (QP1, QP2, QP3) and CP (CP1, CP2, CP3), all without post-harvest treatment, as well as the sample treated with 4°C RNAlater (RL2) and two samples incubated in 4°C TRIzol Reagent for 10 minutes (TR1, TR2). One sample, treated at 4°C for 10 minutes without snap-freeze in liquid nitrogen (NF1) was outgrouped to all the above-mentioned samples in group A. Group B includes all the four lyophilized samples (LC1, LC2, LQ1, LQ2), and group C includes two snap-frozen samples, which were thawed and incubated at 4°C for 10 min (FT2 and FT3).

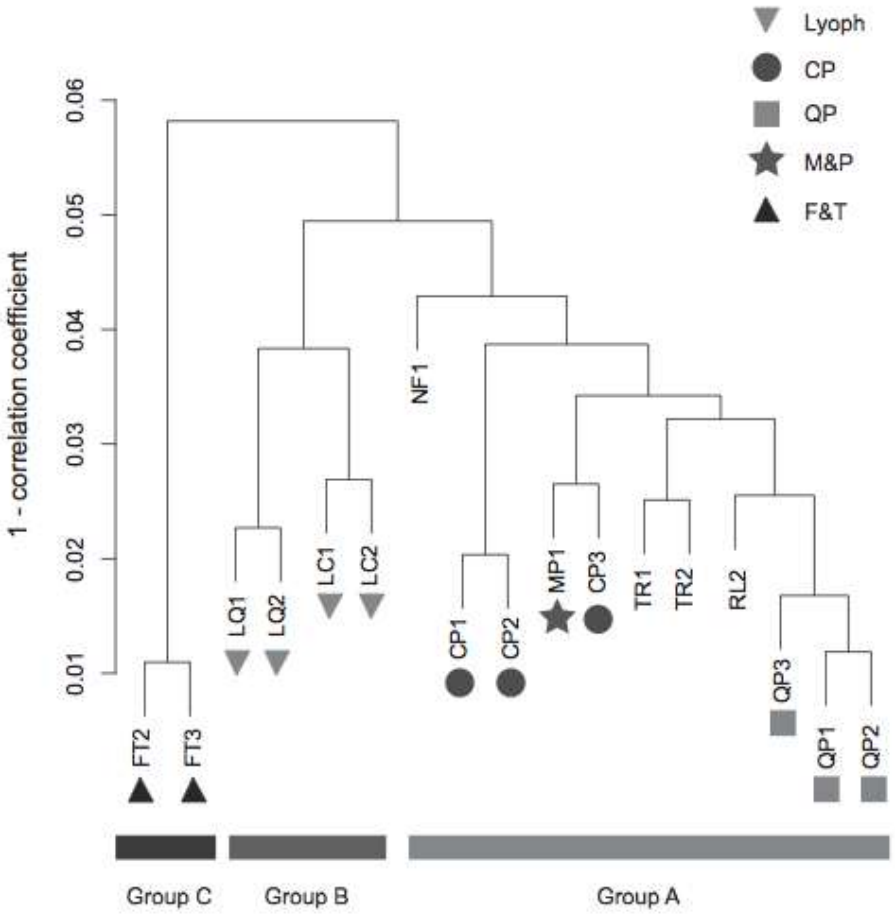

Fig. 1. Hierarchical clustering of cDNA samples with various post-harvest treatments. 17 samples were clustered based on their expression patterns of 12,504 genes. The seven samples used for the optimization of cell disruption methods, CP, QP and M&P were indicated with filled circle, filled square and filled star, respectively. Group B is comprised of lyophilized samples (indicated with filled inverted triangles), and group C is comprised of samples which were thawed and incubated without TRIzol Reagent (indicated with filled triangles). All the remaining samples were found in group A.

Hierarchical clustering showed mRNA profiles to be perturbed by both lyophilization and freeze and thaw. It should be noted that the culture (NF1) incubated at 4°C for 10 minutes without snap-freeze in liquid nitrogen, was found in group A, which was comprised mainly of samples without prolonged post-thaw incubations. This indicates that the observed deviation of mRNA profiles in group C were largely attributable to the freeze-thaw process rather than a response to environmental changes, such as deprivation of nutrient due to transfer of cells from culture medium to a microcentrifuge tube, injury incurred during transfer, or the temperature shift from 23°C to 4°C. The sample TR1 and TR2 incubated at 4°C for 10 minutes with TRIzol Reagent, were closely associated with samples which were not subjected to post-harvest treatments prior to cell disruption. These data indicate that the change in the global mRNA profiles due to freeze and thaw was effectively suppressed when samples were immersed in TRIzol Reagent. In addition, the mRNA profile of the fresh culture treated with RNAlater overnight at 4°C (RL2) was found within group A. This indicates that RNAlater is able to immediately stabilize mRNA in *P. capsici*, and perturbation by the treatment is minimum, if there is any. Four lyophilized samples were found in group B, which is distant from the sample prepared by mortar and pestle (MP1), indicating that lyophilization affects mRNA profiles. Furthermore, samples pulverized at room temperature (LQ1 and LQ2) and those on dry ice (LC1 and LC2) formed discrete subclusters, indicating that sample temperature during pulverization also affects mRNA profiles.

Genes associated with certain biological processes were enriched in lyophilized and frozen-thawed samples

Although repeated attempts were made, *P. capsici* cultures have never survived the process of snap-freeze in liquid nitrogen and rapid thawing or lyophilization, in the conditions used in this research (data not shown). Inviable cells, however, displayed alteration in global mRNA profiles in response to freeze and thaw. It is not clear if the observed alteration is of biological implication or is a part of the natural process of cell decomposition. Elucidation of the mechanism of the alteration in mRNA profiles could yield clues to minimize mRNA noises that occur during sample preparation. We hypothesized that if alteration of mRNA profiles is a consequence of a biological response, then activation/deactivation of groups of genes involved in specific biological processes or metabolic pathways will be observed. Enrichment analysis of functional categories can be used to detect the activation/deactivation of such specific biological processes or metabolic pathways. To test this hypothesis, we conducted gene ontology (GO) enrichment analysis (Subramanian *et al.*, 2005). GO terms describe gene products in terms of their associated biological processes, cellular components and molecular functions in a species-independent manner (Harris *et al.*, 2008). Out of 17,363 *P. capsici* genes on the microarray, at least one GO term was assigned to 7,767 gene models. From microarray mRNA profiling data, we first identified apparently differentially displayed genes with statistical support ($p>0.05$) between groups (Fig. 1 and Table S1). Student's T-test showed that transcript abundances of 7,310 genes were significantly different between groups. Note that because multiple hypothesis correction has not been conducted, these genes include truly significant genes as well as false positives.

GO enrichment analysis revealed that 22 GO terms were enriched in the comparison between group A and group B (Table S2). Memberships of these GO terms are partially overlapped, and they can be summarized into five main terms (Table 3). In response to lyophilization, mRNA transcripts for 1) "alcohol catabolic processes" and 2) "glucose catabolic process" became less abundant, whereas genes for 3) "phosphorylation", 4) "microtubule-based process" and 5) "cell communication" became abundant. Likewise in comparison between group A and group C, fourteen GO terms were enriched. Again, a large part of them has redundant descriptions having overlapped gene sets, and they can be summarized into two main terms. In response to freeze and thaw, mRNA transcripts for 1) "cellular amino acid and derivative metabolic process" became less abundant, whereas those for 2) "microtubule-based process" became abundant.

Table 3. Enriched GO biological processes in response to lyophilization and freeze and thaw (complete list in Table S2)

GO	description[a]	Obs[b]	Exp[b]	Total[c]	*P* value[d]
	A>B				
GO:0006007	glucose catabolic process	15	7.1	41	2.6E-02
GO:0046164	alcohol catabolic process	15	7.7	44	4.5E-02
	B>A				
GO:0016310	phosphorylation	105	63.7	399	2.1E-06
GO:0007017	microtubule-based process	33	13.1	82	2.6E-06
GO:0007166	cell surface receptor linked signal transduction	10	4.0	25	2.7E-02
	A>C				
GO:0006519	cellular amino acid and derivative metabolic process	65	41.4	186	2.2E-03
	C>A				
GO:0007017	microtubule-based process	25	12.5	82	7.3E-03
	C>B				
GO:0034961	cellular biopolymer biosynthetic process	70	33.2	355	4.5E-08
GO:0010467	gene expression	69	35.6	381	2.1E-06

[a] Enriched GO terms between hierarchical clusters defined in Fig. 1.
[b] Observed number and expected number of genes if probabilities of each outcome are independent of the cluster.
[c] Total number of detected genes on the microarray.
[d] *P* values were determined using Fisher's exact test with Benjamini and Hochberg multiple testing correction.

Effect of post-harvest treatments on mRNA profiles of *N. crassa*

In *P. capsici*, we found that RNALater and TRIzol post-harvest treatments preserved RNA integrity and expression profiles, whereas, lyophilization and thawing of samples at 4°C reduced RNA integrity and altered mRNA profiles. In *N. crassa*, methods CP and M&P also yielded RNA with high RQI values (Table 2, RQI 9.7 for both methods). However, unlike *P. capsici*, method QP slightly reduced RNA integrity in *N. crassa* in comparison to methods CP and M&P (RQI 9.4, t-test, p=0.04). Furthermore, when samples were incubated for a longer period of time in TRIzol (treatment 1), a decrease in RNA integrity became more noticeable for method QP (RQI 8.9). As has been observed for *P. capsici*, thawing at 4°C slightly reduced RNA integrity (RQI 9.2, treatment 2). Lyophilization again reduced RQI values in *N. crassa*. The lyophilized sample pulverized at room temperature showed smaller RQI (8.8, treatment 7) than that pulverized on dry ice (RQI 9.2, treatment 6). However, compared to *P. capsici*, reduction of RQI values was less severe. In *N. crassa* both RNALater ICE and RNALater gave heavily degraded RNA (treatment 3 and 4).

Discussion

There is a growing demand for high-throughput RNA isolation, and automated cell disruptors have been gaining popularity (Van der Vorst *et al.*, 2009). Validation of the technology is, however, limited to 1) RNA integrity measured by capillary electrophoresis and 2) mRNA profiles of a handful of genes measured by real time quantitative PCR (Van der Vorst *et al.*, 2009). In this research we first optimized automated cell disruptor protocols for an oomycete plant pathogen *P. capsici* and a filamentous ascomycete *N. crassa* and then inspected RNA quality by capillary electrophoresis for both *N. crassa* and *P. capsici* and by global mRNA profiling for only *P. capsici*.

We found that lyophilization as well as thawing of samples prior to the addition of extraction buffer resulted in significant alteration of mRNA profiles. This finding is notable because thawing is inevitable for the majority of protocols devised with cell disruptors, (e. g. (Kasuga & Glass, 2008; Monteiro *et al.*, 2009; Rautio, 2010)) as well as the hot phenol protocol for frozen yeast cells (e.g. (Castillo *et al.*, 2002; Eelderink-Chen *et al.*, 2010)). It should be emphasized that the effect of thawing, which is evident from microarray mRNA profiling, cannot be explicitly detected by conventional quality control measures.

For method QP for *P. capsici*, it was found that adding TRIzol Reagent to frozen samples could minimize the change of mRNA profiles due to thawing. This indicates that TRIzol Reagent percolates into *P. capsici* cells and ceases enzymatic activity; as a consequence this prevents the alteration of mRNA profiles. In contrast, a 4°C TRIzol treatment was found to compromise RNA integrity in *N. crassa*. This implies that the cell wall of *N. crassa* is less permeable to TRIzol, allowing RNA to degrade. We do not have mRNA profiling data for *N. crassa*, however, it is likely that addition of TRIzol also affects its transcriptome. We, therefore do not recommend the use of method QP unless effects of TRIzol on the preservation of mRNA of particular species or tissue types being investigated has properly been evaluated by means of global mRNA profiling.

RNAlater (Ambion), is an aqueous reagent, and is widely used to stabilize and protect RNA in animal tissue (Mutter *et al.*, 2004). The global mRNA profile of the RNAlater-treated *P. capsici* sample closely resembles that prepared by M&P, indicating that the reagent immediately permeated the tissue and stabilized mRNA. On the other hand, an RNALater treatment resulted in severely degraded RNA from *N. crassa*. This is consistent with our finding that while a 4°C TRIzol treatment successfully preserved RNA integrity in *P. capsici*, the same treatment on *N. crassa* yielded slightly degraded RNA. We again do not recommend RNALater unless its effectiveness has been proved for interrogated species or tissue types.

Lyophilization is another widely used method to cease cellular activity prior to RNA extraction (Leary *et al.*, 1969; Sanchez-Rodriguez *et al.*, 2008). Lyophilized samples can be effectively pulverized on a mortar and pestle or an automated cell disruptor. We found that lyophilization damaged RNA integrity and altered mRNA profiles in *P. capsici*. The adverse effect of lyophilization was also observed for *N. crassa* but to a lesser extent. In lyophilization, samples frozen in liquid nitrogen are placed in a vacuum chamber at room temperature, where gas pressure is typically at 100 mTorr. Because the chamber pressure is below the saturation pressure of water vapor, sublimation will progress, while removal of heat by sublimation keeps the sample frozen. It is possible that a rigid cell wall and increasing concentrations of solutes in the cytosol effectively prevent the cell from complete desiccation, by which the cell maintains residual cellular activity such as de novo transcription and mRNA degradation. GO enrichment analysis revealed that in the lyophilized samples 105 genes with a GO term "phosphorylation", of which 53 were annotated as "kinases", and also 41 genes with a GO term "cell communication" appeared to be increased. It seems that *P. capsici* is capable of activating signal transduction cascades in response to lyophilization. Reactivation of transcriptional machinery likely happens when samples in the vacuum chamber are thawed during lyophilization. Again, the effect of lyophilization, which is evident from microarray mRNA profiling, cannot be detected by conventional quality control measures with certainty.

Thawed samples also showed a large alteration in the transcriptome. A cryoinjury of the cell is likely responsible for the shift of mRNA profiles. During freezing, formation of intracellular ice and shrinkage of cells occur in various ascomycete and oomycete species (Morris *et al.*, 1988), which lead to loss of integrity of cell membrane, organelles, and thus viability. GO

enrichment analysis showed that in response to freeze and thaw, genes for microtubule motors were activated. Lyophilized samples, which were also likely to have incurred cryoinjury, showed activation of genes for microtubule motors. Taken together, activation of microtubule motor might be associated with cell repair.

In summary, we demonstrated that a transcriptome is extremely sensitive to RNA extraction protocols. A brief thawing of samples on ice or in contact with extraction buffer or lyophilization process can significantly alter mRNA profiles. We have analyzed mRNA profiles for only the oomycete *P. capsici*, however, such alteration in transcriptomes is likely to occur for the ascomycete *N. crassa* as well as diverse organisms e.g. bacteria, plants and animals. We recommend the use of a mortar and pestle or frozen-phase cell-disruption method unless application of RNAlater or TRIzol-submerged cell disruption method is proven safe by means of global mRNA profiling.

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