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ORIGINAL PAPER

Influence of mitochondria on gene expression in a citrus cybrid

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Abstract The production of cybrids, combining nucleus of a species with alien cytoplasmic organelles, is a valuable method used for improvement of various crops. Several citrus cybrids have been created by somatic hybridization. These genotypes are interesting models to analyze the impact of cytoplasmic genome change on nuclear genome expression. Herein, we report genome-wide gene expression analysis in leaves of a citrus cybrid between *C. reticulata* cv 'Willowleaf mandarin' and *C. limon* cv 'Eureka lemon' compared with its lemon parent, using a *Citrus* 20K cDNA microarray. Molecular analysis showed that this cybrid possesses nuclear and chloroplast genomes of Eureka lemon plus mitochondria from Willowleaf mandarin and, therefore, can be considered as a lemon bearing foreign mitochondria. Mandarin mitochondria influenced the expression of a large set of lemon nuclear genes causing an over-expression of 480 of them and repression of 39 genes. Quantitative real-time RT–PCR further confirmed the credibility of microarray data. Genes over-expressed in cybrid leaves are predominantly attributed to the functional category "cellular protein metabolism" whereas in the down-regulated none functional category was enriched. Overall, mitochondria replacement affected different nuclear genes including particularly genes predicted to be involved in mitochondrial retrograde signaling. Mitochondria regulate all cell structures even chloroplast status. These results suggest that nuclear gene expression is modulated with respect to new information received from the foreign organelle, with the final objective to suit specific needs to ensure better cell physiological balance.

Keywords Citrus · Cybrid · cDNA microarrays · Gene expression · Transcriptome

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Introduction

Organelles may alter the expression of nuclear genes through a communication termed retrograde signalling. Cytoplasm substitution could have positive effects on important agronomic traits such as cytoplasmic male sterility (CMS) (Prakash et al. 2001; Yamamoto et al. 1997), drought tolerance (Uprety and Tomar 1993), improved disease and pathogen resistance (Banga et al. 1984; Voluevich and Buloichik 1992), starch production (Lossl et al. 2000). On the other hand, substitution of the cytoplasm may manifest abnormal morphology of stems, leaves and flowers (Leino et al. 2003; Newton et al. 2004; Pelletier and Budar 2007), reduced cold tolerance and lack of chlorophyll (Kato et al. 1990; Zubko et al. 2001). Various studies showed that new nucleo–cytoplasmic interactions resulting from cytoplasm replacement, rearrangements or cytoplasmic mutations would be behind these particular phenotypes (Hanson 1991; Allen 2005; Bogdanova 2007).

The interactions between cell genomes represent an important regulatory response mechanism in higher plants (Zubko et al. 2001). However, little attention has been paid to retrograde signalling, and nucleo–cytoplasmic interactions remain poorly understood in higher plants (Hanson 1991; Babiychuk et al. 1995; Atienza et al. 2007).

Microarrays represent a powerful tool to investigate expression of thousands of genes and permits comparison of different biological situations (Aharoni and Vorst 2002). Indeed, compared to other techniques evaluating gene expression (Northern blot, RT–PCR), microarrays permit to work on a larger scale favoring a simultaneous analysis of all transcripts of a genome.

Microarray studies on nucleo–cytoplasmic interactions have been accomplished to get new insights into the mechanisms underlying CMS (Carlsson et al. 2007; Kang et al. 2008). The results showed changes in expression profiles of a wide range of nuclear genes involved in the appearance of the sterility phenotype. Carlsson et al. (2007) observed that nuclear genes coding for proteins involved in the import of proteins into organelles, genes playing a role in "energy" and "protein metabolism", as well as genes involved in cell architecture, are modulated in CMS line compared to *Brassica napus*. The mitochondrial mutant of *Nicotiana sylvestris* also shows a change in the expression of nuclear genes, including overexpression of genes involved in oxidative stress response, namely alternative oxidase (AOX), ascorbate peroxidase (APX) and superoxide dismutase (SOD), and also a very active NAD (P) H dehydrogenase, suggesting increased expression of genes coding for this enzyme (Dutilleul et al. 2003). Yu et al. (2001) analyzed the nuclear gene expression in response to inhibition of the mitochondrial electron transport by antimycin A in *Arabidopsis thaliana* and concluded that the responses are in part mediated by retrograde signalling. It is evident that the mitochondrial genome strongly influences the expression of nuclear genes but, to our knowledge, only the specific analysis of this influence, in particular, biological processes such as CMS or electron transport has been accomplished so far. In contrast, there are no reports on the overall extent of changes in the nuclear genome after mitochondria substitution/replacement, which may explain variable observed phenotypic characters.

Cybrids, combining nucleus of a species with alien cytoplasmic organelles, constitute a very valuable material to understand the effect of nucleo–cytoplasmic interaction at the molecular level. They can be obtained by sexual interspecific crosses followed by recurrent backcrosses or by somatic hybridization. The last method permits to generate new nucleo–cytoplasmic associations without any nuclear modification; somatic cybrids are thus the ideal material to analyze the impact of cytoplasmic genome change on genome expression.

The production of cybrids is used for improvement of various crops such as citrus (Grosser et al. 2000; Guo et al. 2004). For this species which is highly heterozygous, somatic cybridization constitutes a potential approach to breed citrus cultivars for specific cytoplasmic traits, such as seedlessness or tolerance traits, as well as to slightly modify phenological or quality traits for cultivar diversification (Saito et al. 1993; Yamamoto and Kobayashi 1995; Grosser et al. 1996; Moriguchi et al. 1996; Ollitrault et al. 1996; Moriguchi et al. 1997; Moreira et al. 2000; Ollitrault et al. 2000; Tusa et al. 2000; Cabasson et al. 2001; Guo et al. 2004; Cai et al. 2007, 2009). They have generally been obtained as chance product in symmetric hybridization but recently method such as cytoplast fusion has been implemented for cybrid production (Guo et al. 2004; Xu et al. 2006).

Several studies have examined the phenotypic changes in citrus cybrids. It has been shown that cybridization can have an impact on aroma. Fanciullino et al. (2005) found that, for volatile compounds of leaves, the cybrids were very close to their nucleus-giving parent; however, some nucleo–cytoplasmic interactions occurred, such as the synthesis of more monoterpene alcohols and sesquiterpene in the kumquat and clementine cybrid. Tusa et al. (2000) evaluated the resistance to "mal secco" in cybrids between Valencia orange and Femminello lemon. The low mortality observed in cybrids after inoculation, suggests that specific mechanisms of tolerance to "mal secco" could be activated. The evaluation of orange and mandarin cybrids showed positive variation for important agronomic traits such as maturity date and the number of seeds (Grosser et al. 2000). In a previous work on indicators of fruit taste, nutritional and organoleptic quality, we showed that cybridization affected organic acid content although no modification was observed for sugar and carotenoid content (Bassene et al. 2008). A recent leaf proteomics analysis from a pummelo cybrid with Satsuma mandarin mitochondria suggested an alteration in photosynthesis, stress resistance, and metabolism (Wang et al. 2010). Given the available biological resources, citrus constitute one of the few models of woody perennial species to address nucleo–cytoplasmic interaction study.

In this work, we have used the cybrid *C. reticulata* cv 'Willowleaf' + *C. limon* cv 'Eureka' (WLM + EUR) to explore the effect of mitochondria substitution in the regulation of nuclear gene expression. A previous deep molecular analysis has shown that this cybrid possess nuclear and chloroplast genomes of Eureka lemon plus mitochondria from Willowleaf mandarin (Bassene et al. 2008) and, therefore, can be considered as a lemon bearing foreign mitochondria. To get an overview of the molecular

effects associated to the new nucleo–mitochondrial interaction, we analyzed variation in global gene expression between WLM + EUR and Eureka lemon using a genome-wide 20K cDNA microarray.

Materials and methods

Plant material

The experiment was conducted on leaves of Eureka lemon [*Citrus limon* (L.) Burm.] and a cybrid called WLM + EUR produced by symmetric protoplast fusion between Willowleaf mandarin (*Citrus reticulata* Blanco) and [*Citrus limon* (L.) Burm.] (Ollitrault et al. 2000). For both genotypes, mature budwood was grafted in four replicate onto volkameriana rootstock (*Citrus limonia* Obs.). Trees were randomly planted at the same time in the same homogeneous field plot at the Station de Recherche Agronomiques (INRA/CIRAD) in San Giuliano (Corsica, France) in 2002 and subjected to same standard cultivation practices. For the experiment, fully expanded leaves from the last finished sprouting branches were harvested. The effects of tree and branch position were minimized by random sampling.

RNA isolation and labeling

Total RNA was extracted from nitrogen-powdered fully expanded leaves according to the protocol described by Ancillo et al. (2007). Forty micrograms of RNA was labeled by indirect method (Randolph and Waggoner 1997). Reverse transcription, cDNA purification, dye coupling, and fluorescent cDNA purification were accomplished as formerly done by Ancillo et al. (2007).

Microarray hybridization and scanning

A genome-wide 20K cDNA microarray, including 21,081 putative unigenes of citrus (Martinez-Godoy et al. 2008) and developed under the Citrus Functional Genomics Project (CFGP; http://bioinfo.ibmcp.upv.es/genomics/cfgpDB), was used. Microarray hybridization and washing were performed as described by Martinez-Godoy et al. (2008). Four hybridizations (biological replicates) were made, with two of them reverse-labeled (dye switch) with respect to the other two, to correct for dye bias. Afterwards arrays were scanned at 532 nm for the Cy3 and 635 nm for the Cy5 dyes, with a GenePix 4000B scanner (Axon Molecular Devices, Sunnyvale, CA, USA), at a 10 nm resolution and 100% laser power. Photomultiplier tube voltages were adjusted to equal the overall signal intensity for each channel, to increase signal-to-noise ratio and to reduce the number of spots with saturated pixels. Spot intensities were quantified using GenePix Pro 6.0 (Axon Molecular Devices). Spots with a net intensity in both channels below twofold the mean background intensity were considered low-signal spots and removed.

Micro array data analysis

Data were global median normalized using GenePix Pro 6.0 (Axon Molecular Devices) so that the median of the median ratios of every valid spot in the slide was equal to 1. Only probes with valid data in all slides were considered for further analysis (15,694 spots). To detect differentially expressed genes, data were analyzed with the significance analysis of microarray package (Tusher et al. 2001) using two-class unpaired comparison with a false discovery rate (FDR) of 1.7% with no fold change cut-off. The differentially significant expressed gene lists were further analyzed using Blast2GO (Conesa et al. 2005) to find differential distributions of the gene ontology term.

To each putative unigene present on the microarray slide is allocated an AGI name corresponding to the Arabidopsis gene with which it has a high sequence similarity. We used those codes to import differentially expressed genes into the "Mapman visualization tool" (Thimm et al. 2004), which enables simpler and visual enhanced analysis.

Real-time PCR

Real-time PCR (qPCR) amplifications and measurements were performed using an ABI PRISM 7000 sequence detection system and SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, California).

Expression of four selected genes was estimated by quantitative real-time RT–PCR. Primer pairs for each gene were designed based on the corresponding sequences available in the database of the CFGP (http://bioinfo.ibmcp.upv.es/genomics/cfgpDB) and are listed in Table 1. All PCRs were carried out on three different samples and for each one, the experiments were done in triplicate, and means were calculated.

Results

Microarray analysis

To isolate genes differentially expressed in leaves of the cybrid WLM + EUR compared with its lemon parent, a cDNA microarray containing 21,081 putative unigenes was used to analyze their genomes expression profile. Total RNA was extracted from leaves, amplified, labeled, and hybridized to the microarray. Four biological replicates were

Table 1 Changes in gene expression estimated by microarray hybridization and by quantitative real-time RT–PCR

Gene ID	Best blast hit	Primers(1)	Fold change average in RT–PCR(2)	Fold change average in microarray(3)
C04011B01	Aconitase-iron-regulated protein 1	5′-CAGGCTCAGGAGTTGTTACT-3′	3.45	2.9
		5′-GCAGATGCACAGATTTCATCC-3′		
C31101A02	Ribulose bisphosphate carboxylase large chain precursor	5′-CAGTCAATACGTTGCGAACA-3′	2.63	1.85
		5′-TCACTCGTGAAAGAACTGAA-3′		
C02009D08	Putative salt-inducible protein	5′-GTGAAGCTCGTGATGTTTTC-3′	0.42	0.51
		5′-TCTTTACATTGCAAGGCATC-3′		
C20003E08	Ferritin-3, chloroplast precursor	5′-GTGGTCGCTTTTAGATAGTC-3′	5.11	4.41
		5′-CAGGGCAGAAACAGAACTTT-3′		

(1) Primer pair used for gene expression analysis by QRT–PCR, (2) Average fold change of three independent RT-PCR reactions for three independent samples for each treatment, (3) Average fold change of the mean of expression values of four replicas in the microarray

analyzed per species. After quality analysis, 15,694 spots (unigenes) were considered as valid for further analysis.

In total, 519 differentially expressed genes (~3.3%) were revealed as statistically significant with a FDR of 1.7%. Among the 519 genes differentially expressed, 480 were over-expressed in the cybrid compared to lemon while 39 genes were repressed (Supplementary Table 1 additional file). To perform a sequence distribution study, we compare the significant differentially expressed gene list, linked to their functional annotation provided by the Gene Ontology Consortium, to the whole microarray gene set taken as a reference, by applying the Seq Distribution/GO implemented in Blast2GO (Conesa et al. 2005).

Among the genes over-expressed in WLM + EUR, the genes attributed to the functional category "cellular protein metabolism" are among the most highly represented (about 40%) (Fig. 1). Genes in this functional class were found significantly enriched. The other genes are equally distributed in the categories "translation" (about 18%), "transcription" (15%), "regulation" (15%) and "RNA metabolic process" (12%). The class "cellular protein metabolism" is particularly represented by genes that encode ribosomal proteins. The analysis of the cellular localization of proteins encoded by genes over-expressed shows that they are not preferentially located in mitochondria (Fig. 2). All cell structures were affected even genes targeted to chloroplasts and implicated in light reactions and Calvin cycle.

Among the 39 genes repressed in leaves of WLM + EUR compared to Eureka lemon, no functional category was highly represented. Gene enrichment for biological processes shows that no functional class is significantly enriched among the genes repressed in the cybrid.

To provide a comprehensive analysis of cybrid response to the lemon mitochondria substitution, differentially expressed genes were imported into the "Mapman visualization tool" (Thimm et al. 2004), which permits genes to be represented in graphic metabolic pathways with color-coded expression level. Overall, we observed that altered cybrid gene expression depict genes predicted to be involved in mitochondrial retrograde regulation (MRR), such as genes implicated in heat shock (C34001C10, IC0AAA26DH06, C31802C03), pathogen sensing, Ca^{2+} signaling (C34201F01, C08011H05, C31403G05), protein kinases (C06020H08), and nuclear transcription factors (C05076B02 a bZIP, IC0AAA85BB09 a WRKY), are particularly represented in modulated genes (Fig. 3).

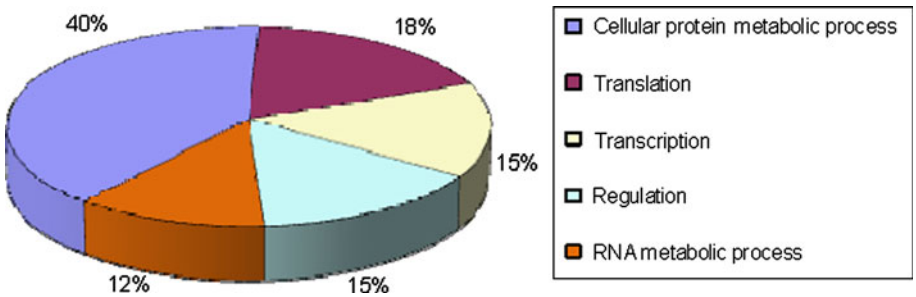

Fig. 1 Classification of genes over-expressed in WLM + EUR cybrid leaves compared to Eureka lemon in biological process GO categories

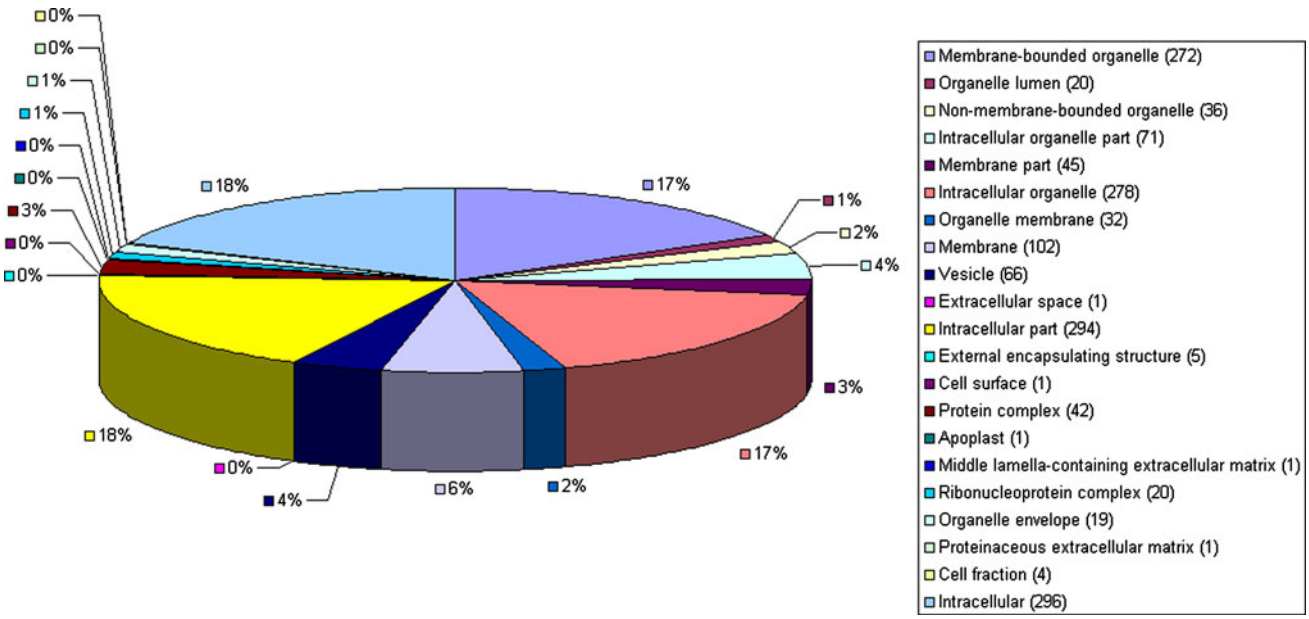

Fig. 2 Classification of genes over-expressed in WLM + EUR cybrid leaves compared to Eureka lemon in cellular GO categories

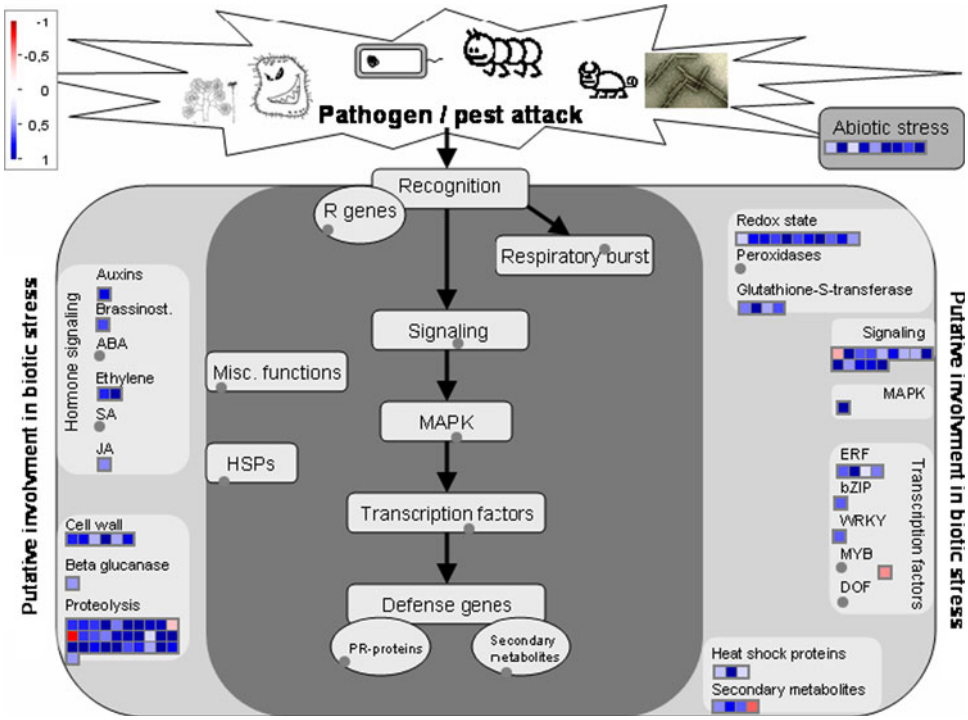

Fig. 3 View of expression of genes putatively involved in stress response. Transcripts with increased abundance in WLM + EUR compared to lemon are in *blue* and those with decreased abundance in *red*

Real-time RT–PCR validation of microarray data

To validate microarray results, differential expression of some genes was confirmed by quantitative real-time RT–PCR using the same RNA preparations analyzed in hybridization experiments (Table 1). The relative accumulation of mRNAs matched that observed by microarray hybridization, although changes detected were not quantitatively identical. This variation is commonly observed in the validation of microarray results by RT–PCR (Allen and Nuss 2004; Lopez et al. 2005) and may be attributed to intrinsic differences between both techniques.

Discussion

Transcriptional differences between the Eureka lemon and WLM + EUR cybrid

It has been shown that modification of mitochondrial genome by mutation or substitution with a foreign one (formation of cybrids or alloplasts) can cause nuclear gene expression changes (Zubko 2004; Rhoads and Subbaiah 2007; Carlsson et al. 2008). This regulation of nuclear gene expression by the mitochondrial genome, called "retrograde signaling", allows the organelles to coordinate their function with the nucleus (Butow and Avadhani 2004; Liu and Butow 2006; Rhoads and Subbaiah 2007; Woodson and Chory 2008). Some microarray studies reveal that an important number of genes change by the effect of this retrograde signaling, but these analyses were mainly focused on specific processes such as CMS or electron transport (Yu et al. 2001; Carlsson et al. 2007; Kang et al. 2008). So, there was an obvious need to examine the overall extent of changes in the nuclear genome after mitochondria replacement. In the present work, we have addressed this question by analyzing the changes in the transcriptome of the cybrid *C. reticulata* cv 'Willowleaf' + *C. limon* cv 'Eureka' (WLM + EUR) with respect to the lemon parent.

Analysis of the cybrid gene expression profile using cDNA microarrays revealed the existence of transcriptional changes compared to the lemon parent. About 3.3% of 15,694 genes analyzed showed changes in their expression patterns. Knowing that the cybrid differs from lemon in its mandarin mitochondria, excluding any somaclonal alteration, which may happen during in vitro culture, these transcriptional changes may be related to the nucleo–mitochondrial interaction. This result is consistent with previous observations in CMS line of *Brassica* (Carlsson et al. 2007). In this previous microarray analysis, they identified a large number of genes differentially expressed in CMS line compared to *B. napus*, which shows that presence of *A. thaliana* recombined mitochondrial DNA in the CMS line strongly influences nuclear gene expression. Therefore, presence of mandarin mitochondria in the cybrid influences the expression of many lemon nuclear genes by causing an over-expression of a large number of them (480 genes) and repression of a more reduced set of genes (39 genes).

Genes over-expressed in cybrid leaves are predominantly involved in protein metabolism

Functional classification of genes up-regulated shows that they are represented in various functional categories. Nevertheless, genes attributed to the functional category "cellular protein metabolism" (about 40%) are the most abundant and the more represented class. The rest are mainly related to transcription, translation, regulation, and RNA metabolism (about the remaining 60%). Regulation of RNA and protein metabolism has a crucial role in several determinant processes in plant development as cell cycle, active growth, aging, or germination efficiency (Rost and Van't Hof 1973; Verma and Marcus 1973; Reuzeau and Cavalie 1997; Mehta et al. 2010; Rattan 2010), but in addition, it appears to have an utmost significance in regulating homeostasis in the adaptive response of the plant to several abiotic stresses such as cold or high salinity (Sahi et al. 2006; Zhu et al. 2007). There seems to exist a coordinated action of ribosomal proteins, RNA binding proteins, and translation and elongation factors along with several accessory proteins that regulate stress-associated translation in controlling various cellular adaptations during the stress response. Different genomic and proteomic analysis on protein localization show that organelles could contain up to several thousands of different proteins (Andersson et al. 2003; Richly and Leister 2004). However, the majority of these proteins (93–99%) are encoded in the nucleus, synthesized in the cytoplasm and then imported into the organelles (Woodson and Chory 2008). This may explain the fact that new nucleo–mitochondrial interaction, by MRR, causes change in expression profiles of nuclear genes involved in protein metabolism to suit the specific needs of the foreign organelle and ensure better physiological balance. Nuclear gene expression is modulated with respect to the new information received from the foreign mitochondria, hence the need of regulations to achieve a new homeostasis in cybrid cells. We may assume that the substitution of lemon mitochondria with that of mandarin during somatic hybridization created disturbance of whole cellular balance, which induces altered gene expression of several functional categories compared to lemon.

Altered cybrid gene expression depict genes putatively involved in MRR

The mitochondria replacement affected different nuclear genes including particularly genes predicted to be involved in mitochondrial retrograde signaling. Among altered genes, we found several that encode glutathione S-transferase (GST), SOD, APX, mitochondrial respiratory chain, and the ascorbate glutathione pathway. GST family members are cytosolic dimeric proteins involved in cellular detoxification of xenobiotics and hydroperoxides (Cholon et al. 1992). Herein, four GST genes (C19005A01, C31502H06, C31303F07, and C04013C01) were up-regulated in cybrid leaves compared to lemon, suggesting an enhanced anti-oxidative activity. The retrograde signaling is known to be effected by metabolic cues such as reactive

oxygen species (ROS) and heat shock proteins (Butow and Avadhani 2004; Liu and Butow 2006; Rhoads and Subbaiah 2007). Cellular response to these dysfunctions then entails the induction of genes encoding proteins involved in cellular adjustments of altered mitochondrial state, such as AOXs and NAD (P) H dehydrogenase, genes coding for enzymes which regulate ROS and redox level (such as glutathione transferase, catalase, APX, …) (Rhoads and Subbaiah 2007; Woodson and Chory 2008). In addition to GST, a picture of MRR in the cybrid leaves is reinforced by over-expression of the Cu/ZnSOD C32013F11, the dehydroascorbate reductase C31402E11 associated with ascorbate–glutathione cycle and the APX C31810H06. The latter enzyme is an important H2O2-detoxifying system in plant cells (Foyer et al. 1994). The foreign mitochondria also affected genes of mitochondrial respiratory chain (C02006G08, C06017D02, C34207A11), heat shock (C34001C10, IC0AAA26DH06, C31802C03), Ca^{2+} signaling (C34201F01, C08011H05, C31403G05), protein kinases (C06020H08) and nuclear transcription factors (C05076B02 a bZIP, IC0AAA85BB09_a WRKY). Ca^{2+} signalling, HSP, protein kinases, and nuclear transcription factors are also predicted to be involved in MRR (Rhoads and Subbaiah 2007; Woodson and Chory 2008). Compounds that react with ROS, may act as primary or secondary signals and may interact with other signaling components, such as kinases and transcription factors. In general, WRKY, bZIP, and Dof transcription factors are good candidates to be involved in plant MRR because they are involved in biotic and abiotic stresses responses, including oxidative stress (Rhoads and Subbaiah 2007). Since Eureka lemon and WLM + EUR were in the same field, subjected to the same cultivation practices and no particular stress were observed for overall plants, these genes variations could be attributed to the effect of the foreign mitochondria on lemon nuclear genome. Our microarray results are consistent with those observed in a recent leaf proteome study from a pummelo cybrid with Satsuma mandarin mitochondria (Wang et al. 2010). The differentially expressed spots were mainly involved in photosynthesis, stress response, anti-oxidative stress, and metabolism.

MRR affected whole cell structures

When altered genes are classified by activity in cellular compartment, it is worthy to notice that the mitochondria substitution affects all cell structures in WLM + EUR. Proteins encoded by modulated genes are widely distributed at the cellular level (Fig. 2). Similar observation was made through induced mitochondria respiration deficiency (Yu et al. 2001). Within the hundreds nuclear genes affected, many of them encode proteins targeted for various cellular compartments including the cytoplasm, nucleus, plastid, and mitochondrion. Herein, the cybrid shows altered expression of genes involved in the Calvin cycle and light reactions, which take place in chloroplast stroma and thylakoids, respectively. In *Chlamydomonas reinhardtii*, the activation of the mitochondria respiratory cytochrome causes increased expression of nuclear genes related to photosynthesis (Matsuo and Obokata 2006). Various studies of mitochondrial and chloroplast mutants have highlighted the control that mitochondria can have on chloroplast function, and vice versa, called "cross talk" (Newton and Coe 1986; Sabar et al. 2000; Newton et al. 2004; Priault et al. 2006). Maize mitochondrial mutant (non-chromosomal stripe) inherits abnormal chloroplast development caused by the mitochondrial mutations (Newton et al. 2004), although the reason is still unknown. There is evidence that in plant cells, mitochondria and chloroplasts have complex forms of metabolic interactions. During photosynthesis, chloroplasts use mitochondrial products (CO_2 and ATP) and provide compounds to mitochondrial respiration (O_2 and malate) (Raghavendra and Padmasree 2003). It is evident that mitochondria replacement caused change in the existing homeostasis between the two organelles with change in redox state (highlighted by the anti-oxidative activity noticed above), which in return affected the chloroplast status as other cell parts (e. g. nucleus, ribosome, plasma membrane, …). In cotton mutant CMS II, the loss of electron transport complex I function revealed effective antioxidant crosstalk and acclimation between the mitochondria and other organelles to maintain whole cell redox balance (Dutilleul et al. 2003).

Conclusion

Large-scale profiling of gene transcripts has allowed us to get a glimpse of the sweeping changes in the plant transcriptome that take place upon mitochondria substitution. Our results involve protein metabolism, and RNA processing and metabolism in the regulation of nuclear gene expression by the mitochondrial genome. The elucidation of the underlying mechanisms demands further investigation and becomes an exciting challenge for the future

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