Pedro Paulo Augusto Fabiano Arantes Pereira Barreto

Alterações no metabolismo energético provocadas pela superexpressão da proteína desacopladora mitocondrial 1 (UCP1) em tabaco induzem biogênese mitocondrial e resposta global a estresses

Alterations on energy metabolism caused by mitochondrial uncoupling protein 1 (UCP1) overexpression in tobacco induce mitochondrial biogenesis and global stress response

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ALTERAÇÕES NO METABOLISMO ENERGÉTICO PROVOCADAS PELA SUPEREXPRESSÃO DA PROTEÍNA DESACOPLADORA MITOCONDRIAL 1 (UCP1) EM TABACO INDUZEM BIOGÊNESE MITOCONDRIAL E RESPOSTA GLOBAL A ESTRESSES

ALTERATIONS ON ENERGY METABOLISM CAUSED BY MITOCHONDRIAL UNCOUPLING PROTEIN 1 (UCP1) OVEREXPRESSION IN TOBACCO INDUCE MITOCHONDRIAL BIOGENESIS AND GLOBAL STRESS RESPONSE

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos necessários à obtenção do título de Doutor em Genética e Biologia Molecular, na área de Bioinformática.

Thesis presented to the Institute of Biology of the State University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Genetics and Molecular Biology, on the area of Bioinformatics.

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RESUMO

A proteína desacopladora mitocondrial 1 (UCP1) é uma proteína mitocondrial codificada pelo núcleo capaz de desacoplar o gradiente eletroquímico usado para a síntese de ATP, dissipando a energia na forma de calor. A descoberta de homólogos e ortólogos da UCP1, sugere outros papéis fisiológicos para estas proteínas. As UCPs podem servir como uma válvula de escape, diminuindo a força protonmotiva (PMF) e reduzindo a produção de ROS em condições desfavoráveis. Plantas superexpressando UCPs se desenvolvem melhor quando submetidas a estresses bióticos e abióticos. Estas plantas demonstraram diminuição na produção de ROS, alteração no estado redox celular, além de um aumento no metabolismo energético e na fotossíntese. Neste trabalho nós investigamos os mecanismos moleculares envolvidos no metabolismo energético celular e resposta a estresses em plantas de tabaco superexpressando a UCP1 de *A. thaliana*. Demonstramos, através de análises moleculares e genômicas, que a superexpressão da UCP1 é capaz de provocar o aumento na respiração desacoplada em mitocôndrias isoladas, diminuir o conteúdo de ATP intracelular, e desencadear um processo de sinalização retrógrada que resulta na indução de genes mitocondriais e genes responsivos a estresses. Esta sinalização retrógrada resultou na indução do processo de biogênese mitocondrial verificado pelo aumento no número e área mitocondrial por célula, além de alterações morfológicas nestas organelas. O processo de biogênese mitocondrial nestas plantas é acompanhado pelo aumento na expressão de um grande número de genes responsivos a estresses, o que resulta no melhor desempenho e reduzida produção de ROS mitocondrial quando submetidas a estresses abióticos. A análise detalhada do transcriptoma de plantas superexpressando UCP1 em comparação com plantas selvagens demonstrou uma forte conexão entre os metabolismos mitocondrial, citoplasmático e cloroplástico para compensar as alterações provocadas pelo aumento na atividade da UCP1. Um grande número de fatores de transcrição ainda não caracterizados foram identificados e podem representar bons alvos para investigações futuras a respeito da regulação da biogênese mitocondrial e do metabolismo energético em plantas. Os resultados contidos nesta tese nos permitem melhor compreender a flexibilidade do metabolismo energético em plantas e identificar possíveis reguladores do processo de biogênese mitocondrial e resposta a estresses em plantas.

ABSTRACT

The mitochondrial uncoupling protein 1 (UCP1) is a nuclear-encoded mitochondrial protein capable of uncouple the electrochemical gradient used for ATP synthesis, dissipating energy as heat. The discovery of UCP1 homologues, and its corresponding orthologues suggest diverse physiological functions for these proteins. UCPs may serve as an escape valve, decreasing the proton motive force (PMF) and preventing ROS production under unfavorable conditions. Plants overexpressing UCPs perform better under biotic and abiotic stresses. These plants show diminished ROS production, alteration of cell redox homeostasis, increased energy metabolism and photosynthesis. In this work we investigated the molecular mechanisms underlying cell energy metabolism and stress response in tobacco plants overexpressing an Arabidopsis thaliana UCP1. We demonstrated through molecular, cellular and genomic tools that UCP1 overexpressing plants is capable of increasing uncoupled respiration of isolated mitochondria, decrease intracellular ATP levels, and trigger a retrograde signaling that resulted in a broad induction of mitochondrial and stress response genes. The retrograde signaling resulted in the induction of mitochondrial biogenesis verified by increased mitochondrial number, area and alterations on mitochondrial morphology. The increased mitochondrial biogenesis in these plants accompanied by the broad increase in the expression of stress responsive genes, may be responsible for the diminished ROS production and the better performance of these plants when submitted to several abiotic stresses. We also performed a detailed analysis of the transcriptome expression of the UCP1 overexpressing plants as compared with the wild type plants. We verified that the UCP1 overexpressing plants exhibited a tight connection between mitochondrial, cytoplasm and chloroplast energy metabolism to accommodate the alterations caused by the increased UCP1 activity. A number of uncharacterized transcription factors seem to be good targets for future investigations on the regulation of plant mitochondrial biogenesis and energy metabolism. The results presented in this work allowed a better understanding of the flexibility of energy metabolism in plants, and the use of this mechanism to identify possible regulators of plant mitochondrial biogenesis and stress response.

SUMÁRIO

A persistência é o caminho do êxito.

(Charles Chaplin)

O objetivo primordial e necessário de toda a sobrevivência deve ser a felicidade.

(Robert Owen)

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INTRODUÇÃO GERAL

A mitocôndria e o metabolismo energético

Entre as vantagens evolutivas presentes em células eucarióticas, a compartimentalização está entre as mais importantes [1]. A localização de determinada via metabólica dentro de um compartimento especializado permite um aumento na eficiência das reações, principalmente pela propiciar uma maior concentração de determinados substratos e enzimas, excluir solutos indesejáveis, e diminuir a distância física entre as reações [2]. Neste sentido, as mitocôndrias são compartimentos celulares presentes na maioria dos organismos eucarióticos especializados em suprir a demanda celular por energia [3].

As principais vias metabólicas localizadas nas mitocôndrias são o Ciclo de Krebs e a Fosforilação Oxidativa (OXPHOS). Enquanto em mamíferos a β-oxidação também ocorre na mitocôndria, este processo, em plantas, é realizado majoritariamente pelos peroxissomos [4]. O metabolismo celular de substratos como glicose e ácidos graxos leva a produção de elétrons na forma de NADH e $FADH_2$, que podem ser produzidos no citoplasma e subsequentemente transportados para a mitocôndria, ou gerados pelas reações do Ciclo de Krebs [5]. Estas moléculas são posteriormente utilizadas por um complexo proteico localizado na membrana interna mitocondrial, denominado Cadeia de Transporte de Elétrons (ETC), que utiliza os elétrons doados pelo NADH e FADH2, assimilando-os a uma molécula de oxigênio molecular (O_2), e produzindo H_2O. Enquanto os elétrons fluem pela ETC, um número determinado de prótons (H+) é transportado da matriz mitocondrial para o espaço intermembrana, estabelecendo um gradiente de prótons através da membrana interna mitocondrial [6]. O gradiente de prótons estabelecido é usado para a síntese de ATP a partir de ADP e fósforo inorgânico (Pi) pelo complexo proteico da ATP sintase, enquanto os prótons são transportados novamente para a matriz mitocondrial [5,6]. Este processo é denominado respiração acoplada, pois o fluxo de prótons para o espaço intermembrana mediado pela ETC está acoplado à reentrada de prótons para a matriz mitocondrial pela ATP sintase. As moléculas de ATP são produzidas, transportadas e utilizadas de acordo com a demanda energética celular.

A demanda energética varia não só entre organismos, mas também entre diferentes tipos celulares e estágios de desenvolvimento [7]. Em plantas, diversos tecidos florais, especialmente nas anteras durante a maturação do grão de pólen, há um grande aumento na demanda por ATP, sendo necessário um aumento na expressão de proteínas mitocondriais que atuam no metabolismo energético, e aumento do numero destas organelas por célula [8-10]. Por outro lado, no tecido adiposo marron (BAT) de alguns mamíferos, o aumento no número de mitocôndrias ocorre em virtude não do aumento na demanda por ATP, mas sim pela necessidade de dissipar energia na forma de calor [11,12]. Novas mitocôndrias surgem a partir da divisão de organelas preexistentes em um mecanismo dinâmico de fusão e fissão, onde mitocôndrias acumulam proteínas e DNA mitocondrial (mtDNA), crescem em tamanho, se fundem a outras mitocôndrias, e posteriormente se dividem [3,13]. Estes processos constituem o ciclo de vida mitocondrial (Figura 1), sendo necessários para a homogeneização do conteúdo mitocondrial através da célula e a remoção de organelas danificadas pelo processo de mitofagia [13]. Células quando expostas à privação, ou excesso de nutrientes, ou quando submetidas a estresses abióticos demonstraram alterações neste ciclo dinâmico, fazendo com que as mitocôndrias passem mais ou menos tempo em cada uma destas etapas ou alterem sua morfologia [13-15].

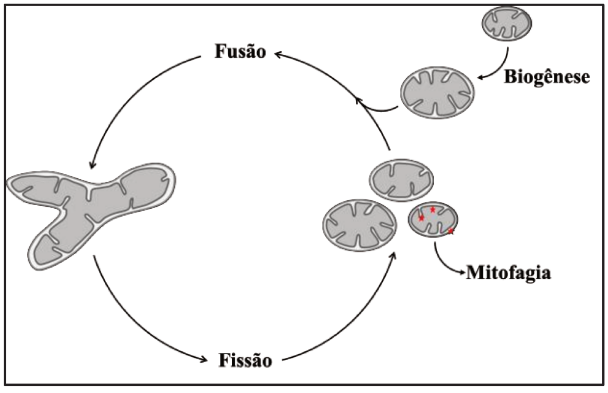

Figura 1. O Ciclo de Vida Mitocondrial. Eventos de fusão e fissão permitem a complementação funcional e genética das mitocôndrias presentes na célula. O processo de síntese de proteínas mitocondriais (biogênese) deve compensar as mitocôndrias eliminadas pelo processo de mitofagia. Um desbalanço entre os eventos de fusão, fissão, biogênese e mitofagia podem causar mudanças substanciais no número, massa, forma e função das mitocôndrias.

O processo de respiração aeróbica tem como consequência inevitável a formação de moléculas extremamente tóxicas denominadas espécies reativas de oxigênio (ROS) [2,6,16,17]. Estas moléculas são produzidas quando elétrons utilizados pela ETC

escapam antes da última etapa do processo de fosforilação oxidativa, reagindo prematuramente com O_2. Levando-se em conta que a mitocôndria é responsável por até 90% do consumo de oxigênio celular, ela é, também, a principal produtora de ROS [2,6,16]. O efeito negativo da compartimentalização da produção de ROS pode ser verificado pela taxa de mutações no DNA mitocondrial (mtDNA), que chega a ser 10 vezes superior à do DNA genômico [2]. No entanto, as mitocôndrias também apresentam a maior capacidade antioxidante entre os compartimentos celulares [17], o que torna estas organelas não apenas compartimentos celulares responsáveis pela produção de energia, mas também por compartimentalizar a produção e a detoxificação de ROS, evitando possíveis danos a outros ambientes celulares, especialmente ao DNA genômico. Por estas razões é fundamental a coexistência de um aparato responsável pela detoxificação de ROS em conjunto com a maquinaria responsável pelas reações do metabolismo energético.

O papel das proteínas desacopladoras mitocondriais

As proteínas desacopladoras mitocondriais (UCPs) localizam-se na membrana interna da mitocôndria e são capazes de, na presença de ácidos graxos, desacoplar a respiração, facilitando a reentrada de prótons na matriz mitocondrial sem a concomitante produçao de energia na forma de ATP [18] (Figura 2).

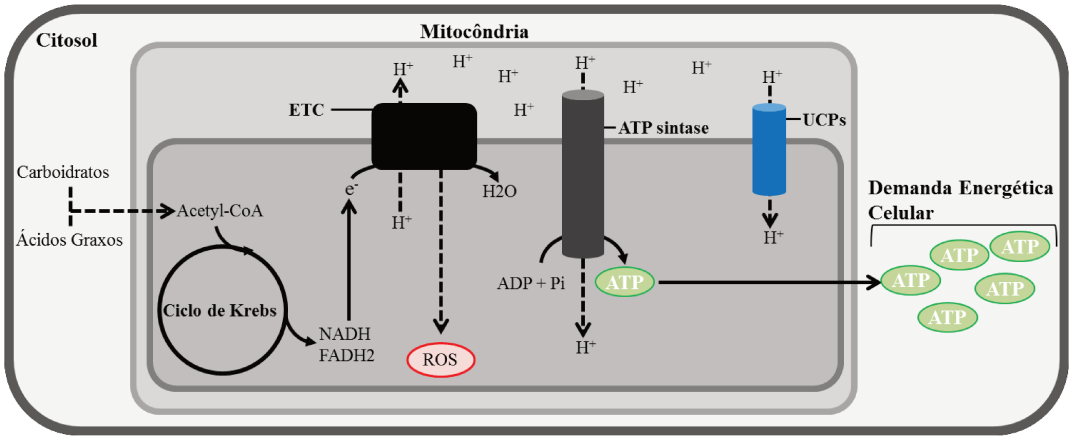

Figura 2. As UCPs são capazes de desacoplar a respiração. O metabolismo celular de carboidratos e ácidos graxos gera elétrons na forma de NADH e FADH2. Estes elétrons são utilizados pela ETC, enquanto prótons são bombeados para o espaço intermembrana, gerando um gradiente eletroquímico através da membrana interna. Parte dos elétrons utilizados pela ETC escapam, reagindo prematuramente com O2 e formando ROS. O gradiente eletroquímico gerado é usado pela ATP sintase para suprir a demanda energética celular, ou dissipado pelas UCPs.

A identificação da UCP1 como sendo específica do BAT em mamíferos fez com que o principal papel fisiológico atribuído a ela fosse o de dissipar energia na forma de calor (termogênese) em resposta a exposição a baixas temperaturas [11]. A UCP1 diminui a eficiência da mitocôndria em gerar ATP, aumentando o gasto energético, e por esta razão tem sido extensivamente estudada em humanos como um possível alvo contra obesidade e diabetes [19]. A superexpressão da UCP1 no BAT e no músculo esquelético em mamíferos é capaz de prevenir o desenvolvimento da obesidade, reduzir o acúmulo de gordura e aumentar a sensibilidade à insulina [20,21]. A identificação de um ortólogo da UCP1 em plantas [22] desencadeou uma busca que revelou que as UCPs constituem uma família multigênica presente em uma grande variedade de organismos eucariotos [23-25].

O ortólogo da UCP1 identificado em plantas demonstrou características semelhantes às apresentada pelas UCP1de mamíferos: desacoplar a respiração na presença de ácidos graxos, ser induzida por ROS e inibida por purinas [22,26]. Estudos demonstraram que os genes que codificam UCPs em plantas são expressos diferencialmente de acordo com o tecido, tipo celular, e tempo de exposição a diferentes estresses abióticos [27,28]. Apesar de ser induzida sob estresse causado por baixas temperaturas e ser altamente expressa em plantas termogênicas, o papel da UCP na termogênese em plantas ainda é objeto de debate [26,29]. A distribuição das UCPs entre diferentes espécies de eucariotos, e o seu padrão de expressão tecido-específico sugerem outras funções fisiológicas para estas proteínas [26,30-32].

Plantas transgênicas de tabaco (*Nicotiana tabacum*) superexpressando a UCP1 de *Arabidopsis thaliana* demostraram ser mais tolerantes a peróxido de hidrogênio (H_2O_2), evidenciando a importância desta proteína na proteção contra estresse oxidativo [33]. Em trabalhos posteriores estas mesmas plantas demonstraram melhor desempenho em comparação com plantas selvagens quando submetidas a estresse salino e osmótico, possivelmente por diminuir a formação de ROS [34]. A proteção contra estresses mediada pela superexpressão de UCPs não parece estar restrita a estresses abióticos, visto que a superexpressão em tomate (*Solanum lycopersicum*) é capaz de conferir tolerância a altas temperaturas, e à infecção por patógenos [35]. Além disso, a melhora do desempenho destas plantas parece não só ser ocasionada pela diminuição na produção de ROS, mas

também por alterar o metabolismo redox da célula como um todo [35]. Outro aspecto interessante é a alteração na capacidade fotossintética observada tanto em plantas superexpressando, como em plantas silenciando a expressão de UCPs [34-36], mostrando que o efeito de sua atividade não se restringe a mitocôndria, mas altera o metabolismo celular de maneira mais ampla.

O principal foco dos trabalhos anteriores realizados em plantas foi relacionar a expressão das UCPs com a diminuição da produção de ROS, mas pouca atenção foi dada a função primária das UCPs, que é dissipar o gradiente de prótons usado para a produção de ATP, e portanto, interferir no gasto energético celular. Mitocôndrias isoladas a partir de plantas de batata (*Solanum tuberosum)* superexpressando UCP demonstraram um aumento não só na respiração desacoplada, mas também no fluxo de carbono nas reações do Ciclo de Krebs [37]. A expressão da UCP pode ser associada a alterações nas principais vias do metabolismo energético celular, como OXPHOS, fotossíntese, e Ciclo de Krebs, portanto é evidente que a sua atividade interfira de alguma maneira na comunicação entre a mitocôndria e outros compartimentos celulares.

A relação entre comunicação mitocôndria-nucleo, biogênese mitocondrial e resistência a estresses

Durante o processo evolutivo a grande maioria dos genes responsáveis por codificar proteínas mitocondriais foi transferido do genoma mitocondrial para o nuclear. Atualmente, estima-se que o proteoma mitocondrial de *Arabidopsis thaliana* seja composto por mais de 1.000 proteínas [15], das quais somente 57 sao codificadas pelo genoma mitocondrial [38]. Assim, as adaptações no metabolismo energético e a biogênese mitocondrial dependem de um mecanismo de comunicação e coordenação da expressão entre os dois genomas [39]. O processo de sinalização onde disfunções na organela levam a alteração na expressão de genes nucleares é conhecido como sinalização retrógrada. Este processo é extensivamente estudado em mamíferos, e sabe-se que concentrações citoplasmáticas de cálcio, ROS, e ATP, além do processo dinâmico de fusão e fissão exercem um importante papel na transdução de sinal entre a mitocôndria e o núcleo [3,13, 17,40,41]. Essa sinalização leva a ativação de reguladores transcricionais presentes no

núcleo que alteram a expressão de genes codificadores de proteínas mitocondriais. Em mamíferos já se sabe que o fator de transcrição PGC1-α é capaz de desencadear o processo de biogênese mitocondrial [12], mas o regulador em plantas que desempenharia essa mesma função ainda é desconhecido [7]. Esta questão se torna ainda mais difícil de ser resolvida pelo fato de diversos processos biológicos e sinais externos alterarem também a comunicação entre a mitocôndria e outros compartimentos celulares, como peroxissomo, retículo endoplasmático e cloroplasto [7] (Figura 3).

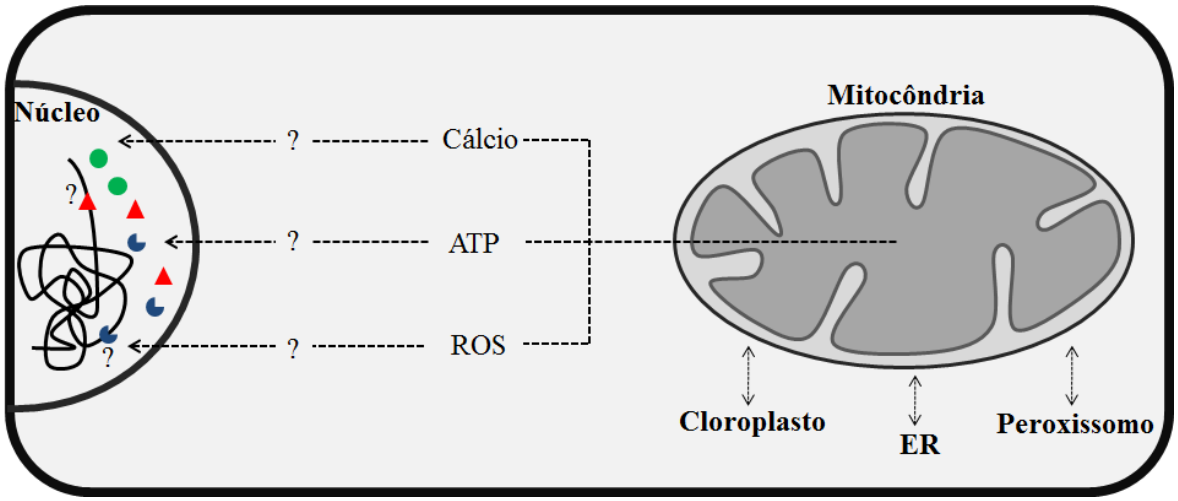

Figura 3. As adaptações no metabolismo energético requerem a comunicação entre mitocôndrias e outros compartimentos celulares. Estima-se que o proteoma mitocondrial seja composto por mais de 1.000 proteínas, sendo a maioria codificada no genoma nuclear. A comunicação entre a mitocôndria e o núcleo pode ser mediada pelos níveis citoplasmáticos de ATP, Cálcio e ROS, que induzem a regulação de genes mitocondriais no núcleo através de fatores de transcrição. Em plantas, pouco se sabe acerca dos reguladores que mediam esse processo. A identificação destes reguladores é dificultada pelo fato que alterações no metabolismo celular também alterarem a comunicação entre a mitocôndria e outras organelas.

Geralmente o termo "biogênese mitocôndrial" é definido como o processo pelo qual novas mitocôndrias são formadas dentro da célula. Levando em conta que novas mitocôndrias se originam a partir de organelas preexistentes, este termo é mais bem definido como o processo que leva a síntese e a montagem de novos componentes mitocondriais, o que requer a expressão coordenada de ambos os genomas [7]. O aumento no número de mitocôndrias nem sempre está relacionado ao aumento na concentração de proteínas mitocondriais, visto que em certas condições onde há disponibilidade excessiva

de nutrientes, as mitocôndrias se fragmentam sem que haja alteração em sua composição, ou na expressão gênica [13]. Neste sentido, os exemplos mais bem estudados em plantas onde o processo de biogênese mitocondrial são durante o desenvolvimento floral [8-10] e germinação [42,43], ou sob a influência de fatores externos como disponibilidade de nutrientes [15] e estresses abióticos [44]. Até o momento pouco se conhece sobre fatores de transcrição que regulam este processo de sinalização retrógrada em plantas, embora vários estudos publicados recentemente sugerem o papel de fatores de transcrição pertencentes às famílias bZIP [45], WRKY [46], TCP [47] e NAC [48] neste processo.

A relação entre a sinalização retrógrada e resistência a estresses já foi bastante estudada em organismos mais simples como *Saccharomyces cereviseae* [49,50] *e C. elegans* [51]. Nestes organismos foi demonstrado que pequenas perturbações na função mitocondrial podem ser sentidas e compensadas pela expressão de genes nucleares, e que a ativação desta resposta é capaz de extender o tempo de vida desses organismos [52]. Neste contexto o termo mitohermese foi cunhado, e se refere ao fenômeno pelo qual estresses mitocondriais moderados, causados por inúmeras situações distintas, desencadearem uma ampla e diversa adaptação nuclear e citoplasmática [52]. Apesar desta resposta variar de acordo com o tipo de estresse, aparentemente ela é capaz de induzir um estado de citoproteção, resultando em adaptações metabólicas e bioquímicas em curto e longo prazo [52] (Figura 4).

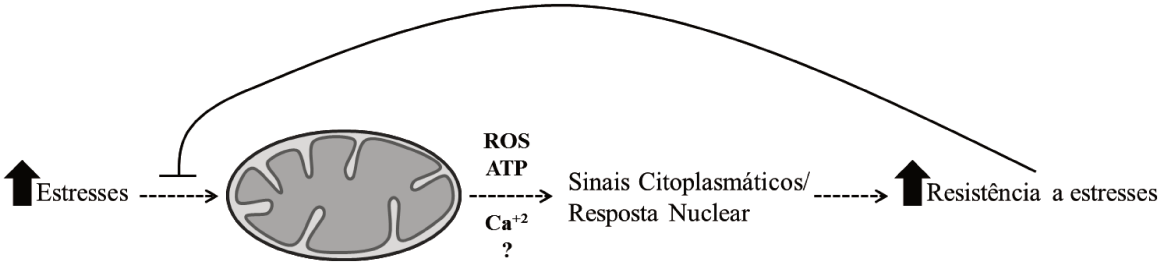

Figura 4. O conceito de mitohermese. Diversos estresses endógenos ou exógenos podem causar perturbações na função mitocondrial. Essas perturbações são transmitidas pelo citoplasma através de mecanismos pouco conhecidos que podem envolver ROS, ATP, Cálcio, ou outros mediadores. Esta sinalização citoplasmática e as mudanças transcricionais subsequentes no núcleo induzem várias vias citoprotetoras. O aumento na resistência a estresses permite a proteção a um amplo espectro de estresses subsequentes.

O exemplo mais bem estudado de mitohermese se refere ao processo de biogênese mitocondrial mediado pelo fator de transcrição PGC1-α. Neste processo, o estresse energético causado por alterações na demanda metabólica, ou na disponibilidade de nutrientes, leva a uma ampla ativação de genes mitocondriais em conjunto com uma potente maquinaria de resistência a estresse oxidativo [53].

OBJETIVOS

Os objetivos do presente trabalho foram:

1) Elucidar os mecanismos moleculares envolvidos no melhor desempenho sob condições de estresse apresentado por plantas transgênicas de *Nicotiana tabacum* superexpressando a UCP1 de *Arabidopsis thaliana*.

2) Identificar as principais vias metabólicas alteradas nas plantas transgênicas e reguladores que possivelmente atuam no mecanismo compensatório induzido pela superexpressão da UCP1.

RESULTADOS

Os resultados deste trabalho e a metodologia utilizada estão apresentados na forma de dois manuscritos, um deles ja publicado na revista *BMC Plant Biology* e o segundo submetido à revista *Plant Science*.

Capítulo 1 - Overexpression of UCP1 in tobacco induces mitochondrial biogenesis and amplifies a broad stress response

Overexpression of UCP1 in tobacco induces mitochondrial biogenesis and amplifies a broad stress response

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Abstract

Background

Uncoupling protein one (UCP1) is a mitochondrial inner membrane protein capable of uncoupling the electrochemical gradient from adenosine-5′-triphosphate (ATP) synthesis, dissipating energy as heat. UCP1 plays a central role in nonshivering thermogenesis in the brown adipose tissue (BAT) of hibernating animals and small rodents. A UCP1 ortholog also occurs in plants, and aside from its role in uncoupling respiration from ATP synthesis, thereby wasting energy, it plays a beneficial role in the plant response to several abiotic stresses, possibly by decreasing the production of reactive oxygen species (ROS) and regulating cellular redox homeostasis. However, the molecular mechanisms by which UCP1 is associated with stress tolerance remain unknown.

Results

Here, we report that the overexpression of UCP1 increases mitochondrial biogenesis, increases the uncoupled respiration of isolated mitochondria, and decreases cellular ATP concentration. We observed that the overexpression of UCP1 alters mitochondrial bioenergetics and modulates mitochondrial-nuclear communication, inducing the upregulation of hundreds of nuclear- and mitochondrial-encoded mitochondrial proteins. Electron microscopy analysis showed that these metabolic changes were associated with alterations in mitochondrial number, area and morphology. Surprisingly, UCP1 overexpression also induces the upregulation of hundreds of stress-responsive genes, including some involved in the antioxidant defense system, such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione-S-transferase (GST). As a consequence of the increased UCP1 activity and increased expression of oxidative stress-responsive genes, the UCP1-overexpressing plants showed reduced ROS accumulation. These beneficial metabolic effects may be responsible for the better performance of UCP1-overexpressing lines in low pH, high salt, high osmolarity, low temperature, and oxidative stress conditions.

Conclusions

Overexpression of UCP1 in the mitochondrial inner membrane induced increased uncoupling respiration, decreased ROS accumulation under abiotic stresses, and diminished cellular ATP content. These events may have triggered the expression of mitochondrial and stress-responsive genes in a coordinated manner. Because these metabolic alterations did not impair plant growth and development, UCP1 overexpression can potentially be used to create crops better adapted to abiotic stress conditions.

Keywords

UCP1, Mitochondria, Oxidative stress, Biogenesis, Plant, Stress response

Background

Mitochondrial uncoupling protein one (UCP1) is a nuclear-encoded protein located in the mitochondrial inner membrane. In the presence of fatty acids, UCP1 uncouples the electrochemical gradient from adenosine-5′-triphosphate (ATP) synthesis, dissipating energy as heat [1]. Mammalian UCP1 has long been investigated in brown adipose tissue (BAT) for its role in thermogenesis and the regulation of reactive oxygen species (ROS) production [2–4].

UCP1 has also been found in plants [5]. Similar to its mammalian orthologs, plant UCP1 belongs to a multigenic family whose members are expressed in a time- and tissue-dependent manner and in response to low temperature [6–8]. Plant UCPs have also been shown to be involved in thermogenesis regulation in skunk cabbage [9] and climacteric increases in respiration in fruits [10], but the widespread presence of this protein in eukaryotic organisms suggests that it may have other functions [8]. The overexpression of *Arabidopsis thaliana UCP1* (*AtUCP1*) in tobacco plants resulted in increased tolerance to oxidative stress [11]. In addition, the tobacco plants overexpressing *AtUCP1* exhibited faster germination under control and stressful conditions, improved performance under drought and salt stresses, and increased rates of photosynthesis [12]. The mechanism underlying this increased stress protection is generally associated with decreased ROS production [11, 12], but a recent study performed on *Solanum lycopersicum*

(tomato) plants overexpressing a UCP gene suggest a wider role for UCPs' protective mechanisms by altering cell redox homeostasis and antioxidant capacity [13]. These previous studies regarding UCP overexpression, along with a study of an insertional knockout of AtUCP1 [14], suggest that UCPs may alter metabolism more globally, modulating mitochondrial, chloroplastic and cytosolic metabolism.

The role of mitochondria in energy metabolism and the stress response implies that this organelle communicates with other cellular compartments. Alteration of mitochondrial function modulates the expression of UCP1 and other nuclear-encoded mitochondrial proteins, including alternative oxidase (AOx) and type II NAD(P)H dehydrogenase, through mitochondria-to-nucleus signaling [15, 16]. Mitochondria-to-nucleus communication has been extensively investigated in mammalian models, in which a signaling process coordinates the expression of genes encoded by the mitochondrial and nuclear genomes through mechanisms known as anterograde (nucleus to organelle) and retrograde (organelle to nucleus) signals [17]. The cytosolic concentrations of Ca^{2+} and ATP, together with the mitochondrial fission/fusion dynamics and ROS production, play central roles in the antero/retrograde signaling pathways and in the regulation of mitochondrial proliferation [18]. Both anterograde and retrograde signaling have been implicated in mitochondrial biogenesis [19, 20], a process that is well documented in mammalian models [4] but poorly understood in plants [21]. In plants, the upregulation of nuclear-encoded mitochondrial genes have been seen during inflorescence development, especially during flowering [22–24], but the molecular components involved in the regulation of this process remains elusive. Recently, it has been shown that the pentatricopeptide proteins [25] and the transcription factors bZIP [26], WRKY [27], TCP [28] and NAC [29] may contribute to the retrograde regulation of mitochondrial/nucleus communication in plants.

In the present work, we investigated the mechanism of UCP1 action in the stress response. We used molecular, cellular and genomic tools to investigate the molecular and cellular events resulting from the overexpression of *AtUCP1* in transgenic tobacco plants. We show that *AtUCP1* overexpression increases uncoupled respiration, decreases cellular ATP concentration, alters mitochondrial morphology, and triggers retrograde

signaling, activating the expression of mitochondrial- and nuclear-encoded mitochondrial proteins. These changes are accompanied by a broad induction of stress-responsive genes that may help the *AtUCP1*-overexpressing cells reduce the levels of ROS and perform better under stress conditions. The results are discussed in the context of the link between mitochondrial biogenesis and the stress response in plants.

Results

Overexpression of the *Arabidopsis UCP1* (*AtUCP1*) in tobacco shows no phenotypic alterations independent of its transgene expression levels

The *AtUCP1* gene, encoding the *Arabidopsis* ortholog of mammalian UCP1 [6], was cloned under the control of the 35S CaMV promoter and transformed into tobacco plants [11]. Two transgenic events, P49 and P07, which presented intermediary and high *AtUCP1* expression, respectively, were chosen for this study. Although UCP1 acts directly in the mitochondrial respiratory chain by uncoupling the electron transport from ATP synthesis, no apparent phenotypic alterations were observed in the P49 and P07 transgenic lines compared with the wild-type (WT) plants (Figure 1A). This result diverges from previous findings showing increased shoot dry mass in plants overexpressing the same UCP1 [12], but it is consistent with observations of tomato plants overexpressing a UCP, which did not exhibit growth stimulation [13]. The addition of sucrose to the growth medium may have altered mitochondrial metabolism and biogenesis [24]. In the data presented in Figure 1A, we did not add sucrose to the nutrient solution.

The transgenic lines P49 and P07 presented 2- to 8-fold increases in *AtUCP1* expression compared to WT plants in the two leaves sampled (Figure 1B). Immunoblotting using an anti-*At*UCP1 polyclonal antibody, which recognizes the tobacco and *Arabidopsis* UCP1, showed that the *At*UCP1 protein (approximately 32 kD) was weakly detectable in WT leaves but accumulated at high levels in P49 and P07 transgenic lines (Figure 1C).

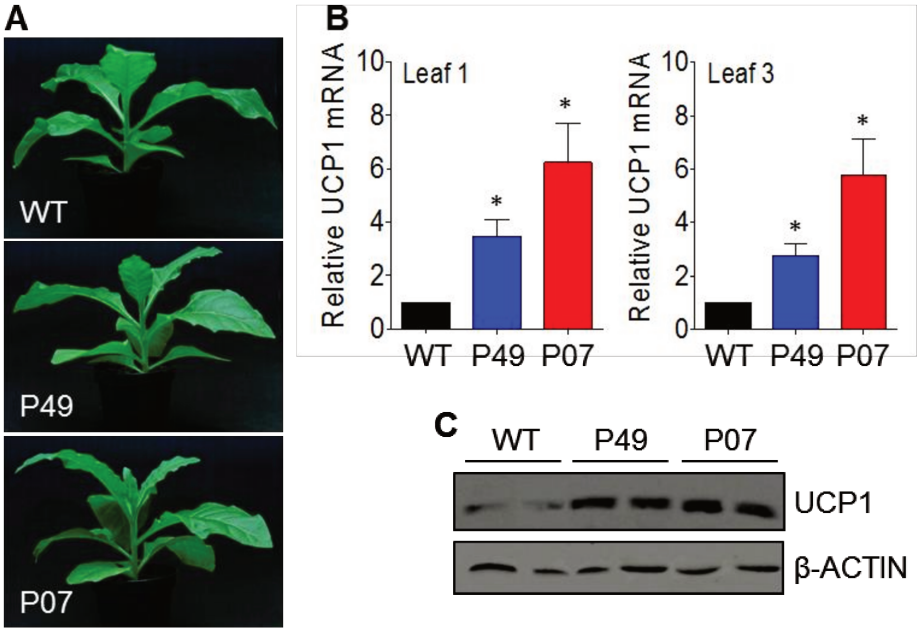

Figure 1. Transgenic tobacco overexpressing *AtUCP1* exhibits a normal plant phenotype independent of the UCP1 expression level. (A) Wild-type (WT) and *AtUCP1* transgenic lines P49 and P07 grown for 12 weeks in 50:50 soil/vermiculite mixtures fertilized with half-strength MS medium not supplemented with sucrose. **(B)** Transcript abundance of *AtUCP1* in L1 and L3 leaves of WT and transgenic lines P49 and P07. **(C)** Immunoblot analysis of L3 leaves of WT and transgenic lines P49 and P07 using an anti-*AtUCP1* antibody. The loading control was examined using an anti-β-actin antibody. Panel **(A)** is representative of an experiment using 12 plants for each genotype (n = 12). Panel **(B)** is representative of an experiment with 6 plants for each genotype (n = 6). Panel **(C)** is representative of an experiment conducted in triplicate. *p < 0.05 compared with the control. Error bars, mean ± s.e.m.

AtUCP1 overexpression triggers mitochondria biogenesis through retrograde signaling

Young and mature leaves differ in their responses to cold stress and photosynthetic activity [30, 31]. Therefore, we examined whether young or more expanded leaves would respond differently to *AtUCP1* overexpression. P07, P49 and WT plants were grown for 12 weeks, and apical leaves varying from youngest (L1) to oldest (L5) (Figure 2A) were sampled. Mesophyll protoplasts were prepared from L3, stained with the mitochondria-selective probe MitoTracker Red CM-H2XRos (Invitrogen, Carlsbad, CA, USA) and analyzed with fluorescent confocal microscopy. Both P49 and P07 transgenic lines showed 1.5- to 2-fold increases in the MitoTracker Red fluorescence signal compared

with WT, indicating that UCP1 overexpression led to an increase in the mitochondrial number and/or volume (Figure 2B, C, and D). The *Arabidopsis* mitochondrial DNA (mtDNA) encodes 57 proteins involved in mitochondrial metabolism and mtDNA maintenance [32]. In mammals, mtDNA replication is strongly associated with cellular demand for ATP [33]. To investigate whether the increased MitoTracker Red fluorescence signal observed in the P49 and P07 transgenic lines is associated with alteration in mtDNA, we used the *MATR* maturase gene to quantify its mtDNA content relative to the WT line. qRT-PCR quantification of *MATR* in total DNA extracted from L1 to L5 leaves revealed an estimated mtDNA increase of 2- to 5-fold in the P49 and P07 transgenic lines, respectively, compared with that of WT plants (Figure 2E). Because mitochondrial biogenesis requires the coordinated expression of both nuclear and mitochondrial genomes [17, 24], we examined whether *AtUCP1* overexpression results in the upregulation of other nuclear- and mitochondrial-encoded mitochondrial proteins. q-PCR of total RNA extracted from L1 to L5 leaves from P49, P07 and WT plants was used to quantify relative mRNA content from genes encoding a selected set of nuclear- and mitochondrial-encoded mitochondrial proteins. Independent of leaf age, the P49 and P07 transgenic lines presented 2- to 5-fold increases in the transcript abundance of NADH dehydrogenase (NADH-DeH) and NADH iron-sulfur proteins 2 and 7 (NADH IS2-7), which are components of mitochondrial respiratory chain complex I [34] (Figure 2F). The increases in both nuclear-encoded (NADH-DeH and NADH-IS7) and mitochondrial-encoded (NADH-IS2) components of complex I indicate that mitochondrial/nuclear coordination is necessary for bioenergetic adaptation. mIncreased transcript abundance in P49 and P07 transgenic lines was also observed for the molecular chaperone GRPe and for the adenine nucleotide exchange factor of heat shock protein-70 (HSP70) that is involved in mitochondrial targeting of proteins [35] (Figure 2F and Additional file 1: Figure S1A-D). We also observed that the P07 transgenic line showed 2- to 5-fold increases in the protein levels of cytochrome oxidase subunit II (COXII), isocitrate dehydrogenase (IDH), voltage-dependent anion channel-1 (VDAC1), and alternative oxidase (AOX), which are proteins associated with both oxidative stress [36] and mitochondrial metabolism [37–39] (Figure 2G). These data indicate that *AtUCP1* overexpression triggered retrograde signaling, leading to increased

transcription and translation of mitochondrial proteins encoded by both the nuclear and mitochondrial genomes, culminating in increased mitochondrial biogenesis.

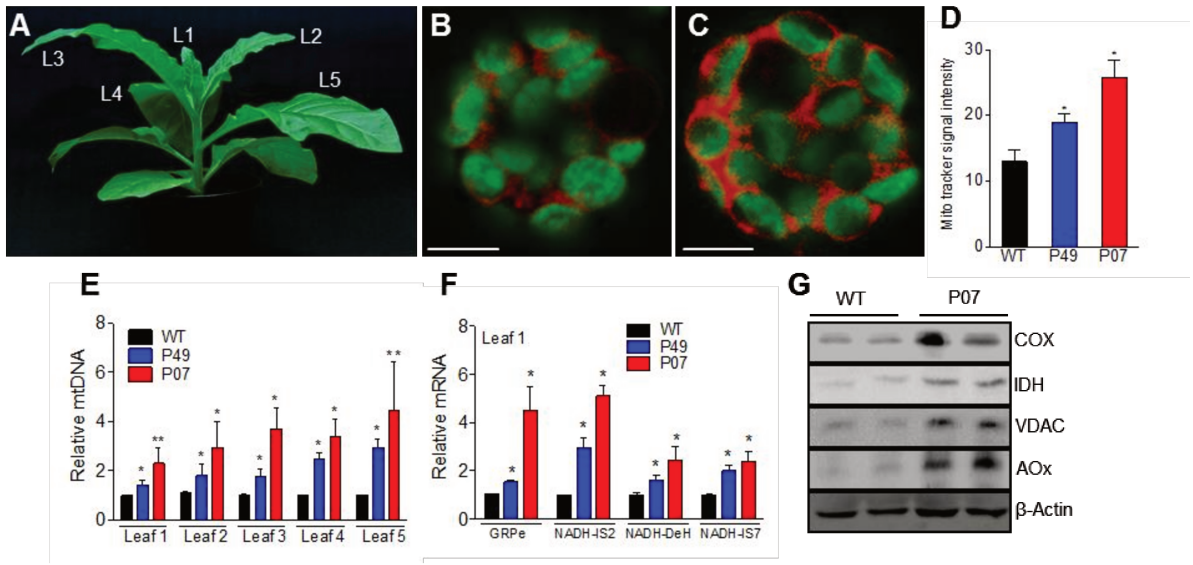

Figure 2. Overexpression of *AtUCP1* in tobacco induces mitochondrial biogenesis through retrograde signaling. **(A)** Five leaves, from youngest (L1) to oldest (L5), were sampled from WT and transgenic lines P49 and P07. **(B** and **C)** Mesophyll protoplasts isolated from WT **(B)** and P07 **(C)** L3 leaves stained with MitoTracker Red. The panels are representative of 50 protoplasts evaluated for each genotype. Scale bars = 5 μm. **(D)** Quantification of the MitoTracker Red signal in a population of protoplasts isolated from the L3 leaves of WT and transgenic lines P49 and P07 (n = 50). **(E)** Relative mtDNA content of WT and transgenic lines P49 and P07 in leaves L1 to L5. **(F)** Relative mRNA content of genes involved in targeting proteins to mitochondria (GRPe chaperone) and energy metabolism (NADH-DeH, NADH-IS2, and NADH-IS7) in L1 leaves of WT and transgenic lines P49 and P07. **(G)** Immunoblot analysis of nuclear- (IDH, VDAC and AOX) and mitochondrial-encoded (COXII) proteins involved in energy metabolism, transport, and stress response in the L1 leaves of WT and transgenic line P07. The data presented in panels **E** and **F** are representative of four biological replicates (n = 4). *$p < 0.05$ and **$p < 0.1$ compared with WT. Error bars, mean ± s.e.m.

AtUCP1 overexpression increases uncoupling respiratory capacity and decreases cellular ATP content

Although *AtUCP1* overexpression protects tobacco plants from oxidative, osmotic and drought stress, no previous data indicate whether UCP1 does, in fact, uncouple ATP synthesis in these plants [11]. However, previous work has demonstrated that mitochondria isolated from *Solanum tuberosum* (potato) plants overexpressing a UCP show increased uncoupled respiration [40]. Here, we demonstrate that crude mitochondria

isolated from L3 leaves of WT and P07 plants retained their respiratory properties: ADP and FCCP can efficiently increase oxygen consumption when compared to basal respiration (Figure 3A). Additionally, transgenic plants showed decreased adenosine 5′-diphosphate (ADP)-dependent respiration and increased uncoupling respiration in comparison to WT plants (Figure 3B). There was no significant difference in the oxygen consumption in the presence of carbonyl cyanide-*4*-trifluoromethoxy phenylhydrazone (FCCP) between P07 and WT mitochondria (Figure 3B). The increased uncoupling respiration of P07 compared with WT mitochondria is evident in a comparison of the leak ratios (uncoupled/maximum oxygen consumption) in P07 and WT (Figure 3B). The increased uncoupling respiration in the P07 transgenic line resulted in 20–35% decreased cellular ATP concentration in the L3 leaves compared with the WT (Figure 3C).

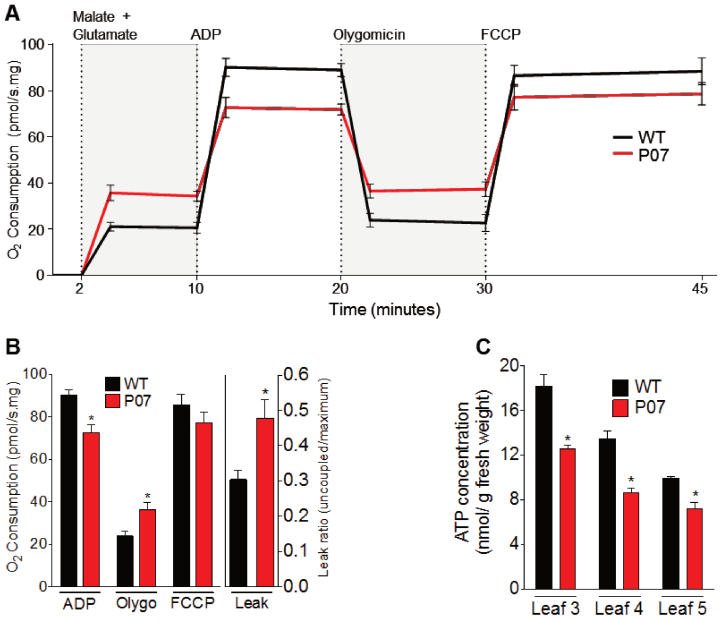

Figure 3. Overexpression of *AtUCP1* increases mitochondrial uncoupled respiration, resulting in decreased cellular ATP content. (A) Time-course of respiration measurements on isolated mitochondria from WT and P07 plants. Malate (10 mM) and glutamate (5 mM) were added to the respiration medium to provide substrates for the electron transport chain. ADP (800 µM) was used to stimulate ADP-dependent respiration, and the ATP synthase inhibitor oligomycin (5 µM) was used to measure oxygen consumption due to uncoupled respiration. Maximum oxygen consumption was measured in the presence of FCCP (2 µM). **(B)** Average oxygen consumption of WT and P07 isolated mitochondria in the presence of ADP, oligomycin and FCCP. The leak ratio was calculated as the ratio between oxygen consumption in the presence of oligomycin and that in the presence of FCCP. **(C)** Decreased ATP levels in the L3, L4 and L5 leaves of the P07 transgenic line compared with WT. The data are representative of six biological replicates (n = 6). *p < 0.05 and **p < 0.1 compared with WT. Error bars, mean ± s.e.m.

Altered mitochondrial metabolism due to increased UCP1 activity leads to changes in mitochondrial number, volume and morphology

Mitochondrial fission and fusion dynamics, as well as mitochondrial morphology, have been shown to be directly affected by modulations in the energy demand and nutrient supply in human cells [41]. The fine regulation of these parameters is linked to the adaptation of mitochondrial architecture to metabolic demand. To address whether *AtUCP1* overexpression affects mitochondrial morphology, we performed transmission electron microscopy (TEM) of mesophyll cells of L2 leaves from P07 and WT plants. Approximately 20 micrograph fields for each genotype were analyzed for mitochondrial number and area using ImageJ [42]. Images from P07 leaves revealed increases of 1.6-fold in mitochondrial number and 1.4-fold in mitochondrial volume, resulting in an overall augmentation of 2.2-fold in the total mitochondrial area (Figure 4A, B, and C). Surprisingly, and perhaps for the first time, we observed a novel mitochondrial morphology in plant cells: donut-shaped mitochondria (Figure 4D). This morphology has been observed in human cells during treatment with FCCP or subjection to hypoxic stress [43]. These treatments decrease oxygen availability for ATP production. This unusual morphology has also been observed during reoxygenation and FCCP washout, when cells reoxygenate and ATP levels are partially restored. When ATP levels were completely restored, the donut-shaped mitochondria fragmented, generating new mitochondria. These findings suggest that *AtUCP1* overexpression triggers a retrograde signal that promotes mitochondrial biogenesis. The alterations in both mitochondrial architecture and morphology may be due to metabolic stress imposed by the higher activity of the electron transport chain, thus increasing oxygen consumption, together with the reduced ATP synthesis caused by the *At*UCP1 overexpression. The donut-shaped mitochondrial morphology might be associated with the fusion/fission dynamics but may also be important to alleviate the tension imposed by the increased mitochondrial volume [43].

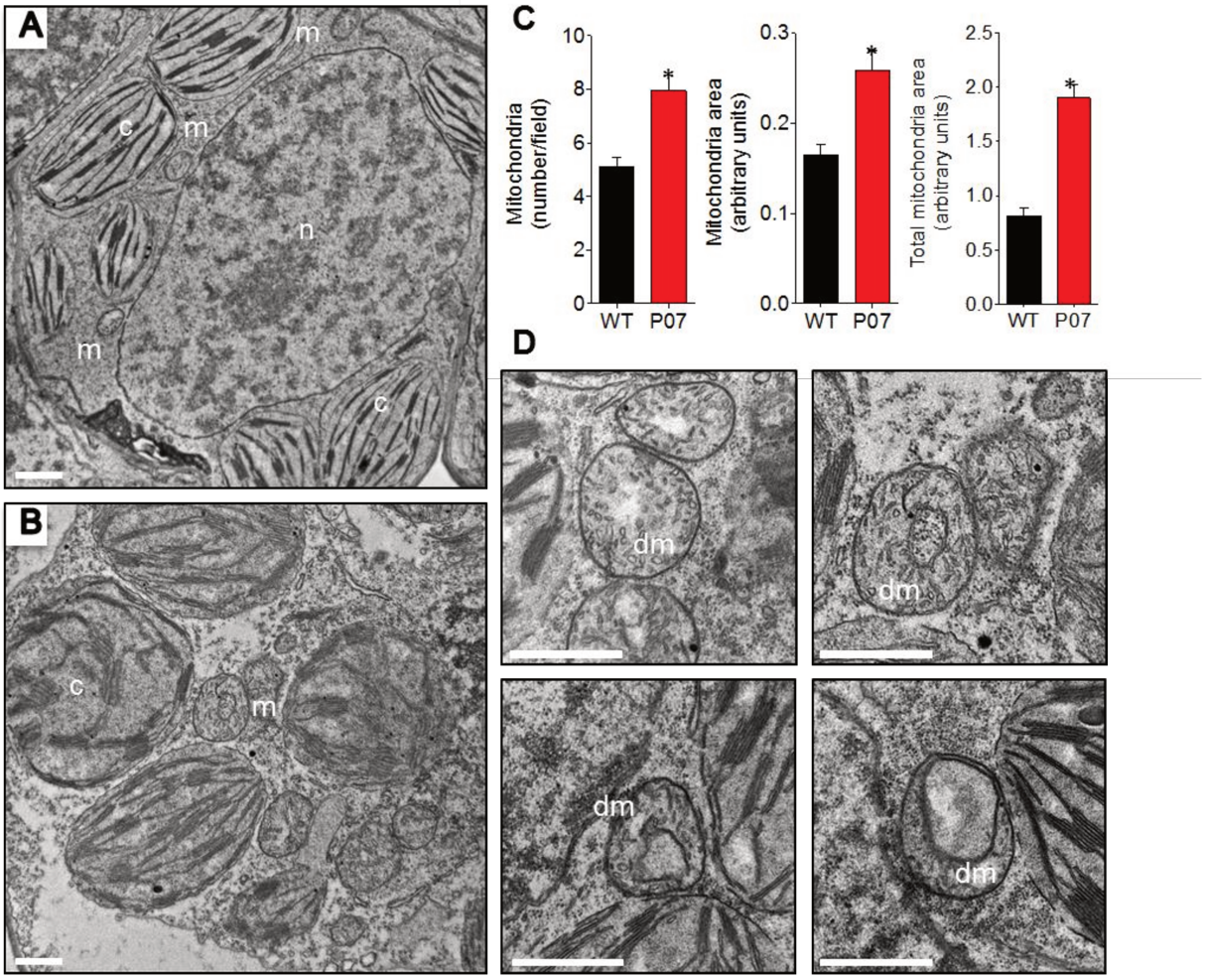

Figure 4. Overexpression of *AtUCP1* increased mitochondrial biogenesis through donut formation/fragmentation. **(A)** Representative TEM micrograph of WT L2 leaves. **(B)** Representative TEM micrograph of P07 L2 leaves. **(C)** Quantification of the mitochondrial number, area, and total area occupied by mitochondria in TEM micrograph field images of WT and P07 plants. **(D)** Close-up of the donut-shaped mitochondria at different developmental stages in P07 transgenic cells. Image analysis was performed on 20 field images (n = 20) for each genotype. *p < 0.05 compared with WT. Error bars, mean ± s.e.m. Scale bars, 1 μm.

AtUCP1 overexpression induces broad transcriptomic activation of mitochondrial and stress-responsive genes

It was unexpected that the overexpression of a single protein would result in mitochondrial biogenesis because the mitochondrial proteome is composed of over 2000 proteins [24]. Therefore, the induction of mitochondrial biogenesis would require the expression of an array of genes encoding these proteins. To examine the extent of

retrograde signaling activation due to *AtUCP1* overexpression, we conducted a global transcriptomic analysis of L3 leaves from P07 and WT plants. The transcripts were annotated using UniProt [44] and categorized into the Clustering of Orthologous Groups (COG) database [45]. We used TAIR subcellular prediction of proteins [46] to identify a total of 705 nuclear-encoded mitochondrial transcripts that have significantly higher RPKM values in the P07 transgenic line and 165 that have higher expression levels in WT plants (Figure 5A, red dots). In addition, we identified 12 mitochondrial-encoded proteins, all of which were more than 2-fold (significantly) upregulated in the P07 transgenic line (Figure 5A, blue dots). The most representative mitochondrial upregulated proteins include those associated with energy production, pentatricopeptide proteins, proteins associated with mitochondrial transcription and translation, and a large number of unknown proteins (Figure 5B and Additional file 2: Table S1). Proteins with unknown subcellular prediction were submitted to further analysis using TargetP [47]. This analysis allowed us to identify another 43 proteins predicted to be targeted to mitochondria with fold-changes higher than 2.0 in P07 plants (Additional file 3: Table S2). Among several proteins with unknown function, we identified possible orthologs of one of the mitochondrial calcium uniporters (MCUs) and a mitochondrial sodium/calcium exchanger (MCX). One of these proteins (Solyc04g079910.2.1 in Additional file 3: Table S2), which is 2.5-fold upregulated in P07, contains a coiled-coil domain on the C-terminal region, which is found among the MCUs, and has a strong sequence similarity with the *Arabidopsis thaliana* (*At*MCU), *Mus musculus* (MmMCU) and *Homo sapiens* (HsMCU) mitochondrial calcium uniporters. The other protein (Solyc07g042000.2.1 in Table 2) contains a sodium/calcium-exchanging domain whose sequence is predicted to be targeted to mitochondria. Among the upregulated proteins in the P07 transgenic line, we also identified several proteins associated with calcium signaling, mitochondrial import machinery and mitochondrial fission and fusion processes, along with transcripts related to lipid metabolism, which may act to support UCP1 activity and cell metabolism (Additional file 4: Table S3). Several transcription factors that may play a role in mitochondrial retrograde signaling were also identified (Additional file 4: Table S3). Surprisingly, we also identified 1071 genes responsive to abiotic stress, 72% (770) of which have increased expression in the P07

transgenic line compared with WT (Figure 5C, red dots). Among these stress-associated proteins, we identified large numbers associated with heat, osmotic, cold, drought, salt, oxidative and cadmium stress (Figure 5D). We also observed 1.4- to 3.4-fold increases in the transcript levels of genes involved in antioxidant mechanisms, including SOD, GPX and GSTs, among the upregulated stress-responsive genes (Additional file 5: Table S4).

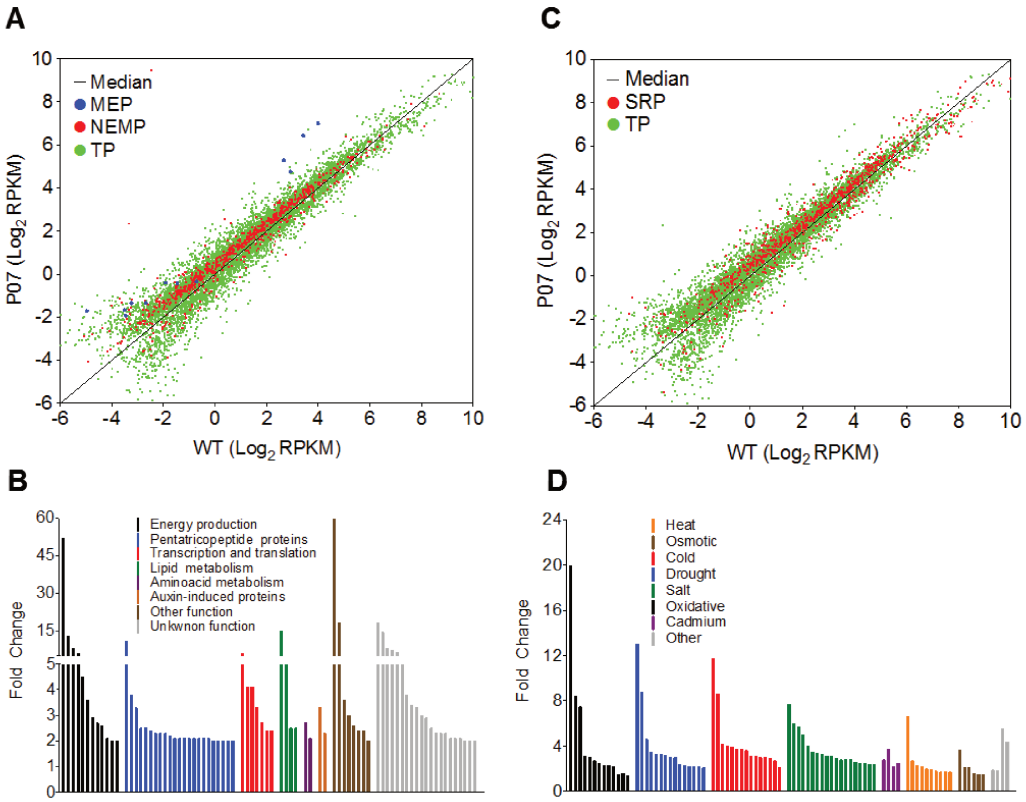

Figure 5. Transcriptome analysis reveals a global upregulation of mitochondrial and stress-responsive genes in transgenic P07 compared with WT. (A) Scatterplot of $\log_2$ RPKM showing nuclear- (Red circle symbol) and mitochondrial (Blue circle symbol)-encoded mitochondrial proteins that were upregulated in the P07 *AtUCP1* transgenic line compared with WT. The green dots represent the total differentially expressed genes. **(B)** Examples of genes associated with nuclear and mitochondrial-encoded mitochondrial proteins that were upregulated in the P07 transgenic line compared with WT. **(C)** Scatterplot of $\log_2$ RPKM showing stress-responsive (Red circle symbol) genes that were upregulated in *AtUCP1* transgenic lines compared with WT. The green dots represent the total differentially expressed genes. **(D)** Examples of genes associated with abiotic stress responses that were upregulated in the P07 transgenic line compared with WT. Normalization was performed by applying $\log_2$ to the RPKM values of WT and the P07 transgenic line. All the represented transcripts showed significantly different expression between the genotypes ($p < 0.05$). The RPKM means were calculated using four biological replicates ($n = 4$) of L3 leaves from each genotype.

UCP1 transgenic line performed better under abiotic stresses

In addition to the previous observation of increased tolerance to oxidative, salt, and drought stresses [11, 12], we observed that the P07 UCP1 overexpressor performed better under low and high pH, hyperosmotic stress, and oxidative stress (Figure 6A). Additionally, in the presence of free fatty acids, the P07 transgenic line also exhibited improved performance under low temperature, a result that support the thermogenic role of plant UCP1 (Figure 6A). Because UCP1 activity in mouse brown adipose tissue, potato tuber and tobacco leaves can reduce superoxide accumulation [12, 40, 48], we examined whether this process also occurs in the P07 transgenic line but used an approach that detects ROS accumulation only within mitochondria. When subjected to salt, oxidative and hyperosmotic stress, mitochondrial ROS accumulation was decreased in the P07 transgenic line compared with WT plants in both leaf discs and roots (Figure 6B and C).

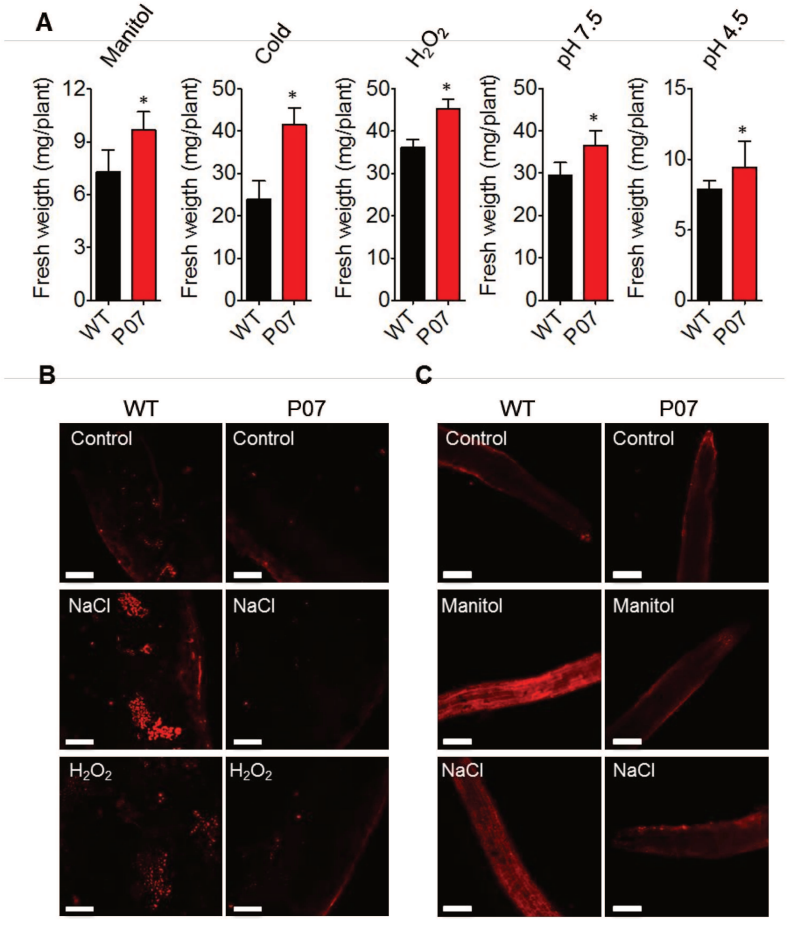

Figure 6. *AtUCP1*-overexpressing plants performed better under abiotic stress, most likely by decreasing ROS accumulation. (A) Two-week-old WT and P07 plantlets were plated on half-strength MS medium supplemented with 80 mM mannitol (hyperosmotic stress) or 10 mM H_2O_2 (oxidative stress), pH 7.5 and pH 4.5 for 22 days under a day/light regime of 16 h at 100 μE m^{-2} s^{-1} and 8 h in darkness. For the low-temperature treatment, the seeds were germinated and grown for 30 days on half-strength MS medium supplemented with 50 μM:10 μM linoleic acid:bovine serum albumin. Plant growth assays were performed using four biological replicates (n = 4) on plates containing 5 plants for each treatment. *p < 0.05 and **p < 0.1 compared with WT. Error bars, mean ± s.e.m. Scale bars, 200 μm. **(B)** Leaf discs (0.8 cm) or **(C)** root tips isolated from WT and the P07 transgenic line were incubated in half-strength MS medium supplemented with 100 mM NaCl, 100mM mannitol, or 10 mM H_2O_2 for 20 h. Control treatments were performed using unsupplemented half-strength MS medium. Tissues were stained with MitoSox red and visualized with fluorescent confocal microscopy.

Discussion

The mitochondrial respiratory chain is composed of four macromolecular complexes through which free energy is conserved by coupling electron transport to the formation of a proton motive force by complexes I, III, and IV, which is then dissipated by F1FO-ATPase (complex V) for ATP synthesis [49]. In the BAT, electron transport is uncoupled from ATP synthesis by UCP1 for heat production [4]. However, BAT is a specialized tissue dedicated to thermogenesis regulation whose main characteristic is a dramatic increase in mitochondria number and UCP1 expression [50]. In other cell types, however, decreased ATP concentration may compromise cell metabolism [51]; therefore, cells somehow sense the cytoplasmic ATP concentration as a signal to change gene expression to quickly readjust the mitochondrial architecture and cell metabolism [18, 52]. In this work, we show that the overexpression of UCP1 increases uncoupled respiration and decreases cellular ATP concentration. There is evidence demonstrating that mitochondria isolated from potato plants overexpressing UCP exhibit increased uncoupling respiration [40], but the present study represents the first time that the effect of UCP on intracellular ATP level has been quantified in plants. It is possible that the enhanced uncoupled respiration and its consequent negative effect on the ATP production trigger a signal to compensate for the energy metabolism and maintain ATP at safe levels. Consistent with this view is the remarkable upregulation of electron transport chain components, including NADH-DeH, NADH-IS2-7, and COX, observed in the P07 overexpressor. The coordinated upregulation of the electron transport chain components encoded by the nuclear and

mitochondrial genomes occurs in tissues with high metabolic demands and increased mitochondrial biogenesis [21, 50, 53], such as flowers [22–24, 54] and germinating seeds [55].

Mitochondrial biogenesis was observed using multiple approaches, including MitoTracker RED fluorescence imaging, quantitation of mtDNA and TEM. In all cases, these methods allowed us to conclude that the overexpression of *AtUCP1* resulted in increased mitochondrial number and volume. To assess whether the upregulation of mitochondrial genes was restricted to the electron transport chain components, we performed global transcriptome sequencing and observed the upregulation of hundreds of genes encoding mitochondrial proteins. All of the identified transcripts encoded on the mtDNA were upregulated in transgenic plants, which is consistent with the increased mtDNA content. For instance, the ATP synthase gene encoded by the mtDNA was found to be upregulated by 8.1-fold, and two of its nuclear subunits were increased by 2.7- and 3.6-fold. This upregulation was not restricted to the components involved in the oxidative phosphorylation but also included genes that participate in the tricarboxylic acid cycle (TCA). The key control point of the TCA is the enzyme IDH [56], which showed increased protein levels in P07 plants (Figure 2G). In fact, experiments performed with mitochondria isolated from potato plants demonstrated that UCP1 activity facilitates higher TCA flux by decreasing membrane potential and, consequently, ROS production [40].

Transcriptional regulation is the main regulatory factor in the expression of nuclear-encoded genes associated with respiration [21], but the vast majority of proteins targeted to the mitochondria are transported post-translationally by tightly regulated machinery [57]. Among these proteins, HSP70 is involved in the transport from the cytoplasm to the mitochondrial outer membrane, whereas the mitochondrial TOM and TIM translocases are responsible for transport inside the organelle [58]. Interestingly, all of these proteins were upregulated in the P07 *AtUCP1* overexpression line. The increased expression of components of the mitochondrial energy production machinery, along with the increased mitochondrial number of transgenic cells, may alter cellular energy homeostasis. Therefore, the mitochondria may consume more substrates in an attempt to boost ATP synthesis; for that purpose, these substrates must be supplied from other

sources. This need might explain the increased photosynthetic rate exhibited by these plants under control conditions [12]. Transcriptome sequencing provides an important tool to investigate this possibility. We did not observe differential expression patterns between WT and P07 plants regarding chloroplast-targeted proteins (data not shown), but further analysis of the transcriptome might provide new insights into this issue.

The adaptation to changes in energy supply and demand influences not only mitochondrial gene expression but also mitochondrial architecture and morphology [41]. Alterations in mitochondrial morphology during starvation have already been observed in *Arabidopsis thaliana* cells cultured without a carbon source [24]. In human cells, treatment with the chemical uncoupler FCCP causes an increase in oxygen consumption and a decrease in ATP generation, provoking mitochondrial fragmentation and the appearance of donut-shaped mitochondria [43]. This fragmentation may occur to support an increase in respiration in an attempt to elevate ATP levels [41], whereas the formation of the donut-shaped morphology may be a component of a protective mechanism that helps to preserve the organelles from damage under conditions of metabolic stress [41, 43]. When FCCP is washed out of these cells, the donut morphology persists until the ATP levels are completely restored, at which point the donuts fragment and new mitochondria arise. The mechanisms underlying the donut formation in UCP1-overexpressing cells might be explained by an analogy with human cells. Increased UCP1 activity decreases the membrane potential that is used for ATP synthesis, provoking an increase in mitochondrial number to balance the ATP levels in transgenic plants. Mitochondria are responsible for at least 90% of cell oxygen consumption [59]. We therefore expect that an increase in respiratory chain machinery and mitochondrial number, together with the constant leak caused by UCP1, would result in increased oxygen consumption per molecule of ATP generated. Thus, this metabolic stress caused by UCP1 overexpression appears sufficient to allow the formation of donut mitochondria in plant cells.

Communication between mitochondria and the nucleus is required for the adaptations that we observed in P07 transgenic plants. Although several important regulators of chloroplastic retrograde signaling have been extensively studied [60], relatively little is known about the regulators of mitochondrial retrograde signaling in

plants. Several recently published studies suggest roles for the WRKY, bZIP, TCP, and NAC transcription factor families in activating the expression of nuclear-encoded mitochondrial proteins in plants [26–29]. These transcription factors recognize elements that are overrepresented in the regulatory region of mitochondrial genes and that are involved in several biotic and abiotic stress responses [21]. The colocalization of proteins in mitochondria and the nucleus appears to be an important topic that is very little explored in plants. For example, an *Arabidopsis thaliana* pentatricopeptide protein (PNM1) [25] was identified in both mitochondria and nucleus that may act to coordinate the expression between the two genomes. The increased expression of transcription factors that belong to the WRKY and TCP families, along with the upregulation of a large number of pentatricopeptide proteins in transgenic plants, presents the opportunity to further explore new candidates involved in mitochondrial retrograde signaling. The expression of most transcriptional regulators is further linked to cellular signals and environmental cues [21]. The most studied cellular signals involved in mitochondria-nucleus signaling are ATP, calcium and ROS [18]. Although the role of mitochondria in calcium metabolism has been extensively studied in mammals, little is known about calcium signaling by plant mitochondria [61]. Mitochondria in mammals are able to sequester cytoplasmic Ca^{+2}, functioning as a transient calcium store for protective mechanisms [18, 62]. Additionally, the Ca^{+2} levels inside the organelle are positively correlated with increased ATP production [63], although Ca^{+2} overaccumulation is linked to the induction of apoptosis in both mammals and plants [61]. Consistent with this relation, it is very interesting that we found two genes upregulated in transgenic plants that are possible orthologs, an MCU and an MCX, along with several genes involved in calcium signaling (Additional file 3: Tables S2 and Additional file 4: Table S3). The MCUs and MCXs have been extensively studied in animal cells, and their expression is tightly linked with mitochondrial calcium transport [64, 65].

The main focus of previous studies regarding UCP1 overexpression in plants has been ROS production [11–13, 40]. Both potato and tobacco transgenic plants presented decreased superoxide production under abiotic stresses [11, 12, 40]. Additionally, tomato plants overexpressing UCP1 demonstrate not only an alteration on ROS production but also

a possible role of UCP1 in regulating the cellular redox homeostasis [13]. The plants analyzed in the present study showed increased expression not only of the antioxidant defense system, including GPX, GSTs and SOD, but also of an array of stress-responsive genes. This effect may help explain the positive impact of *AtUCP1* overexpression on the tolerance to multiple abiotic stresses. Consistent with previous data, we show in this work that *AtUCP1* overexpression protects plants from a number of abiotic stresses, including high pH, low pH and cold. The fact that transgenic tobacco performs better at low temperatures when the medium is supplemented with fatty acids reinforces the thermogenic role of UCP1 in plants. The importance of ROS in cell signaling is well known [66]; thus, the results regarding decreased mitochondrial ROS production under oxidative, osmotic and salt stresses also reinforce the importance of this molecule in UCP1-mediated retrograde signaling.

Together, these data indicate that *AtUCP1*-overexpressing lines suffer metabolic stress caused by the increased uncoupling respiration. Chronic overexpression of UCP1 can induce mitochondrial biogenesis in mammals [67]. Therefore, we propose that in the transgenic plants, the lower ATP level acts as a key element in the retrograde signaling, promoting a broad increase in the expression of both mitochondrial genes and stress-related genes. This pattern is reflected in the decreased ROS production and the better performance of these plants under various stresses. These findings advance our understanding of stress-tolerance mechanisms, mitochondrial biogenesis and bioenergetic adaptation in plants and therefore might assist in the implementation of biotechnological tools for the development of abiotic stress-tolerant plants. The overall regulation of stress-responsive genes indicates a link between UCP1-activated mitochondrial biogenesis and the increased stress response exhibited by the transgenic plants.

Conclusions

In this work, we present strong evidence that the overexpression of UCP1 protein affects the mitochondrial dynamics at both structural and metabolic levels, leading to an increased mitochondria number and volume per cell. It could be argued that these changes are a consequence of the overpopulation of UCP1 protein at the inner

mitochondrial membrane, thereby leading to abnormal mitochondrial structure and function. We interpret the results presented in this manuscript as a beneficial effect of the overexpression of UCP1 that triggers a retrograde signaling process, signaling the nuclear and mitochondrial genomes to increase the production of mitochondrial proteins. Consequently, more mitochondrial membrane is produced. The increase in UCP1, both because of the overproduction of the protein and the increased mitochondrial number and volume, leads to increased uncoupled respiration, decreasing the cellular ATP concentration. This change may trigger metabolic stress and enhances a strong stress response. Because it does not affect plant growth and development, this mechanism might be used to create crops better adapted to abiotic stress conditions.

Methods

Plant materials and growth conditions

Tobacco (*Nicotiana tabacum*) ecotype SR1 overexpressing *AtUCP1* was obtained as previously described [11]. In this work, we used two independent transgenic lines, P49 and P07, which express *AtUCP1* at intermediate and high levels, respectively. Transgenic and wild-type (WT) plants were grown under a light/dark cycle of 16 h at 100 μE m^{-2} s^{-1} and 8 h in darkness for 12 weeks on 50:50 soil/vermiculite mixtures at 24°C. The plants were fertilized weekly with half-strength MS medium not supplemented with sucrose. For the cellular and molecular analysis, samples of the five youngest leaves (L1 to L5) of each genotype were collected, frozen in liquid nitrogen, and stored at −80°C. For the other experiments, seeds were grown directly in experimental medium, as described in the figure legends.

qRT-PCR

For mitochondrial DNA quantification, total DNA was isolated from WT, P49, and P07 plants using the plant DNAzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For the mRNA expression analysis, total RNA was isolated from leaf tissue using the NucleoSpin Mini RNA/Protein Kit (Macherey-Nagel, Duren, Germany). cDNA was synthesized using the Revertaid First Strand cDNA Synthesis Kit

(Fermentas, Vilnius, Lithuania) according to the manufacturer's protocol. qRT-PCR was performed using an ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA) with SYBR Green (Applied Biosystems). The reactions were performed at least in triplicate for four biological replicates. The mRNA expression and mtDNA content analysis were normalized using *ACTIN1* as an internal reference. Values are presented as the mtDNA content or transcript abundance of P49 and P07 relative to WT. Student's t-test was performed to determine significance ($p < 0.05$). Primers based on the *MATR* gene were used for mitochondrial DNA quantification [68].

The following primers were designed for amplification:

ACTIN1

FW: 5′-ACTGTCCACGAGGTCCGG-3′

RV: 5′-TGTCGGATCTTGCGCGGC-3′

AtUCP1

FW: 5′-TTGAGCAAGAAAATTCTTGCTG-3′

RV: 5′-AGGCGGAAGGAAAATTAGC-3′

NADH-DeH

FW: 5′-GGCTGAAGCGCGAGAAGAC-3′

RV: 5′-CAGGGCAGGCTTCTTGGC-3′

NADH-IS2

FW: 5′-GTGAAGTGGCGTGGCAAAC-3′

RV: 5′-TTGTGGATCGCGAAGGGAG-3′

NADH-IS7

FW: 5′-CGCAGTTGCGAAGCGAAAC-3′

RV: 5′-GGGCCATCGCGACAGAGA-3′

GRPe chaperone

FW: 5′-AAACCTTGGCTTGTGACCCA-3′

RV: 5′-TCATTCGGCCAGCTGAAGTT-3′

Immunoblot analysis

Total soluble proteins were extracted from WT, P49 and P07 plants by grinding frozen leaf tissue in 5x protein sample buffer (40 mM Tris, pH 7.4; 30 mM NaCl; 10 mM β-mercaptoethanol; 0.1% Triton X-100; 5 mM benzamidine). The proteins were separated on 12% SDS-PAGE gels, transferred to nylon membranes, and blotted using the following polyclonal antibodies raised against *Arabidopsis* proteins (Agrisera, Vännäs, Sweden): COXII, VDAC (At3g01280), AOx1/2 (At2g22370 and At5g64210), IDH (At4g35260 and At2g27130), and ACTIN (At2g37620, At3g18780, At3g53750, At5g59370, At2g42100, At5g09810, At1g49240, At3g12110, and At3G46520). The bands were detected using an ImageQuant LAS500 (GE Healthcare, Little Chalfont, UK) with the SuperSignal West Pico chemiluminescent substrate (Thermo-Scientific, Waltham, MA, USA).

Mitochondrial quantification in leaf mesophyll protoplasts by MitoTracker Red staining

Tobacco mesophyll protoplasts were isolated from WT, P49, and P07 plants as previously described [69], with minor modifications. Leaf strips (0.1 cm in length) were vacuum-infiltrated and incubated for 3 h in an enzyme solution (20 mM MOPS, pH 5.7; 0.4 M d-mannitol; 20 mM KCl; 10 mM $CaCl_2$; 0.1% BSA; 1.5% cellulase; 0.4% macerozyme). After digestion, the suspension was filtered through 75 μm nylon mesh and centrifuged at 100 x g for 2 min. The protoplasts were resuspended in W5 buffer (2 mM MOPS, pH 5.7; 154 mM NaCl; 125 mM $CaCl_2$; 5 mM KCl), incubated on ice for 30 min, and subsequently resuspended in MMG solution (4 mM MOPS, pH 5.7; 0.4 M mannitol; 15 mM $MgCl_2$). The protoplasts were then stained with 250 nM MitoTracker Red CM-H2XRos (Invitrogen) for 30 min in the dark and washed in MMG solution before viewing. MitoTracker Red CM-H2XRos fluorescence was detected at Ex/Em of 543/589 nm and chlorophyll auto fluorescence was detected at Ex/Em 543/645 nm using a confocal microscope (LSM780-NLO; Zeiss, Oberkochen, Germany) equipped with a 40× oil-immersion objective. The image analysis was performed for 40 protoplasts per sample using ImageJ 1.44p [42]. MitoTracker Red CM-H2XRos fluorescence intensity was normalized using the chlorophyll autofluorescence intensity.

Mitochondrial superoxide detection by MitoSox staining

Leaf discs (0.8 cm in diameter) isolated from L3 leaves were treated with half-strength MS medium (control) or half-strength MS medium supplemented with 100 mM NaCl (salt stress) or 10 mM H_2O_2 (oxidative stress) for 20 h under constant light and agitation. For root analysis, plants grown for 3 weeks on half-strength MS medium were transferred to petri dishes containing fresh half-strength MS medium not supplemented (control) or supplemented with 100 mM NaCl (salt stress) or 100 mM mannitol (hyperosmotic stress) and kept under constant light for 20 h. The leaf discs and roots were stained with 5 μM MitoSox-Red (Invitrogen) for 25 min and washed with half-strength MS medium before viewing. Fluorescence was detected at Ex/Em of 510/580 nm using a confocal microscope (LSM780-NLO; Zeiss) equipped with a 10× objective.

Transmission electron microscopy

Leaf strips (1–2 mm in length) from L2 leaves of WT and P07 plants were submerged and vacuum infiltrated for at least 2 h in fixative solution (2.5% glutaraldehyde, 2.5% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4). The samples were washed in 0.1 M cacodylate buffer and postfixed in a solution containing 1% osmium tetroxide (OsO_4) and 1.5% potassium ferrocyanide ($KFeCN_6$) for 1 h. The samples were then washed 3 times in water and incubated in 1% uranyl acetate in maleate buffer for 1 h; this was followed by 3 washes in maleate buffer and subsequent dehydration in a graded ethanol series (50%, 70%, and 90%, 10 min each; 100%, 2 × 10 min). The samples were then placed in propylene oxide for 1 h and infiltrated in a 1:1 mixture of propylene oxide and TAAB 812 Resin mixture (Marivac Canada, St. Laurent, Canada). The samples were embedded in TAAB 812 Resin mixture and polymerized at 60°C for 48 h. Ultrathin sections of approximately 90 nm were cut using a Reichert Ultracut-S microtome, placed onto copper grids, stained with lead citrate, and examined using a Tecnai G2 Spirit BioTWIN transmission electron microscope. The images were recorded with an AMT 2 k CCD camera. The mitochondrial number and area were quantified on 20 field images representing each genotype at 4800× direct magnification. The image analysis was performed using ImageJ 1.44p [42].

ATP measurement

ATP assays were performed on L4 and L5 leaves isolated from four biological replicates of WT and P07 plants. ATP extraction was performed as previously described [70], with minor modifications. The leaves were ground in liquid nitrogen, and 30 mg of leaf tissue was homogenized with 300 µL of 0.1 M HCl for 5 min. The homogenate was centrifuged at 20,000 x g for 10 min, and the supernatant was centrifuged using a microconcentrator Ultra-15 (Millipore, Billerica-USA) at 14,000 x g for 20 min. The ATP content was determined by using an ATP assay kit (Invitrogen) and a GloMax luminometer (Promega, Fitchburg, WI, USA).

Measurement of mitochondrial respiration

Measurements of mitochondrial respiration were performed using crude isolated mitochondria from six biological replicates of WT and P07 plants. The isolation of crude mitochondria was performed as previously described [71], with minor modifications. All steps were performed at 4°C. Approximately 5 g of leaves were cut with a razor blade and ground in 20 mL of grinding buffer (0.3 M sucrose, 60 mM TES, 10 mM EDTA, 10 mM KH_2PO_4, 25 mM disodium pyrophosphate, 1 mM glycine, 1% (w/v) PVP-40, 1% (w/v) BSA, 50 mM sodium ascorbate, 20 mM cysteine, pH 8.0). The extract was filtered through two layers of 20 µm nylon mesh and one layer of Miracloth (Millipore, Billerica, MA, USA) and centrifuged twice at 2500 x g for 5 min to remove the debris. The resulting supernatant was centrifuged at 15,000 x g for 15 min, and the resulting pellet was gently resuspended in 20 mL of wash buffer (0.3 M sucrose, 10 mM TES, 2 mM EDTA, 10 mM KH_2PO_4, pH 7.5) and centrifuged at 15,000 x g for 15 min. The pellet containing the crude mitochondria was resuspended in 200 µL of wash buffer. The respiratory measurements were performed using an Oxygraph-2 k respirometer (Oroboros, Innsbruck, Austria) at 25°C in 2 mL of mitochondria-containing assay buffer (0.3 M sucrose, 10 mM TES, 10 mM KCl, 2 mM $MgSO_4$, 5 mM KH_2PO_4, 0.1% (w/v) BSA, 2 µM EGTA, pH 7.5). Malate (5 mM) and glutamate (10 mM) were used as substrates to stimulate respiration. ADP (800 µM) was added to measure ADP-dependent respiration. Oligomycin (5 µM) was used as an F1FO ATP synthase inhibitor to measure oxygen consumption due to uncoupling activity,

and the chemical ionophore carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP) (2 μM) was used to determine the maximum oxygen consumption.

Transcriptome sequencing, assembly, and mapping

Total RNA isolated from four biological replicates of WT and P07 L3 leaves was used to create single-end RNASeq libraries using the Illumina TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The libraries were sequenced in four lanes of an Illumina HiSeq 2000 for 75 cycles. A total of 42.7 Gb of sequence was generated for the 8 libraries, with a minimum of 27.9 million and a maximum of 113.2 million reads for each library (Additional file 6: Table S5). The Illumina reads were filtered to remove adapters and low-quality reads (reads <70% bases with quality ≥ Q20) using AdapterRemoval, the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit), and Perl scripts. After the filtering step, the reads were subjected to digital normalization using the diginorm software (https://github.com/ged-lab/2012-paper-diginorm). The resulting 49.5 million reads were assembled with the Trinity [72] assembler, generating 271,750 transcript contigs. High-quality reads (569 million reads) were mapped to Trinity contigs using Bowtie [73], and the RPKM [74] values were calculated. Because the *N. tabacum* genome is not yet completely sequenced and the scaffolds of *N. benthamiana* are estimated to cover only 79% of its genome, we used the complete genome sequence of *Solanum lycopersicum* [75], a close relative of *N. tabacum*, as a template. To identify protein-coding genes, all 271,750 contigs were used as queries in BLASTn and BLASTx searches against both the non-redundant set of 34,727 tomato-coding sequences (CDSs) and predicted protein sequences. The 271,750 contigs were compared to the Solgenomics tomato genome predicted protein database using BLASTx (E-value $<1e^{-5}$). Of the total contigs, 134,752 mapped to the tomato genome, resulting in the identification of 20,045 distinct orthologs for the tomato CDS models. As representative contigs, we chose the three best matching contigs with higher average RPKM values (P07 and WT) for each tomato CDS. An F-test was used to calculate whether the samples had equal or unequal variances, and the appropriate Student t-test was then applied to calculate the significant difference between the average RPKM of P07 versus

WT in a contig-wise manner. The contigs identified as differentially expressed were selected for the next steps. The fold change value for the representative transcript was calculated as the ratio between the average RPKM values in P07 and WT. The subcellular annotation of the defined contigs was determined by BLASTx queries against the TAIR [46] protein database (release 10) and TargetP [47]. Gene function data were obtained from the existing annotations of the tomato genome. The tomato protein dataset was annotated against UniProt [44] and mapped against the COG (Eukaryotic Clusters of Orthologous Groups) [45] database with BLASTp, and COG functional categories were assigned only if the two best hits had the same COG function. Stress-related genes were mapped using BLASTx against the tomato proteome in the Stress Responsive Transcription Factor Database (STIFDB) [76], and annotations were assigned to the corresponding representative tobacco contig.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL database under the following accession numbers: *At*UCP1 (NM_115271.4), Actin1 (EU938079.1), NADH-DeH (Y09109.1), NADH-IS2 (M77225.1), NADH-IS7 (L16810.1), GRPe chaperone (AF098636.1), *At*MCU (NP_119075), *Mm*MCU (XP_006513531), *Hs*MCU (NP_612366), *At*CCX3 (NP_566474).

Transcriptome sequencing data are available from Sequence Read Archive (SRA), which is accessible through NCBI BioProject ID: PRJNA211804 under the experiment ID SUB287723.

Competing interests

The authors declare that they have no competing interests.

Author contributions

P.B. and P.A. designed the experiments, analyzed the data, and wrote the paper. P.B. performed the experiments. V.O. and I.A.P.N. performed the bioinformatics analysis

of the RNASeq data. I.G.M. developed the transgenic plants. All authors read and approved the final manuscript.

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References

1. Nedergaard J, Ricquier D, Kozak LP: **Uncoupling proteins: current status and therapeutic prospects.** *EMBO Rep* 2005, **6:**917–921.

2. Krauss S, Zhang CY, Lowell BB: **The mitochondrial uncoupling-protein homologues.** *Nat Rev Mol Cell Biol* 2005, **6:**248–261.

3. Andrews ZB, Diano S, Horvath TL: **Mitochondrial uncoupling proteins in the CNS: in support of function and survival.** *Nat Rev Neurosci* 2005, **6:**829–840.

4. Kajimura S, Seale P, Spiegelman BM: **Transcriptional control of brown fat development.** *Cell Metab* 2010, **11:**257–262.

5. Vercesi AE, Martins LS, Silva MAP, Leite HMF, Cuccovia IM, Chaimovich H: **PUMPing plants.** *Nature* 1995, **375:**24.

6. Maia IG, Benedetti CE, Leite A, Turcinelli SR, Vercesi AE, Arruda P: **AtPUMP: an Arabidopsis gene encoding a plant uncoupling mitochondrial protein.** *FEBS Lett* 1998, **429:**403–406.

7. Borecky J, Nogueira FTS, Oliveira KAP, Maia IG, Vercesi AE, Arruda P: **The plant energy-dissipating mitochondrial systems: depicting the genomic structure and the**

expression profiles of the gene families of uncoupling protein and alternative oxidase in monocots and dicots. *J Exp Bot* 2006, **57:**849–864.

8. Vercesi AE, Borecký J, Maia IG, Arruda P, Cuccovia IM, Chaimovich H: **Plant uncoupling mitochondrial proteins.** *Annu Rev Plant Biol* 2006, **57:**383–404.

9. Ito-Inaba Y, Hida Y, Mora H, Inaba T: **Molecular identity of uncoupling proteins in thermogenic skunk cabbage.** *Plant Cell Physiol* 2008, **49:**1911–1916.

10. Jezek P, Borecký J, Zácková M, Costa AD, Arruda P: **Possible basic and specific functions of plant mitochondrial uncoupling protein (pUCP).** *Biosci Rep* 2001, **21:**237–245.

11. Brandalise M, Maia IG, Borecký J, Vercesi AE, Arruda P: **Overexpression of plant mitochondrial uncoupling protein in transgenic tobacco increases tolerance to oxidative stress.** *J Bioenerg Biomembr* 2003, **35:**205–209.

12. Begcy K, Mariano ED, Mattiello L, Nunes AV, Mazzafera P, Maia IG, Menossi M: **An *Arabidopsis* mitochondrial uncoupling protein confers tolerance to draught and salt stress in transgenic tobacco plants.** *Plos One* 2011, **6:**e23776.

13. Chen S, Liu A, Zhang S, Li C, Chang R, Liu D, Ahammed GJ, Lin X: **Overexpression of mitochondrial uncoupling protein conferred resistance to heat stress an *Botrytis cinerea* infection in tomato.** *Plant Physiol Biochem* 2013, **73:**245–253.

14. Sweetlove LJ, Lytovchenko A, Morgan M, Nunes-Nesi A, Taylor NL, Baxter CJ, Eickmeier A, Fernie AR: **Mitochondrial uncoupling protein is required for efficient photosynthesis.** *Proc Natl Acad Sci U S A* 2006, **103:**19587–19592.

15. Rhoads DM, Umbach AL, Subbaiah CC, Siedow JN: **Mitochondrial reactive oxygen species: contribution to oxidative stress and interorganellar signaling.** *Plant Physiol* 2006, **141:**357–366.

16. Rhoads DM, Subbaiah CC: **Mitochondrial retrograde regulation in plants.** *Mitochondrion* 2007, **7:**177–194.

17. Woodson JD, Chory J: **Coordination of gene expression between organellar and nuclear genomes.** *Nat Rev Genet* 2008, **9:**383–395.

18. Ryan MT, Hoogenrad MJ: **Mitochondrial-nuclear communications.** *Annu Rev Biochem* 2007, **76:**701–722.

19. Pesaresi P, Schneider A, Kleine T, Leister D: **Interorganellar communication.** *Curr Opin Plant Biol* 2007, **10:**600–606.

20. Jung HS, Chory J: **Signaling between chloroplasts and the nucleus: can a systems biology approach bring clarity to a complex and highly regulated pathway.** *Plant Physiol* 2009, **152:**453–459.

21. Welchen E, Garcia L, Mansilla N, Gonzalez DH: **Coordination of plant mitochondrial biogenesis: keeping pace with cellular requirements.** *Front Plant Sci* 2013, **4:**551.

22. Zabaleta E, Heiser V, Grohmann L, Brennicke A: **Promoters of nuclear-encoded respiratory chain complex I genes from Arabidopsis thaliana contain a region essential for anther/pollen-specific expression.** *Plant J* 1998, **156:**49–59.

23. Binder S, Brennicke A: **Gene expression in plant mitochondria: transcriptional and post-transcriptional control.** *Philos Trans R Soc Lond B Biol Sci* 2003, **3586:**181–188.

24. Giegè P, Sweetlove LJ, Congat V, Leaver CJ: **Coordination of nuclear and mitochondrial genome expression during mitochondrial biogenesis in Arabidopsis.** *Plant Cell* 2005, **17:**1497–1512.

25. Hammani K, Gobert A, Hleibieh K, Choulier L, Small L, Giegè P: **An *Arabidopsis* dual-localized pentatricopeptide repeat protein interacts with nuclear proteins involved in gene expression regulation.** *Plant Cell* 2011, **23:**730–740.

26. Roschzttardtz H, Fuentes I, Vásquez M, Corvalán C, León G, Gómez I, Araya A, Holuique L, Vicente-Carbajosa J, Jordana X: **A nuclear gene encoding the iron-sulfur subunit of mitochondrial complex II is regulated by B3 domain transcription factors during seed development in *Arabidopsis thaliana*.** *Plant Physiol* 2009, **150:**84–95.

27. Van Aken O, Zhang B, Law S, Narsai R, Whelan J: **AtWKRY40 and AtWRKY63 modulate the expression of stress-responsive nuclear genes encoding mitochondrial and chloroplast proteins.** *Plant Physiol* 2013, **162:**254–271.

28. Giraud E, Ng S, Carrie C, Duncan O, Low J, Lee CP, Van Aken O, Millar AH, Murcha M, Whelan J: **TCP transcription factors link the regulation of genes encoding mitochondrial proteins with the circadian clock in *Arabidopsis thaliana*.** *Plant Cell* 2010, **22:**3921–3934.

29. De Clercq I, Vermeirssen V, Van Aken O, Vandepoele K, Murcha MW, Law SR, Inzé A, Ng S, Ivanova A, Rombaut D, Van de Cotte B, Jaspers P, Van de Peer Y, Kangasjärvi J, Whelan J, Van Breusegem F: **The membrane-bound NAC transcription factor ANAC013 functions in mitochondrial retrograde regulation of the oxidative stress response in Arabidopsis.** *Plant Cell.* 2013, **25:**3472–3490.

30. Ölçer H, Lloyd JC, Raines CA: **Photosynthetic capacity is differentially affected by reductions in sedoheptulose-1,7-biphosphatase activity during leaf development in transgenic tobacco plants.** *Plant Physiol* 2001, **125:**982–989.

31. Rogalski M, Schöttler MA, Thiele W, Schulze WX, Bock R: **Rpl33, a nonessential plastid-encoded ribosomal protein in tobacco, is required under cold stress conditions.** *Plant Cell* 2008, **20:**2221–2237.

32. Unseld M, Marienfeld JR, Brandt P, Brennicke A: **The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotide.** *Nat Genet* 1997, **15:**57–61.

33. Dickinson A, Yeung KY, Donoghue J, Baker MJ, Kelly RDW, McKenzie M, Jhons TJ, St John JC: **The regulation of mitochondrial DNA copy number in glioblastoma cells.** *Cell Death Differ* 2013, **20:**1644–1653.

34. Klodmann J, Senkler M, Rode C, Braun HP: **Defining the protein complex proteome of plant mitochondria.** *Plant Physiol* 2011, **157:**587–598.

35. Padimam M, Reddy VS, Beachy RN, Fauquet CM: **Molecular characterization of a plant mitochondrial chaperone GrpE.** *Plant Mol Biol* 1999, **39:**871–881.

36. Fiorani F, Umbach AL, Siedow JN: **The alternative oxidase of plant mitochondria is involved in the acclimation of shoot growth at low temperature. A study of Arabidopsis AOX1a transgenic plants.** *Plant Physiol* 2005, **139:**1795–1805.

37. Smolková K, Jezek P: **The role of mitochondrial NADPH-dependent isocitrate dehydrogenase in cancer cells.** *Int J Cell Biol* 2012, **212**:1-12.

38. Robert N, D'Erfurth I, Marmagne A, Erhardt M, Allot M, Boivin K, Gissot L, Monachello D, Michaud M, Duchêne AM, Barbier-Brygoo H, Maréchal-Drouard L, Ephritikhine G, Filleur S: **Voltage-dependent-anion-channels (VDACs) in Arabidopsis have a dual localization in the cell but show a distinct role in mitochondria.** *Plant Mol Biol* 2012, **78:**431–446.

39. Leadsham JE, Sanders G, Giannaki S, Bastow EL, Hutton R, Naeimi WR, Breitenbach M, Gourlay CW: **Loss of cytochrome c oxidase promotes RAS-dependent ROS production from the ER resident NADPH oxidase, Yno1p, in yeast.** *Cell Metab* 2013, **18:**279–286.

40. Smith AMO, Ratcliffe G, Sweetlove LJ: **Activation and function of mitochondrial uncoupling protein in plants.** *J Biol Chem* 2004, **279:**51944–51952.

41. Liesa M, Shirihai OS: **Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure.** *Cell Metab* 2013, **17:**491–506.

42. Schneider CA, Rasband WS, Eliceir KW: **NIH image to ImageJ: 25 years of image analysis.** *Nat Methods* 2012, **9:**671–675.

43. Liu X, Hajnóczky G: **Altered fusion dynamics underlie unique morphological changes in mitochondria during hypoxia-reoxygenation stress.** *Cell Death Differ* 2011, **18:**1561–1572.

44. The UniProt Consortium: **Update on activities at the universal protein resource (UniProt) in 2013.** *Nucleic Acids Res* 2013, **41:**D43–D47.

45. Tatusov RL, Fedorova ND, Jackson JD, Jacobs AR, Kiryutin B, Koonin EV, Krylov DM, Mazumder R, Mekhedov SL, Nikoslkaya AN, Rao BS, Smirnov AS, Sverdlove AV, Vasudevan S, Wolf YI, Yin JJ, Natale DA: **The COG database: an updated version includes eukaryotes.** *BMC Bioinformatics* 2003, **4:**41.

46. Lamesch P, Berardini TZ, Li D, Swarbreck D, Wilks C, Sasidharan R, Muller R, Dreher K, Alexander DL, Garcia-Hernandez M, Karthikeyan AS, Lee CH, Nelson WD, Ploetz L, Singh S, Wensel A, Huala E: **The Arabidopsis information resource (TAIR): improved gene annotation and new tools.** *Nucleic Acids Res* 2012, **40:**1202–1210.

47. Emanuelsson O, Brunak S, Heijne VJ, Nielsen H: **Locating proteins in the cell using TargetP, SignalP, and related tools.** *Nat Protoc* 2007, **2:**953–971.

48. Oelkrug R, Kutschke M, Meyer CW, Heldmaier G, Jastroch M: **Uncoupling protein 1 decreases superoxide production in brown adipose tissue mitochondria.** *J Biol Chem* 2010, **285:**21961–21968.

49. Vafai SB, Mootha VK: **Mitochondrial disorders as windows into an ancient organelle.** *Nature* 2012, **491:**374–383.

50. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM: **Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1.** *Cell* 1999, **98:**115–124.

51. Miyoshi N, Oubrahim H, Chock PB, Stadtman ER: **Age-dependent cell death and the role of ATP in hydrogen-peroxide-induced apoptosis and necrosis.** *Proc Natl Acad Sci USA* 2006, **103:**1727–1731.

52. Moghadam AA, Ebrahimie E, Taghavi SM, Niazi A, Babgohari MZ, Deihimi T, Djavaheri M, Ramezani A: **How the nucleus and mitochondria communicate in energy production during stress: nuclear MtATP6, an early-stress responsive gene, regulates the mitochondrial F_1F_0-ATP synthase complex.** *Mol Biotechnol* 2013, **54:**756–769.

53. Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, Spiegelman BM: **Transcriptional co-activator PGC-1 alpha drives the formation of slow twitch muscle fibres.** *Nature* 2002, **418:**797–801.

54. Peters K, Niessen M, Peterhansel C, Spath B, Holzle A, Binder S, Marchfelder A, Braun HP: **Complex I-complex II ratio strongly differs in various organs of *Arabidopsis thaliana*.** *Plant Mol Biol* 2012, **79:**273–284.

55. Law SR, Narsai R, Taylor NL, Dellanoy E, Carrie C, Giraud E, Millar AH, Small I, Whelan J: **Nucleotide and RNA metabolism prime translational initiation in the earliest events of mitochondrial biogenesis during Arabidopsis germination.** *Plant Physiol* 2012, **158:**1610–1627.

56. Berg JM, Tymoczko JL, Stryer L: **Biochemistry**. In *The citric acid cycle.* 5th edition. New York: W H Freeman; 2002.

57. Pfanner N, Geissler A: **Versatility of the mitochondrial protein import machinery.** *Nat Rev Mol Cell Biol* 2001, **2:**339–349.

58. Schleiffe E, Becker T: **Common ground for protein translocation: access control for mitochondria and chloroplasts.** *Nat Rev Mol Cell Biol* 2011, **12:**48–59.

59. Searcy DG: **Metabolic integration during the evolutionary origin of mitochondria.** *Cell Res* 2003, **13:**229–238.

60. Kleine T, Leister D: **Retrograde signals galore.** *Front Plant Sci* 2013, **4**:1-3.

61. Stael S, Wurzinger B, Mair A, Mehlmer N, Vothknecht UC, Teige M: **Plant organellar calcium signaling: an emerging field.** *J Exp Bot* 2012, **63:**1525–1542.

62. Clapham DE: **Calcium signaling.** *Cell* 2007, **131:**1047–1058.

63. Tarasov AI, Griffiths EJ, Rutter GA: **Regulation of ATP production by mitochondrial Ca + 2.** *Cell calcium* 2012, **52:**28–35.

64. Alam MR, Groschner LN, Parichatikanond W, Kuo L, Bondarenko AI, Rost R, Waldeck-Weiermaier M, Mali R, Graier WF: **Mitochondrial Ca2+ uptake 1 (MICU1) and mitochondrial Ca + 2 uniporter (MCU) contribute to metabolism secretion coupling in clonal pancreatic β-cells.** *J Biol Chem* 2012, **287:**34445–34454.

65. Palty R, Silverman WF, Herschfinkel M, Caporale T, Sensi SL, Parnis J, Nolte C, Fishman D, Shoshan-Barmatz D, Herrmann S, Khananshvili D, Sekler I: **NCLX is an essential component of mitochondrial Na^{+}/Ca^{+2} exchange.** *Proc Natl Acad Sci U S A* 2010, **107:**436–441.

66. Mailloux RJ, Harper ME: **Mitochondrial proticity and ROS signaling: lessons from the uncoupling proteins.** *Trends Endocrinol Metab* 2012, **23:**451–458.

67. Si Y, Palani S, Jayaraman A, Lee K: **Effects of forced uncoupling protein1 expression in 3T3-L1 cells on mitochondrial function and lipid metabolism.** *J Lipid Res* 2007, **48:**826–836.

68. Wang DY, Zhang Q, Liu Y, Lin ZF, Zhang SX, Sun MX, Sodmergen: **The levels of male gametic mitochondrial DNA are highly regulated in angiosperms with regard to mitochondrial inheritance.** *Plant Cell* 2010, **22:**2402–2416.

69. Yoo SD, Cho YH, Sheen J: **Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis.** *Nat Protoc* 2007, **2:**1565–1572.

70. Ogawa K, Hatano-Iwasaki A, Yanagida M, Iwabuchi M: **Level of glutathione is regulated by ATP-dependent ligation of glutamate and cysteine through photosynthesis in Arabidopsis thaliana: mechanism of strong interaction of light intensity with flowering.** *Plant Cell Physiol* 2004, **45:**1–8.

71. Keech O, Dizengremel P, Gardestrom P: **Preparation of leaf mitochondria from *Arabidopsis thaliana*.** *Physiol Plant* 2005, **124:**403–409.

72. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind N, di Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A: **Full-length transcriptome assembly from RNA-seq data without reference genome.** *Nat Biotechnol* 2011, **29:**644–652.

73. Wagner GP, Kin K, Lynch VJ: **Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples.** *Theory Biosci.* 2012, **131:**281–285.

74. Langmead B, Trapnell C, Pop M, Salzberg SL: **Ultrafast and memory-efficient alignment of short DNA sequences to the human genome.** *Genome Biol* 2009, **10:**R25.

75. Tomato Genome Consortium: **The tomato genome sequence provides insights into fleshy fruit evolution.** *Nature* 2012, **485:**635–641.

76. Naika M, Shameer K, Mathew OK, Gowda R, Sowdhamini R: **STIFDB2: An updated version of plant stress-responsive transcription factor database with additional stress signals, stress-responsive transcription factor binding sites and stress-responsive genes in Arabidopsis and rice.** *Plant Cell Physiol* 2013, **54:**1–15.

Additional files

Additional file 1: Figure S1 Tobacco plants overexpressing *AtUCP1* show an increased expression of nuclear-encoded mitochondrial genes.

Additional file 2: Table S1 Classification of mitochondrial genes upregulated in P07 compared to WT. The classification was based on a TAIR annotation of protein subcellular prediction.

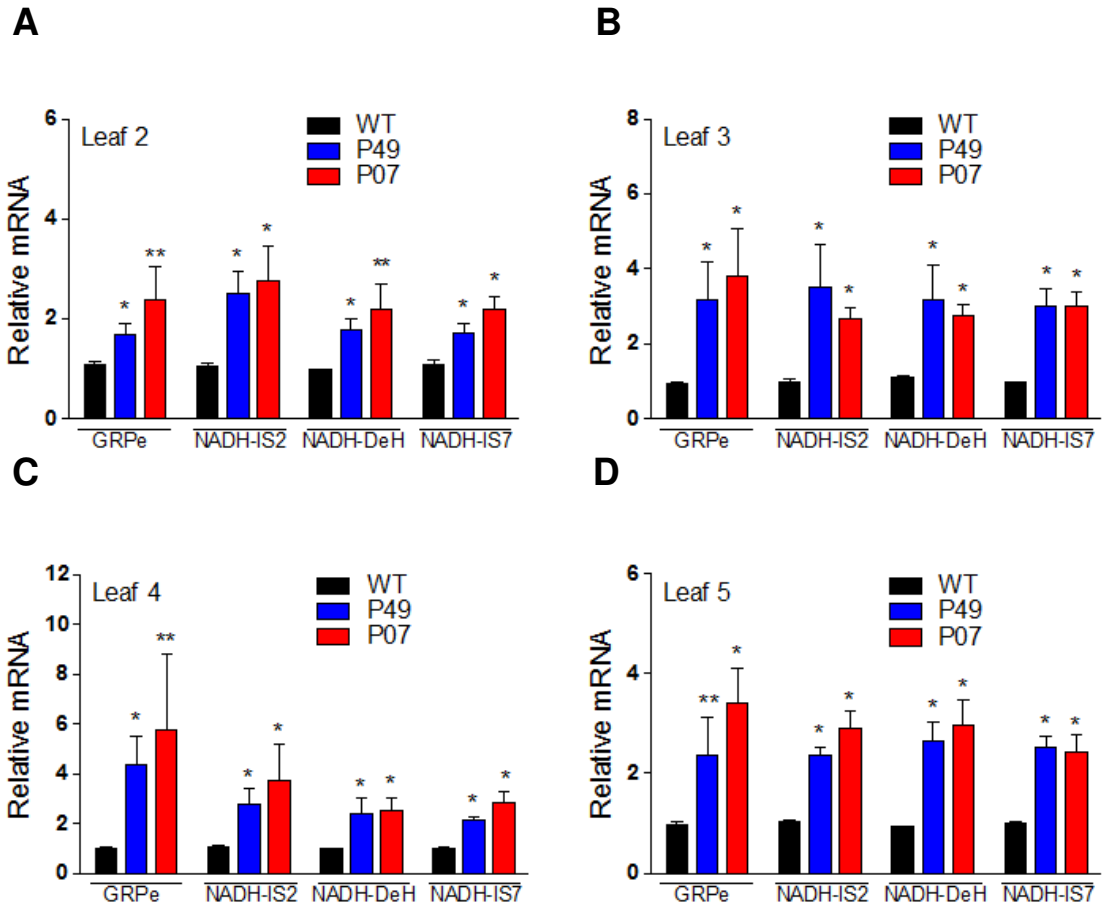
Additional file 3: Table S2 Predicted mitochondrial genes upregulated with ≥ 2-fold change in P07 compared with WT. The prediction was based on TargetP.

Additional file 4: Table S3 Classification of selected genes involved in different metabolic processes and significantly upregulated in P07 compared with WT. The classification was based on COG.

Additional file 5: Table S4 Abiotic stress-responsive genes upregulated in P07 compared with WT. The classification was based on STIFDB and shows the most upregulated genes involved in different types of abiotic stresses.

Additional file 6: Table S5 Summary of RNASeq data filtered and mapped with Bowtie.

Additional File 1: Figure S1 - Tobacco plants overexpressing *AtUCP1* show an increased expression of nuclear-encoded mitochondrial genes. (**A**) Leaf 2, (**B**) Leaf 3, (**C**) Leaf 4 and (**D**) Leaf 5. *, $p < 0.05$, **, $p < 0.1$, compared with wild type. Error bars, mean ± s.e.m.



Additional File 2: Table S1. Classification of mitochondrial genes upregulated in P07 compared to WT. The classification was based on a TAIR annotation of protein subcellular prediction.

Classification/Uniprot Description	*S. lycopersicum* ID	Fold Change	Encoded by
Energy Production			
Mitochondrial glycoprotein family protein	Solyc06g065800.2.1	52,1	Nucleus
Mitochondrial 2-malate carrier	Solyc05g051400.2.1	13,0	Nucleus
Atp Synthase α subunit	Solyc09g056100.1.1	8,1	mtDNA
Cytochrome c oxidase subunit 1	Solyc11g063610.1.1	5,9	Nucleus
Monooxygenase FAD-binding protein	Solyc08g063130.2.1	4,5	Nucleus
ATP synthase β subunit	Solyc11g039980.1.1	3,6	Nucleus
NADH-quinone oxidoreductase subunit D	Solyc01g065780.1.1	2,9	mtDNA
ATP synthase α subunit	Solyc03g043610.1.1	2,7	Nucleus
Cytochrome c oxidase subunit VC	Solyc01g079420.2.1	2,6	Nucleus
NADH dehydrogenase subunit 5	Solyc00g013140.1.1	2,1	Nucleus
Cytochrome c biogenesis	Solyc00g020040.1.1	2,0	Nucleus
Formate dehydrogenase	Solyc02g086880.2.1	2,0	Nucleus
Pentatricopeptide			
PCMP-H61	Solyc03g006990.1.1	10,7	Nucleus
PCMP-E42	Solyc01g080190.2.1	3,8	Nucleus
PCMP-H26	Solyc03g115800.1.1	3,3	Nucleus
PCMP-H45	Solyc07g006990.1.1	2,5	Nucleus
PCMP-E98	Solyc11g008970.1.1	2,5	Nucleus
PCMP-E101	Solyc03g118080.2.1	2,4	Nucleus
PCMP-H24	Solyc05g006460.1.1	2,3	Nucleus
PCMP-E33	Solyc04g016540.1.1	2,3	Nucleus
PCMP-E86	Solyc09g007040.1.1	2,3	Nucleus
Pentatricopeptide repeat protein	Solyc02g086230.1.1	2,2	Nucleus
Pentatricopeptide repeat protein	Solyc01g100340.2.1	2,1	Nucleus
Pentatricopeptide repeat protein	Solyc02g085650.2.1	2,1	Nucleus
PCMP-E64	Solyc01g100800.1.1	2,1	Nucleus
Pentatricopeptide repeat protein	Solyc07g053380.1.1	2,1	Nucleus
Pentatricopeptide repeat protein	Solyc10g081880.1.1	2,1	Nucleus
Pentatricopeptide repeat protein	Solyc04g005460.1.1	2,1	Nucleus
PCMP-H12	Solyc10g084540.1.1	2,1	Nucleus
PCMP-E91	Solyc10g007940.1.1	2,1	Nucleus
PCMP-H89	Solyc05g023900.1.1	2,0	Nucleus
PCMP-E83	Solyc01g099000.1.1	2,0	Nucleus
Pentatricopeptide repeat protein	Solyc01g088490.2.1	2,0	Nucleus
PCMP-E77	Solyc01g112200.2.1	2,0	Nucleus
PCMP-H24	Solyc02g076700.1.1	2,0	Nucleus
Transcription and Translation			
Ribosomal protein S4	Solyc00g021640.2.1	5,9	Nucleus
50S Ribossomal Protein L20	Solyc02g069840.2.1	4,1	Nucleus
50S ribosomal protein L2	Solyc12g035840.1.1	4,1	Nucleus

Intron Maturase type II	Solyc11g056270.1.1	3,3	Nucleus
Ribosomal protein S3	Solyc05g045830.1.1	2,7	Nucleus
30S ribosomal protein S13	Solyc11g056310.1.1	2,4	Nucleus
tRNA dimethylallyltransferase	Solyc01g080150.2.1	2,4	Nucleus
Lipid Metabolism			
GNS1/SUR4 membrane family protein	Solyc02g089860.1.1	15,0	Nucleus
ER glycerol-phosphate acyltransferase	Solyc07g056320.2.1	5,0	Nucleus
Enoyl-(Acyl-carrier-protein) reductase II	Solyc01g105370.2.1	2,5	Nucleus
Hydroxymethylglutaryl-CoA synthase	Solyc08g007790.2.1	2,5	Nucleus
Aminoacid Metabolism			
Proline dehydrogenase	Solyc02g089630.2.1	2,7	Nucleus
Lysine ketoglutarate reductase	Solyc02g078820.2.1	2,1	Nucleus
Auxin-induced			
ARGOS	Solyc12g096570.1.1	3,3	Nucleus
Auxin-induced SAUR-like protein	Solyc03g033590.1.1	2,3	Nucleus
Other function			
Cation diffusion facilitator 9	Solyc12g017350.1.1	231,6	Nucleus
Lycopene Beta Cyclase	Solyc06g074240.1.1	18,0	Nucleus
Cell wall-associated hydrolase	Solyc11g044610.1.1	3,6	mtDNA
DVL1	Solyc02g065570.1.1	3,0	Nucleus
Tetratricopeptide-like protein	Solyc06g065910.2.1	2,6	Nucleus
Major facilitator superfamily transporter	Solyc04g073960.2.1	2,4	Nucleus
Peroxissomal protein Mpv17/PMP22	Solyc06g053550.2.1	2,4	Nucleus
Extracellular ligand-gated ion channel	Solyc07g064900.2.1	2,0	Nucleus
Function Unknown			
Unknown Protein	Solyc08g081850.1.1	18,2	Nucleus
Unknown Protein	Solyc05g045730.1.1	14,4	mtDNA
Unknown Protein	Solyc11g045440.1.1	8,0	mtDNA
Uncharacterized mitochondrial protein	Solyc12g042340.1.1	7,2	Nucleus
Uncharacterized mitochondrial protein	Solyc10g048060.1.1	6,2	mtDNA
Unknown Protein	Solyc03g058300.1.1	5,0	mtDNA
Unknown Protein	Solyc11g030910.1.1	3,8	mtDNA
Unknown Protein	Solyc08g036520.2.1	3,4	Nucleus
Unknown Protein	Solyc00g014840.1.1	3,3	Nucleus
CM0545.320.nc protein	Solyc11g066010.1.1	3,0	Nucleus
Unknown protein	Solyc12g036570.1.1	2,9	mtDNA
Os10g0422600 protein	Solyc07g042190.2.1	2,5	Nucleus
Uncharacterized mitochondrial protein	Solyc03g005630.2.1	2,3	Nucleus
Unknown Protein	Solyc03g064040.1.1	2,3	mtDNA
Unknown Protein	Solyc08g061450.1.1	2,3	Nucleus
Unknown Protein	Solyc02g088130.1.1	2,1	Nucleus
Unknown Protein	Solyc05g006610.2.1	2,1	Nucleus
Unknown Protein	Solyc03g063790.1.1	2,1	mtDNA
Uncharacterized mitochondrial protein	Solyc00g126010.1.1	2,0	Nucleus
Unknown Protein	Solyc07g042330.1.1	2,0	Nucleus
Unknown Protein	Solyc03g098130.2.1	2,0	Nucleus

Aditional File 3: Table S2. Predicted mitochondrial genes upregulated with ≥ 2-fold change in P07 compared with WT. The prediction was based on TargetP.

Uniprot description	***S. Lycopersicum* ID**	**Fold Change**
Sister chromatid cohesion 1 protein 1	Solyc06g074870.1.1	16,2
Unknown Protein	Solyc03g083760.1.1	15,9
Unknown Protein	Solyc00g005860.1.1	6,0
Late embryogenesis abundant protein	Solyc02g062770.1.1	5,5
Unknown Protein	Solyc03g058300.1.1	5,0
60S ribosomal protein L33-B	Solyc03g096360.2.1	4,7
Unknown Protein	Solyc00g281110.1.1	4,6
Wound induced protein	Solyc07g054790.1.1	4,6
Helicase-like protein	Solyc10g045660.1.1	4,5
NBS-LRR class disease resistance protein	Solyc07g056190.2.1	4,2
Ulp1 peptidase-like	Solyc08g016280.1.1	4,1
Os11g0198100 protein	Solyc02g078180.2.1	4,0
Unknown Protein	Solyc06g074570.1.1	4,0
Protein phosphatase 2C	Solyc01g087460.1.1	3,9
UDP-glucosyltransferase HvUGT5876	Solyc04g016200.1.1	3,9
Unknown Protein	Solyc11g030910.1.1	3,8
Unknown Protein	Solyc06g075120.1.1	3,5
Polyprotein	Solyc08g016700.1.1	3,5
Pol polyprotein	Solyc00g131710.1.1	3,4
Unknown Protein	Solyc07g032350.1.1	3,4
Phosphatase 2C family protein	Solyc10g084410.1.1	2,9
NADH-quinone oxidoreductase subunit D	Solyc01g065780.1.1	2,9
Unknown Protein	Solyc00g020030.1.1	2,8
CM0216240nc protein	Solyc02g083120.1.1	2,8
Nbs-lrr resistance protein	Solyc07g055380.1.1	2,8
Xyloglucan endotransglucosylase/hydrolase 3	Solyc07g006850.1.1	2,7
Unknown Protein	Solyc08g079810.2.1	2,6
Unknown Protein	Solyc00g010810.1.1	2,6
Coiled-coil domain-containing protein 109A	Solyc04g079910.2.1	2,5
Sodium/calcium exchanger protein	Solyc07g042000.2.1	2,4
Unknown Protein	Solyc04g051390.2.1	2,3
Endonuclease/exonuclease/phosphatase	Solyc11g062160.1.1	2,3
Lysine ketoglutarate reductase	Solyc11g008930.1.1	2,3
Ankyrin repeat-containing protein	Solyc02g068670.1.1	2,3
Auxin responsive SAUR protein	Solyc07g066560.1.1	2,2

Pentatricopeptide repeat-containing protein	Solyc09g005710.1.1	2,2
Unknown Protein	Solyc09g098510.2.1	2,2
Metallophosphoesterase	Solyc07g005120.2.1	2,0
Phosphatase 2C family protein	Solyc01g107310.2.1	2,0
Cc-nbs-lrr resistance protein	Solyc05g044490.2.1	2,0
Mitochondrial translocase TIM14	Solyc06g072330.2.1	2,0
alpha/beta fold family protein	Solyc11g007030.1.1	2,0
Transposase	Solyc02g050190.1.1	2,0

Additional File 4: Table S3. Classification of selected genes involved in different metabolic processes and significantly upregulated in P07 compared with WT. The classification was based on COG.

UNIPROT description	*S. lycopersicum* ID	FC	TAIR ID	Reference
Lipid Metabolism				
AP2-like transcription factor	Solyc08g076380.1.1	19,6	AT3G54320	Plant J. **60**, 933-47 (2009).
Fatty acyl coA reductase	Solyc06g074390.2.1	6,9	AT4G33790	Plant Physiol. **142**, 866-77 (2006).
4-coumarate CoA ligase-like	Solyc02g088710.2.1	6,6	AT3G21240	Plant J. **19**, 9-20 (1999).
Epoxide hydrolase 3	Solyc02g078570.2.1	5,0	AT4G02340	-
Patatin-like protein 3	Solyc02g065090.2.1	4,6	AT4G37060	-
Cytochrome P450	Solyc10g007860.2.1	3,6	AT1G05160	Proc. Natl. Acad. Sci. U.S.A. **98**, 2065-70 (2001).
Fatty acyl coA reductase	Solyc11g067190.1.1	3,6	AT3G44550	-
Niemann-Pick C1 protein	Solyc04g082490.2.1	3,3	AT4G38350	-
Acyl-protein thioesterase 2	Solyc08g067160.2.1	3,3	AT5G20060	-
Lipase-like protein	Solyc02g077000.2.1	2,7	AT1G06250	-
Acyl-CoA synthetase II	Solyc02g037490.1.1	2,3	AT5G16370	Plant Cell. **19**, 3170-93 (2007).
Squalene synthase	Solyc10g054130.1.1	2,1	AT4G34640	Proc. Natl. Acad. Sci. U.S.A. **92**, 2328-32 (1995).
Fatty-acid--CoA ligase	Solyc12g009040.1.1	2,1	AT3G06860	Biochem. Soc. Trans. **28,** 95-9 (2000).
Acetyl-CoA C-acetyltransferase	Solyc07g045350.2.1	2,0	AT5G47720	-
Patatin-like protein 3	Solyc05g056030.2.1	2,0	AT2G39220	-
Oxysterol-binding protein	Solyc11g013190.1.1	2,0	AT4G08180	Trends Plant Sci. **9**, 378-84 (2004).
Calcium Signaling and Transportation				
Calcium protein kinase	Solyc01g096820.2.1	4,3	AT5G12180	Plant J. **59**, 528-39 (2009).
Sodium/calcium exchanger	Solyc07g006370.1.1	3,2	AT5G17850	Plant Physiol. **126**, 1646-67 (2001).
Calcium-transporting ATPase 1	Solyc09g082890.1.1	2,9	AT3G22910	J. Biol. Chem. **277**, 9840-52 (2002).
Sodium/calcium exchanger	Solyc07g042000.2.1	2,4	AT3G14070	Plant Physiol. **148**, 1474-86 (2008).
Calcium-transporting ATPase 1	Solyc02g090560.2.1	2,3	AT4G29900	Plant Physiol. **159**, 798-809 (2012)
Calcineurin B-like protein	Solyc08g077770.2.1	2,3	AT4G17615	Plant Cell. **15**, 1833-45 (2003).
C2 domain-containing protein	Solyc07g048040.1.1	2,3	AT5G55530	-
Calcium-binding protein	Solyc10g061870.1.1	2,2	AT1G64850	-

Calcium-transporting ATPase 1	Solyc04g016260.2.1	2,1	AT2G41560	Plant Physiol. **124**, 1814-27 (2000).
Calcium transactivator	Solyc04g009820.2.1	2,1	AT3G57330	Plant Physiol. **154**, 1158-71 (2010).
Calcium/proton exchanger	Solyc07g056110.2.1	1,8	AT3G13320	FEBS Lett. **579**, 2648-56 (2005).
Calcium protein kinase	Solyc01g096350.2.1	1,8	AT2G46700	Biochem. Biophys. Res. Commun. **342**, 119-26 (2006).
Calcium protein kinase 3	Solyc01g112250.2.1	1,6	AT4G35310	-
Calcium protein kinase	Solyc02g032820.2.1	1,6	AT4G04720	Mol Plant. **4**, 83-96 (2011).
C2 domain-containing protein	Solyc07g008070.1.1	1,6	AT2G33320	-
Calcium protein kinase 8	Solyc11g065660.1.1	1,6	AT5G12480	-
MPTP Opening				
VDAC Protein	Solyc07g008350.2.1	1,4	AT3G01280	Plant Mol Biol. **78**, 431-46 (2012).
VDAC Protein	Solyc02g092440.2.1	1,4	AT5G15090	Plant Mol Biol. **78**, 431-46 (2012).
Ornithine transporter	Solyc10g009090.2.1	1,9	AT2G33820	Plant J. **33,** 1027-35 (2003).
Mitochondrial carrier protein	Solyc09g011360.2.1	1,8	AT3G53940	-
Mitochondrial carrier protein	Solyc06g082290.2.1	1,8	AT2G30160	-
Mitochondrial carrier protein	Solyc01g095510.2.1	1,6	AT4G27940	Planta. **226**, 1031-9 (2007).
Mitochondrial carrier protein	Solyc12g008770.1.1	1,5	AT4G28390	Eur. J. Biochem. **269**, 3172-81 (2002).
Mitochondrial ADP/ATP carrier	Solyc07g053830.2.1	1,4	AT5G13490	Eur. J. Biochem. **269**, 3172-81 (2002).
ADPATP carrier protein 1	Solyc08g063030.2.1	1,4	AT4G01100	Plant Physiol. **148**, 1797-808 (2008).
Mitochondrion-Microtubule association				
Misato homolog 1	Solyc01g100190.2.1	1.4	AT4G37190	Exp Cell Res. **313**, 1393-404 (2007).
Fission Proteins				
FIS1a	Solyc11g065100.1.1	1,4	AT3G57090	Mol Plant. **1**, 1036-47 (2008).
FIS1b	Solyc12g099770.1.1	1,3	AT5G12390	Mol Plant. **1**, 1036-47 (2008).
Dynamin-2[a]	Solyc01g103120.2.1	1,7	AT1G59610	Proc. Natl. Acad. Sci. U.S.A. **107**, 6094-9 (2010).
Dynamin-1[a]	Solyc01g095970.2.1	1,4	AT5G42080	Plant Cell. **15**, 899-913 (2003).
WRKY Transcription Factors				
WRKY transcription factor 73	Solyc06g070990.2.1	19,9	AT5G15130	-
WRKY transcription factor 6	Solyc05g050340.2.1	2,8	AT4G11070	-
WRKY transcription factor 23	Solyc08g081610.2.1	2,3	AT4G01250	Mol. Cells. **31**, 303-

				13 (2011).
WRKY transcription factor 5	Solyc07g055280.2.1	2,3	AT1G29280	-
WRKY transcription factor 5	Solyc02g072190.2.1	2,0	AT3G58710	-
WRKY transcription factor	Solyc07g051840.2.1	1,9	AT1G62300	Plant Cell. **21**, 3554-66 (2009).
Transcription factor WRKY	Solyc02g080890.2.1	1,8	AT4G04450	-
WRKY transcription factor	Solyc04g051540.2.1	1,7	AT4G39410	-
DNA-binding WRKY VQ	Solyc02g078030.1.1	1,6	AT1G28280	Plant Physiol. **159**, 810-25 (2012).
WRKY transcription factor 32	Solyc01g104550.2.1	1,5	AT1G68150	-
TCP Transcription Factors				
TCP family transcription factor	Solyc08g080150.1.1	3,1	AT2G45680	-
Transcription factor TCP	Solyc06g069460.1.1	1,9	AT3G02150	Plant Cell. **20**, 2293-306 (2008).
TCP13	Solyc03g115010.1.1	1,6	AT1G30210	Plant Cell. **17**, 2693-704 (2005).
CYCLOIDEA	Solyc10g008780.1.1	1,5	AT2G31070	Plant J. **71**, 99-107 (2012).
TCP3	Solyc07g053410.2.1	1,5	AT1G53230	-
bZIP Transcription Factors				
BZIP transcription factor	Solyc01g079480.2.1	1,4	AT3G62420	Plant Physiol. **150**, 84-95 (2009).
Import Machinery				
TXR1	Solyc08g006120.2.1	1,7	AT3G59280	-
PAM16	Solyc06g068820.2.1	1,5	AT5G61880	-
TIM23-2	Solyc03g121860.1.1	1,4	AT1G72750	Plant Cell. **24**, 2675-95 (2012).
OXA1	Solyc08g008320.2.1	1,4	AT5G62050	Plant Cell Physiol. **41**, 1157-63 (2000).
Metaxin 2	Solyc07g009320.2.1	1,4	AT2G19080	Plant Cell. **19**, 3739-59 (2007).

Additional File 5: Table S4. Abiotic stress-responsive genes upregulated in P07 compared with WT. The classification was based on STIFDB and shows the most upregulated genes involved in different types of abiotic stresses.

Stress/UNIPROT Description	*S. Lycopersicum* ID	FC	TAIR ID	Reference
Heat				
Heat shock protein	Solyc01g099660.2.1	6,6	AT5G42020	Plant Cell Physiol. **37**:862-5 (1996).
Heat shock protein	Solyc11g066060.1.1	2,7	AT3G12580	Plant Physiol. **126**, 789-800 (2001).
Heat stress transcription factor A3	Solyc08g062960.2.1	2,3	AT5G03720	Plant J. **53**, 264-74 (2008).
Heat shock protein	Solyc11g066100.1.1	2,2	AT3G12580	Plant Physiol. **126**,789-800 (2001).
Heat shock protein	Solyc03g082920.2.1	2,0	AT1G09080	Plant Cell Physiol. **37**, 862-5 (1996).
Chaperone protein dnaJ 2	Solyc02g077670.2.1	1,9	AT2G20560	-
Chaperone protein DnaJ	Solyc02g014860.2.1	1,8	AT4G39960	-
Heat stress transcription fator	Solyc03g026020.2.1	1,7	AT4G36990	Mol. Gen. Genet. **258**, 269-78 (1998).
Cytochrome P450	Solyc07g062500.2.1	1,7	AT3G14620	-
Alpha-amylase	Solyc03g095710.2.1	1,7	AT4G25000	Plant Cell Environ. **30**, 388-98 (2007).
Osmotic				
GID1-like gibberellin receptor	Solyc06g008870.2.1	3,6	AT3G63010	-
Protein kinase 2	Solyc03g095510.2.1	2,1	AT3G08720	Plant Cell. **18**, 477-90 (2006).
Calcium-transporting ATPase 1	Solyc04g016260.2.1	2,1	AT2G41560	Plant Physiol. **124**, 1814-27 (2000).
U-box domain-containing protein	Solyc05g056500.1.1	1,6	AT3G19380	-
Chorismate mutase 2	Solyc11g017240.1.1	1,5	AT5G10870	-
Folate/biopterin transporter	Solyc12g005140.1.1	1,5	AT5G54860	-
Cold				
Unknown Protein	Solyc10g050220.1.1	11,7	AT4G33980	-
TPX-2	Solyc07g052530.2.1	8,6	At1g05260	Plant J. **32**, 13-24 (2002).
ABC transporter G family	Solyc01g101070.2.1	4,2	AT3G53480	-
Pyruvate decarboxylase 1	Solyc09g005110.2.1	4,0	AT4G33070	-
Pyruvate decarboxylase	Solyc10g076510.1.1	3,9	AT4G33070	-
Receptor-like protein kinase	Solyc04g006930.2.1	3,7	AT4G18670	-
UDP-glucosyltransferase	Solyc00g227860.1.1	3,7	AT2G22590	-
Mate efflux family protein	Solyc11g010380.1.1	3,6	AT5G65380	-
TCP family transcription fator	Solyc08g080150.1.1	3,1	AT2G45680	-
Serpin	Solyc04g079470.2.1	3,1	AT1G47710	J. Mol. Biol. **364**, 625-36 (2006).
CONSTANS zinc finger protein	Solyc02g079430.2.1	3,0	AT4G27310	-
UDP-glucosyltransferase	Solyc12g096080.1.1	3,0	AT4G34135	-
Multidrug resistance protein mdtK	Solyc04g074840.2.1	2,9	AT5G65380	-
Omega-6 fatty acid desaturase	Solyc12g044950.1.1	2,7	AT3G12120	Plant Cell Physiol. **48**, 856-65 (2007).

Beta-1 3-glucanase	Solyc04g016470.2.1	2,1	AT3G57260	J. Exp. Bot. **57**,1537-46 (2006).
Drought				
Mitochondrial malate carrier	Solyc05g051400.2.1	13,0	AT4G24570	-
ABC transporter G family	Solyc04g070970.2.1	8,8	AT5G06530	Mol. Cell Proteomics. **6**, 1198-214 (2007).
Neutral invertase like protein	Solyc01g111100.2.1	4,6	AT4G34860	-
Timing of CAB expression-like	Solyc03g115770.2.1	3,5	AT5G61380	EMBO J.**28**,3745-57 (2009).
F-box family protein	Solyc12g006130.1.1	3,3	AT3G07870	-
Acyl-protein thioesterase 2	Solyc08g067160.2.1	3,3	AT5G20060	-
Kinase family protein	Solyc02g091170.2.1	3,2	AT4G18950	-
Hydroxycinnamoyl CoA	Solyc07g005760.2.1	3,0	AT5G48930	-
Zinc finger A20 and AN1	Solyc01g086970.2.1	3,0	AT3G12630	-
N-acetyltransferase	Solyc08g068780.1.1	2,4	AT2G39030	-
Homeobox leucine zipper protein	Solyc03g082550.2.1	2,3	AT2G46680	Plant J. **10**, 375-81 (1996).
Zinc finger A20 and AN1	Solyc10g083460.1.1	2,2	AT3G52800	-
F-box family protein	Solyc09g072680.1.1	2,2	AT3G07870	-
CHY zinc finger family protein	Solyc08g067960.2.1	2,2	AT5G25560	-
Prephenate dehydratase	Solyc06g074530.1.1	2,1	AT1G08250	-
NaCl				
FAD-binding protein	Solyc02g070070.1.1	7,7	AT1G30760	-
Galactinol synthase	Solyc02g084980.2.1	6,0	AT2G47180	Plant Physiol. **147**,1251-63 (2008).
Ammonium transporter	Solyc04g050440.2.1	5,7	AT1G64780	-
Epoxide hydrolase 3	Solyc02g078570.2.1	5,0	AT3G51000	-
Chaperone protein dnaj	Solyc11g071830.1.1	4,0	AT3G44110	-
Ninja-family protein 3	Solyc02g088910.2.1	3,5	AT3G29575	-
Glutathione transferase	Solyc08g062570.1.1	3,4	AT1G74590	-
Protein kinase	Solyc04g064590.1.1	3,3	AT1G53570	-
Glutathione S-transferase 12	Solyc03g116130.1.1	3,1	AT1G74590	-
Hydroxycinnamoyl transferase	Solyc05g052680.1.1	3,1	AT2G39980	-
N-benzoyltransferase	Solyc05g052670.1.1	2,9	AT2G39980	-
Auxin responsive protein	Solyc01g097290.2.1	2,8	AT3G04730	-
Unknown Protein	Solyc12g087960.1.1	2,8	AT4G38060	-
CBL-interacting protein kinase 7	Solyc12g010130.1.1	2,8	AT4G30960	Plant J. **58**, 778-90 (2009).
Wound-induced protein 1	Solyc09g014940.1.1	2,6	AT5G01740	-
Glucose transporter member 5	Solyc03g093400.2.1	2,5	AT5G61520	-
Wound-induced protein 1	Solyc06g050760.1.1	2,5	AT3G10985	-
Ethylene responsive regulator	Solyc12g056980.1.1	2,4	AT1G78080	FEBS J. **276**,6624-35 (2009).
NF-X1 finger transcription factor	Solyc03g118420.2.1	2,4	AT1G10170	FEBS Lett. **580**, 4851-6 (2006).
Oxidative				
Cevi-16	Solyc02g064970.2.1	19,9	AT5G39580	-
Class I heat shock protein	Solyc10g086680.1.1	8,4	AT1G07400	Plant J. **41**,212-20 (2005).
Blue copper protein	Solyc07g008130.2.1	7,5	AT5G20230	Plant Physiol. **122**,

				657-65 (2000).
PO2	Solyc10g084240.1.1	3,1	AT2G37130	-
ClpB chaperone	Solyc03g115230.2.1	3,0	AT1G74310	Plant J. **48**, 535-47 (2006).
Cytochrome P450	Solyc02g084570.2.1	2,7	AT4G36220	Plant Physiol. **109**, 1159-66 (1995).
Proline dehydrogenase	Solyc02g089630.2.1	2,5	AT3G30775	Plant Physiol. **116**, 409-18 (1998).
POD-5	Solyc03g044100.2.1	2,3	AT3G49960	-
GH3 family protein	Solyc10g011660.2.1	2,3	AT2G46370	Plant Physiol. **134**, 1536-45 (2004).
FBP-5	Solyc11g072920.1.1	2,2	AT5G06720	-
Superoxide dismutase	Solyc02g082590.2.1	1,5	AT1G08830	Plant Cell. **18**, 2051-65 (2006).
Glutathione peroxidase	Solyc12g056240.1.1	1,4	AT1G63460	-
Mitochondrial carrier protein	Solyc01g095510.2.1	1,6	AT4G27940	Planta. **226**, 1031-9 (2007).
Cadmium				
Pyruvate kinase	Solyc09g008840.2.1	2,8	AT3G52990	Proteomics. **6**, 2180-98 (2006).
Chitinase	Solyc10g055800.1.1	3,7	AT3G12500	Proteomics. **6**, 2180-98 (2006).
Multidrug resistance protein 2	Solyc10g055770.1.1	2,2	AT3G12500	Proteomics. **6**, 2180-98 (2006).
Enoyl-reductase II	Solyc01g105370.2.1	2,5	AT5G64250	J. Exp. Bot.**57**, 4003-13 (2006).
Other				
WRKY transcription factor	Solyc07g051840.2.1	1,9	AT1G62300	Plant Cell.**21**, 3554-66 (2009)
NAC domain transcription factor	Solyc04g078670.2.1	1,8	AT5G63790	Plant Physiol. **149**, 1724-38 (2009).
ELF4-like protein	Solyc06g051680.1.1	5,5	AT2G40080	Plant Physiol.**133**, 1530-8 (2003).
Homeobox-leucine zipper protein	Solyc08g078300.2.1	4,4	AT4G16780	Plant J. **4**, 469-79 (1993).

Additional File 6: Table S5 - Summary of RNASeq data filtered and mapped with Bowtie.

Library	Sequenced reads	Filtered reads	Mapped reads	% of mapped reads
P07P1F3	28.427.445	27.905.560	20.912.405	74,94
P07P2F3	100.466.818	90.289.253	66.222.859	73,35
P07P3F3	67.479.470	65.760.027	48.887.230	74,34
P07P4F3	57.941.595	56.154.368	41.363.058	73,66
WTP1F3	115.484.011	113.267.169	79.782.022	70,44
WTP2F3	61.768.888	55.603.556	39.791.178	71,56
WTP3F3	85.125.227	83.066.501	59.319.951	71,41
WTP4F3	52.352.176	50.748.225	36.352.829	71,63
Total	**569.045.630**	**542.794.659**	**392.631.532**	**72,33**

Capítulo 2 - UCP1 overexpression in tobacco alters organellar signaling and reconfigures cellular energy metabolism

UCP1 overexpression in tobacco alters organellar signaling and reconfigures cellular energy metabolism

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Abstract

Uncoupling mitochondrial proteins (UCPs) dissipates the proton gradient that is used for ATP production. The overexpression of UCP1 in plants induces mitochondrial biogenesis, reduces ROS production, increases net photosynthesis rates and amplifies the abiotic stress response. Here, we present a detailed analysis of the transcriptomes of UCP1-overexpressing tobacco plants (P07) compared with those of wild-type plants. In depth RNA sequencing allowed for the identification of 2,237 up-regulated and 991 down-regulated genes in P07 compared with wild type. We observed a global up-regulation of the abiotic stress response, fatty acid metabolism and anaerobic respiration. Interestingly, a large number of the proteins encoded by differentially expressed genes (DEGs) were targeted to the chloroplast. The alterations in energy metabolism were accompanied by the activation of signaling and regulatory mechanisms involving a set of protein kinases and transcriptional regulators. Among the up-regulated transcription factors were HRE2 and WRI1, which are important regulators of responses to hypoxia and lipid biosynthesis, respectively. The data show that UCP1 overexpression imposes strong alterations in mitochondrial activity that alter organellar signaling and readapt cellular metabolism. This overexpression results in plants that perform better under unfavorable conditions.

Highlights:

- UCP1 overexpression provokes a stressful environment inside the cell
- Cellular adaptation includes the induction of genes that encode mitochondrial and chloroplastic proteins
- UCP1 overexpression induces a reconfiguration of cellular energy metabolism
- These adaptations lead to better performance under stressful conditions

Keywords:

UCP1, oxidative stress, biogenesis, transcriptome, mitochondria, chloroplast, stress response.

1. Introduction

Mitochondria and chloroplasts are complex cytoplasmic organelles that originated from the incorporation of prokaryotic organisms into primordial eukaryotic cells [1-4]. The nuclear genome has been estimated to encode 800–2000 genes that originated from cyanobacteria and gave rise to chloroplasts and more than 1000 genes that originated from α-proteobacteria and gave rise to mitochondria [2-4]. Although extensive gene transfer occurred from these organelles to the nucleus, both of these organelles preserved a small number of genes within their genomes that are essential for energy metabolism and photosynthesis [5,6].

Plants are regularly exposed to adverse environmental conditions that alter the functions of the mitochondria and chloroplasts, triggering a feedback mechanism that induces nuclear gene expression to restore cellular and organellar functions [7]. This mechanism is referred to as retrograde regulation (RR) and is far less studied in plants than in non-plant models [8]. This knowledge gap is due in part to the complexity of plant cells and the fact that several proteins are dual-localized to the mitochondria and chloroplasts, which are interconnected organelles [8,9]. In non-plant models, mild mitochondrial stress is thought to trigger RR and induce nuclear and cytoplasmic adaptations, which in turn make the organism more tolerant to subsequent stressful conditions [10]. The mammalian PGC1-α is a classical example of a gene that plays a role in this process because it is able to induce mitochondrial biogenesis along with potent antioxidant machinery in response to certain metabolic disturbances [11]. Pentatricopeptide proteins [12] and members of the transcription factor families bZIP [13], WRKY [14], TCP [15] and NAC [7] have been suggested to contribute to mitochondrial RR in plants during stressful conditions. Recently, we showed that the overexpression of mitochondrial uncoupling protein 1 (UCP1) in tobacco provokes metabolic stress that triggers RR by decreasing cellular ATP concentrations. This decrease induces the expression of an array of nuclear- and mitochondrial-encoded genes, along with a large number of stress-responsive genes, resulting in better plant adaptations to several abiotic stresses [16].

UCPs comprise families of nuclear-encoded proteins that are localized to the mitochondrial inner membrane [17,18]. In the brown adipose tissue (BAT) of animals and in the presence of free fatty acids, UCP1 uncouples the electrochemical gradient from ATP synthesis, dissipating energy as heat [19]. The identification of UCP1 homologs in other human tissues [17] and orthologs in several eukaryotic organisms [18] suggests that other physiological functions exist for these proteins in addition to thermogenesis. UCPs may serve as escape valves, decreasing the proton motive force (PMF) and preventing reactive oxygen species (ROS) production under unfavorable conditions or when the cellular demand for ATP is low [17,18,20]. In fact, the overexpression of mammalian UCP members results in the reduction of cellular ROS production [21,22]. In plants, members of the UCP family have been associated with several physiological roles, including thermogenesis regulation [23], abiotic stress responses [16,24-27] and climacteric increases in respiration [28]. Plant UCP1 genes, along with other UCP family members, are expressed in a time- and tissue-dependent manner and upregulaed by cold stress [18,29,30]. The overexpression of UCPs protects plants from biotic [27] and abiotic stresses, including oxidative, salt, drought, cold, and high and low pH stresses [16,24-27,31]. In addition, UCP-overexpressing plants also exhibit increased photosynthetic capacities [26,27], which is consistent with the observation that a UCP1 insertional knockout in *Arabidopsis thaliana* negatively affected chloroplast metabolism [32].

Here, we present a detailed analysis of transcriptomic profiling in tobacco plants overexpressing *A. thaliana* UCP1 (*At*UCP1) compared with their wild-type counterparts. This analysis focused on elucidating the altered cellular metabolism and increased stress tolerance that has been observed to occur in *At*UCP1 overexpressors in addition to identifying possible regulators that mediate organellar communication in plants.

2. Materials and Methods

2.1. Plant Materials

The tobacco (*Nicotiana tabacum*) ecotype SR1 overexpressing *At*UCP1 was obtained as previously described [31]. In this work, we used the transgenic line P07, which expresses high levels of *At*UCP1 [16,31]. P07 and wild-type plants were grown for 12

weeks at 24°C at a photoperiod of 16 h of light (100 µE $m^{-2}s^{-1}$) and 8 h of darkness. For the RNA-seq and quantitative real-time polymerase chain reaction (qRT-PCR) analyses, samples of the third youngest leaf for each genotype were collected, frozen in liquid nitrogen and stored at -80°C. Four biological replicates were sampled for each genotype.

2.2. Preparation of cDNA library and transcriptome sequencing

Total RNA was extracted from wild type and P07 frozen leaves, and the Illumina TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA, USA) was used to create single-end RNA-seq libraries as previously described [16]. The libraries were sequenced on four lanes using the Illumina HiSeq 2000 sequencing system for 75 cycles. The Illumina reads were filtered to remove adapters and low quality reads (reads with less than 70% of the bases possessing quality scores of ≥ Q20) using AdapterRemoval [33], the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) and Perl scripts. After the filtering step, the reads were subjected to digital normalization using the Diginorm software (https://github.com/ged-lab/2012-paper-diginorm). The resulting reads from the last cleaning step were assembled with the Trinity assembler [34]. The sequence data are available in the Sequence Read Archive (SRA), which is accessible through NCBI BioProject ID PRJNA211804 under the experiment ID SUB287723.

2.3. Mapping and annotation of differentially expressed genes

High quality reads were mapped to the Trinity contigs using the Bowtie software [35], and reads per kilobase per million (RPKM) [36] mapped reads values were calculated for the contigs as the expression values. An RPKM cut-off value of 0.1 was set to indicate contig expression. Because the *N. tabacum* genome has not yet been completely sequenced, and the scaffolds of *N. benthamiana* are estimated to cover only 79% of its genome, we used the complete genome sequence of *Solanum lycopersicum* [37], which is a close relative of *N. tabacum,* to map and annotate the expressed contigs. To identify the protein-coding genes, all of the contigs were used as queries in BLASTn and BLASTx searches against both the non-redundant set of tomato coding sequences (CDS) and predicted protein sequences. The representative contigs for each gene were selected and submitted to statistical analyses for the identification of the differentially expressed genes

(DEGs) as previously described [16]. The DEGs were selected for the next steps of the analysis. The fold-change (FC) values for the representative transcripts were calculated as the ratios between the average RPKM values in P07 and wild type.

2.4. Functional annotations

Annotations were carried out only for those DEGs that possessed RPKM values that varied by more than 1.5-fold (FC $\geq$ 1.5 or $\leq$ -1.5). Gene function data were obtained from the existing annotations of the tomato genome and by BLASTp searches against the UNIPROT database [38]. The resulting protein dataset was mapped against the COG (Eukaryotic Clusters of Orthologous Groups) [39] database by BLASTp, and COG functional categories were assigned only if the two best hits had the same COG function. The DEGs were also assigned gene ontology (GO) [40] categories and Kyoto Encyclopedia of Genes and Genomes (KEGG) [41] categories using the Blast2GO [42] software. For the GO term enrichment analysis using Fischer's exact test, all of the DEGs were mapped to GO terms and searched for significantly enriched ontologies compared with the whole transcriptome background. Transcription factors were mapped by a BlastP search against the Plant Transcription Factor Database (PlnTFDB 3.0) [43], and Perl scripts were used to identify only the bi-directional best hits (BBH) [44]. The transcription factor annotations were assigned to the corresponding representative tobacco contig.

2.5. qRT-PCR validation

Thirteen contigs that were up- or down-regulated in P07 were used for the experimental expression validation (Supplemental Table S1). Total RNA that was isolated from the P07 and wild-type plants was used for first-strand cDNA synthesis using the Revertaid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's protocol. Real-time PCR was performed using the ABI PRISM 7500 system (Applied Biosystems, Foster City, CA, USA) with SYBR Green dye (Applied Biosystems). The reactions were performed at least in triplicate with four biological replicates, and the results were expressed relative to the expression levels of the *actin1* gene in each sample using the 2ΔΔCT method. All values are presented as fold changes of P07

relative to wild type. Student's t test was performed to determine significant changes ($p < 0.05$).

3. Results

3.1. Read processing, assembly and mapping

The transcriptome profiles of the P07 tobacco plants overexpressing *At*UCP1 compared with their wild-type counterparts were assessed using the leaves of 12-week-old plants. cDNA that was prepared from total RNA from four biological replicates for each genotype was sequenced using the Illumina HiSeq 2000 sequencing platform. A total of 42.7 Gb of sequence data were generated for the 8 libraries with a minimum of 27.9 million and a maximum of 113.2 million reads for each library (Supplemental Table S2). After trimming for low quality reads, the resulting 569 million high quality reads were submitted to digital normalization using the Diginorm software (https://github.com/ged-lab/2012-paper-diginorm), resulting in 49.5 million normalized reads. These normalized reads were assembled into 271,750 contigs with an average length of 1163 base pairs (Table 1). To identify protein-coding sequences, the 271,750 contigs were used as queries in BLASTn and BLASTx searches against tomato CDS and predicted protein sequences. Of the 271,750 contigs, 134,752 mapped to the tomato genome, resulting in the annotation of 20,045 distinct orthologs for the tomato CDS models.

The 569 million high-quality reads were mapped to the 271,750 assembled contigs, and gene expression levels were measured as RPKM values, which ranged from 1×10^{-3} to more than 2×10^{4}. Further analyses were performed only for those genes whose RPKM values were greater than 0.1, that were significantly different between wild type and P07 and that had fold-changes of more than 1.5 (up- or down-regulated) in the P07 plants. Using these criteria, we were able to identify 2,237 genes that were up-regulated and 991 that were down-regulated (Table 1). The gene encoding *At*UCP1 was approximately 1,000-fold up-regulated compared with the endogenous tobacco-encoded UCP1 gene (*Nt*UCP1). The large number of DEGs in the P07 and wild-type plants suggests a global transcriptome-level adaptation due to increased UCP1 expression.

Table 1

Distribution of Trinity generated contigs

Total number of reads		316,023,367
Mean contig length (bp)		1,163
Median contig length (bp)		728
Number of mapped contigs		134,752
Number of annotated unique genes		20,075
Number of up-regulated genes (FC >= 1.5)		2,237
Number of down-regulated genes (FC <= -1.5)		991
Distribution of contig length (bp)		
	200 - 500	96,920
	500 - 1,000	60,142
	1,000 - 1,500	35,142
	1,500 - 2,000	26,331
	≥ 2, 000	53,215
	Total	271,750

3.2. Annotation of DEGs

The DEGs that were annotated by matching with the tomato CDS were used as queries in a BlastP search against the UNIPROT database [38]. To further evaluate the effectiveness of the annotations, we searched the annotated sequences for genes with Cluster of Orthologous Group (COG) classifications [39]. COG annotations allowed for the classifications of 1,679 up-regulated genes and 550 down-regulated genes into 25 functional categories (Supplemental Table S3). Among these categories, the cluster of "general function prediction" comprised the largest up-regulated group (508 genes, 22.7%), followed by "signal transduction mechanisms" (263 genes, 11.7%), "posttranslational modification, protein modification and chaperones" (222 genes, 9.9%), "carbohydrate transport and metabolism" (126 genes, 5.6%) and "transcription" (112 genes, 5%) (Fig. 1, blue bars). Similar distribution patterns among COG categories were found for the down-regulated genes with the exception of the "secondary metabolites biosynthesis, transport and metabolism" category, which comprised 9.6% of the down-regulated categorized genes (Fig. 1, red bars). These data are in accordance with our previous hypothesis that *At*UCP1 overexpression broadly impacts cellular metabolism, requiring global changes in regulatory

mechanisms at both the transcriptional and posttranscriptional levels [16]. Importantly, many of the categorized up- (27.6%) and down-regulated genes (18.5%) corresponded to poorly characterized proteins, most with no function predicted (Fig. 1 and Supplemental Table S3).

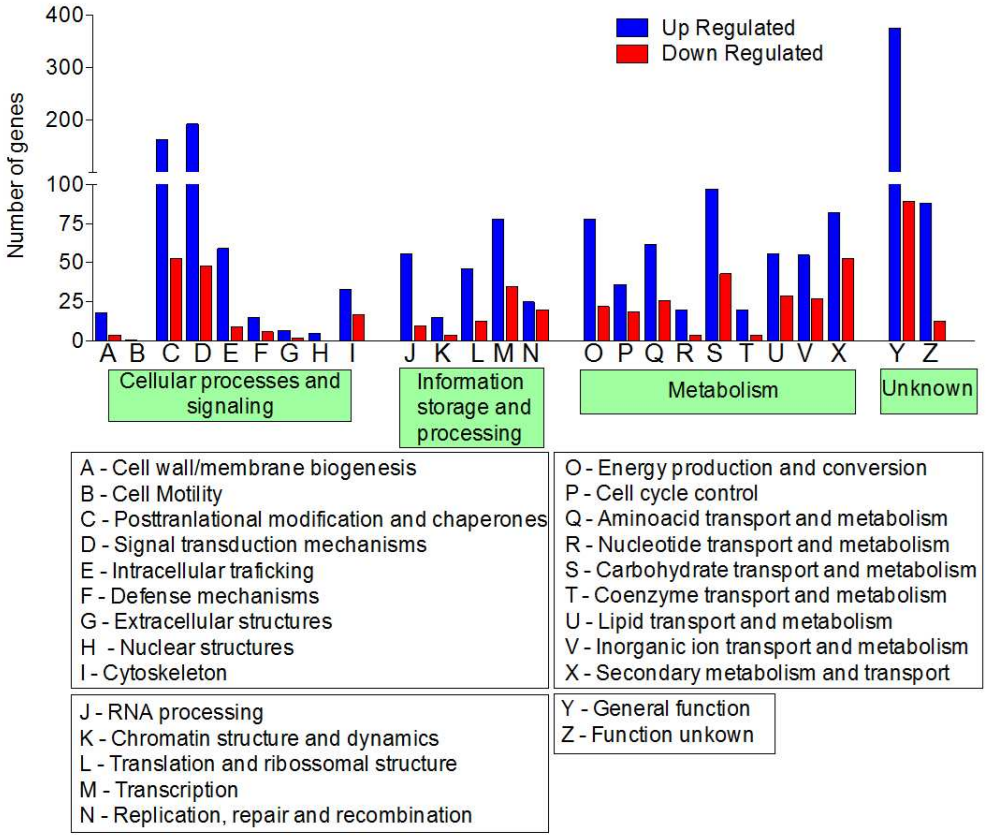

Figure 1. Histogram of COG classifications. DEGs were used as queries against the COG database, yielding 1,679 classifications of up-regulated genes (blue bars) and 550 classifications of down-regulated genes (red bars) into 25 different categories.

Gene ontology (GO) [40] assignments were applied to classify the functions of the DEGs using Blast2GO [42]. A total of 546 genes (375 up- and 171 down-regulated) were not assigned to any GO term (Supplemental Table S4). We were able to assign at least one term to the 1,862 up-regulated genes. The GO assignments are divided into three main categories, including "biological process", "molecular function" and "cellular compartment". The most abundant GO term in the "biological process" category was

“regulation of transcription, DNA-dependent” which comprised 6.9% of the annotated genes. In the “molecular function” category, the ontologies “protein binding” and “ATP binding” were the most highly represented, comprising 22.7% and 12.9% of the annotated genes, respectively, while in the “cellular compartment” category, the most represented terms were “nucleus”, “plasma membrane” and “mitochondrion” (Table 2). Different patterns were found among the down-regulated genes, in which the most representative GO term of the “molecular function” category was “oxidation-reduction process”, comprising 9.1% of the annotated genes; for “cellular compartment”, the most representative terms were “nucleus”, “plasma membrane”, “extracellular region” and “chloroplast” (Table 2).

An enrichment analysis (Fisher’s exact test) [45] was used to identify the most representative ontologies of the up- and down-regulated groups of genes (Table 3). Among the up-regulated genes, the ontologies “stress response” (442 genes), “purine nucleotide binding” (302 genes) and “ATP binding” (243 genes) were the most highly represented. This analysis clearly shows that the overexpression of UCP1 up-regulates genes that are involved in metabolic pathways that are associated with protection against abiotic stresses (Tables 2 and 3). Because ATP and ADP levels are directly associated with energy production and cell signaling [46], the over-representation of the ontologies “ADP binding” (Table 2) and “ATP binding” (Table 3) in P07 suggests that these molecules play an important role in signaling and adaptation to these abiotic stresses, which are imposed by the increased UCP1 expression in the P07 mitochondria. This hypothesis is in accordance with the observation that ATP is the signaling molecule that links the functional state of the mitochondria to nuclear gene expression in budding yeast [47].

Additionally, the GO terms “purine nucleotide binding” and “fatty acid beta-oxidation” were enriched. Prior to the functional identification of UCP1, the addition of purine nucleotides to isolated mitochondria in BAT was shown to reduce high rates of uncoupled respiration [19]. Purine nucleotides, such as ADP and GTP, bind with high affinity to the nucleotide-binding site of UCP1, inhibiting its activity [19,20,48]. However, UCP1 activity is stimulated by fatty acids [17,18,49]; therefore, fine-tuning should occur inside of the cell to maintain and modulate UCP1 activity at proper levels.

Table 2

Comparison of gene ontology (GO) classifications of DEGs in P07 compared with wild type plants

GO-term	Number of Genes	
	Up	Down
Molecular Function		
protein binding	424	122
ATP binding	242	64
DNA binding	122	48
zinc ion binding	115	44
protein serine/threonine kinase activity	114	30
DNA binding transcription factor activity	110	42
nucleic acid binding	62	11
ADP binding	43	2
heme binding	39	24
chromatin binding	33	7
metal ion binding	32	14
Biological Process		
regulation of transcription, DNA-dependent	129	48
protein phosphorylation	122	32
oxidation-reduction process	91	66
response to salt stress	58	23
response to cadmium ion	57	13
defense response	57	7
transmembrane transport	53	18
response to water deprivation	51	12
protein targeting to membrane	50	10
regulation of plant-type hypersensitive response	50	10
response to cold	46	18
Cell compartment		
Nucleus	384	138
plasma membrane	309	121
Mitochondrion	156	22
Cytosol	154	46
Chloroplast	145	61
Plasmodesma	100	38
Golgi apparatus	86	38
extracellular region	70	73
vacuolar membrane	46	9
endoplasmic reticulum	46	15
cell wall	34	21

Table 3

Fischer enrichment analysis of Gene Ontology (GO) data

Ontology	Number of genes
Up-regulated	
response to stress	442
purine nucleotide binding	302
ATP binding	243
kinase activity	173
nucleic acid binding transcription factor activity	110
response to ethylene stimulus	51
regulation of cellular response to stress	50
negative regulation of cell death	39
negative regulation of programmed cell death	37
fatty acid beta-oxidation	22
Down-regulated	
extracellular region	94
chloroplast part	61
Photosynthesis	57
generation of precursor metabolites and energy	54
cell wall	41
cell wall biogenesis	27
chlorophyll metabolic process	25
cation homeostasis	20
regulation of proton transport	12
oxygen binding	10

Interestingly, the ontologies “chloroplast part” and “photosynthesis”, comprising 61 and 57 genes, respectively, were among the enriched terms of the down-regulated genes in the P07 plants (Table 3). The association of UCP1 with photosynthesis has been previously described [32]. In fact, the overexpression of UCP1 increases photosynthetic capacity [26,27]. Thus, we further analyzed the expression of chloroplastic genes to elucidate the effects of UCP1 overexpression on chloroplast metabolism.

3.3. UCP1 overexpression affects chloroplast metabolism

The balance between energy supply and demand largely depends on a tightly regulated signaling network that coordinates three of the most critical processes to plant life, including photosynthesis, photorespiration and dark respiration [50]. Organelles are

sensitive to metabolic imbalances that can induce oxidative stress inside of the cell by promoting the generation of ROS [51]. Thus, it would be reasonable to expect that the metabolic alterations that are promoted by UCP1 overexpression [16] would also affect the expression of genes that encode chloroplast proteins.

The light reactions of photosynthesis convert light energy into chemical energy in the form of ATP and drive the production of NADPH from $NADP^+$. These molecules are not only used in the carbon fixation reactions of the Calvin cycle but also in various metabolic pathways in other organelles [52]. Because there has long been a debate about whether the ATP and NADPH that are required by the Calvin cycle are supplied solely by the photosystem reactions [52], we observed the expression patterns of the genes that were associated with the "photosynthesis" ontology, which was over-represented among the down-regulated genes in P07. Although many genes whose products are targeted to the chloroplast were up-regulated in P07 (Table 3), only two of them encode subunits of the photosynthesis reaction centers. These subunits have unknown functions and are encoded by the chloroplast genome (Fig. 2A and Supplemental Table S5). Conversely, two genes that encode proteins in the photosystem-I (PSI) and -II (PSII) complexes were 1.8- to 2.4-fold down-regulated in the P07 plants (Fig. 2A). Because the overexpression of UCP1 improves photosynthetic capacity [26,27], the ATP and NADPH that are required for carbon fixation would be expected to be partially produced by different pathways at other subcellular locations to compensate for the decreased expression of the genes encoding the PSI and PSII proteins. Thus, we further analyzed the chloroplastic genes that were up-regulated in the P07 plants (Fig. 2B, Supplemental Table S5). In fact, the most highly up-regulated genes that were targeted to the chloroplast were involved in carbon, lipid and amino acid metabolism.

One source of chloroplastic NADPH is the NADP-malic enzymes [53,54], such as NADP-ME3, which was 2.2-fold up-regulated in P07 (Fig. 2B, Supplemental Table S5. This enzyme has been suggested to be involved in fatty acid biosynthesis by producing NADPH for the heightened demands of plants during the later stages of pollen development [54]. The gene encoding the chloroplastic protein plastidic malate dehydrogenase (pMDH) was also up-regulated in P07. This enzyme catalyzes the reversible conversion of

oxaloacetate to malate using either NADH or NADPH as the oxidant or reductant, respectively. The malate valve is suggested to balance the ATP/NADPH ratio in the chloroplast as required by changing metabolic demands [55,56].

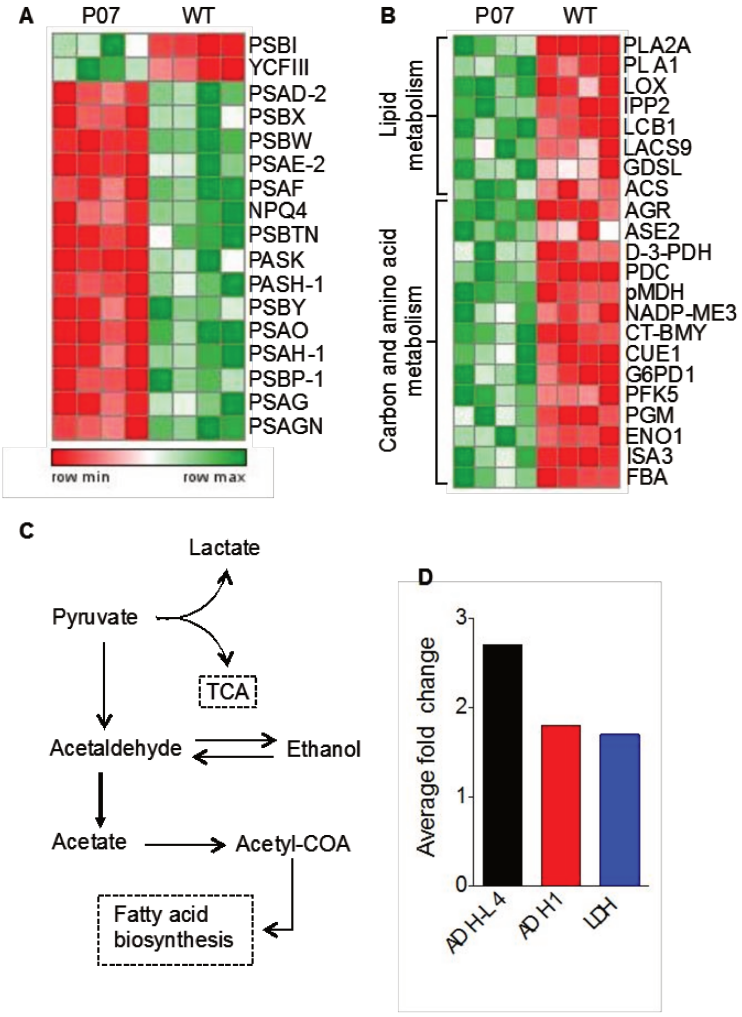

Figure 2. **The overexpression of UCP1 alters the expression of genes encoding chloroplast proteins.** **(A)** Genes targeted to photosystem reaction centers (PSI and PSII) were down-regulated in transgenic plants. **(B)** Carbon, amino acid and lipid metabolism were the best represented categories of up-regulated genes encoding chloroplast proteins. **(C)** Pyruvate may be used as carbon source for both aerobic and anaerobic respiration. Under aerobic conditions, pyruvate flows to the TCA cycle. Under anaerobic conditions, it can be converted to lactate or acetaldehyde, which is further used for alcoholic fermentation. Ethanol and acetaldehyde can also be used for fatty acid biosynthesis. **(D)** The enzymes responsible for anaerobic respiration, alcohol dehydrogenase and lactate dehydrogenase were up-regulated in the UCP1 overexpressor.

Two machineries have historically been involved in providing acetyl-CoA for lipid biosynthesis inside of chloroplasts, the plastidic pyruvate-dehydrogenase complex (pPDHC) [57], which is responsible for the majority of fatty acid biosynthesis [58,59], and plastidic acetyl-CoA synthase (ACS) [60]. The pPDHC complex uses pyruvate that is transported from the cytosol or synthesizes it inside of plastids from 3-phosphoglycerate (PGA) and 2-phosphoenolpyruvate (PEP) [61]. The expression of genes encoding components of the pPDHC complex did not change in P07 compared with wild type, but the gene that encodes the PEP translocator (CUE1) that transports PEP produced in the cytosol to the chloroplasts increased by 2.0-fold in the P07 plants (Supplemental Table S5).

Another pathway that is involved in lipid biosynthesis uses ACS and bypasses the PDHC complex, converting pyruvate into fatty acids [60,62]. It is hypothesized that this pathway is important during fertilization when pollen is undergoing rapid metabolism and growth in a low oxygen environment [63,64]. Under these conditions, plants undergo anaerobic fermentation, in which ACS is used to detoxify the byproducts of fermentation by converting them into fatty acids [63,64]. At normal oxygen concentrations, pyruvate in the cytosol flows into the tricarboxylic acid (TCA) cycle inside of the mitochondria, which ultimately generates ATP by oxidative phosphorylation reactions [17,65]. If cells undergo rapid metabolism, as occurs during pollen grain development [64], oxygen levels decline, and anaerobic fermentation is used to help increase the ATP supply. Pyruvate can be converted into lactate through lactate dehydrogenase (LDH) or ethanol by the subsequent actions of pyruvate decarboxylase and alcohol dehydrogenase (ADH) [62-64]. Ethanol and acetaldehyde, which are toxic byproducts of fermentation, can be used for lipid biosynthesis by ACS (Fig. 2C). Genes encoding all of the enzymes that are involved in the ACS pathway of lipid biosynthesis and anaerobic respiration were up-regulated in the P07 plants (Fig. 2D and Supplemental Table S5).

Additionally, genes coding for 15 chloroplastic ribosomal subunits were up-regulated in P07 (Supplemental Table S6). It is possible that these proteins are involved in post-transcriptional mechanisms that are necessary for chloroplast biogenesis [66,67].

In general, our results clearly show that the increased accumulation of UCP1 in the inner mitochondrial membrane alters central energy metabolism and affects gene expression, particularly that involving cytosolic and chloroplastic metabolism. The overexpression of UCP1 negatively impacts the expression of photosystem subunits and confers positive effects on carbon and lipid metabolism, translation, and surprisingly, anaerobic respiration. The increase in anaerobic respiration together with mitochondrial respiration [16] reflects the metabolic profile of tissues experiencing high-energy demands, such as developing pollen grains.

3.4. KEGG pathway mapping

The KEGG database [41] was used to identify the biological pathways that were altered in P07. Similar to what was revealed by the GO analysis, the "purine metabolism" pathway was the most representative pathway that was altered in P07 (Supplemental Table S7). We focused the analysis on the most highly altered energy metabolism pathways in P07, such as "starch and sucrose metabolism", "glycolysis/gluconeogenesis", "oxidative phosphorylation" and "lipid metabolism" (Supplemental Table S7-A and B). KEGG pathway mapping was used to construct an integrated model describing the effects of UCP1 overexpression on cytosolic, mitochondrial (Fig. 3A) and chloroplastic (Fig. 3B) metabolism.

Starch and sucrose metabolism provide glucose for glycolysis reactions in the cytosol. Starch conversion into glucose is mediated by amylases and isoamylases [68]. Glucose is then metabolized to pyruvate by the subsequent actions of several enzymes. The fate of pyruvate inside of the cell varies; for example, it can be used in anaerobic respiration by producing lactate or acetaldehyde, which are converted into oxaloacetate, which is then fed into the TCA cycle or transported to the mitochondria. There the mitochondrial pyruvate dehydrogenase complex (mPDHC) converts it into acetyl-CoA that is then fed into the TCA cycle. The TCA reactions produce NADH and FADH2, which are used by oxidative phosphorylation machinery (OXPHOS). Most of the genes encoding these enzymes were up-regulated in P07 (Supplemental Table S8-A), which is in accordance with previous reports of increased intermediary metabolites that are involved in

the TCA cycle [24] in addition to the increased expression of genes encoding the OXPHOS machinery [16].

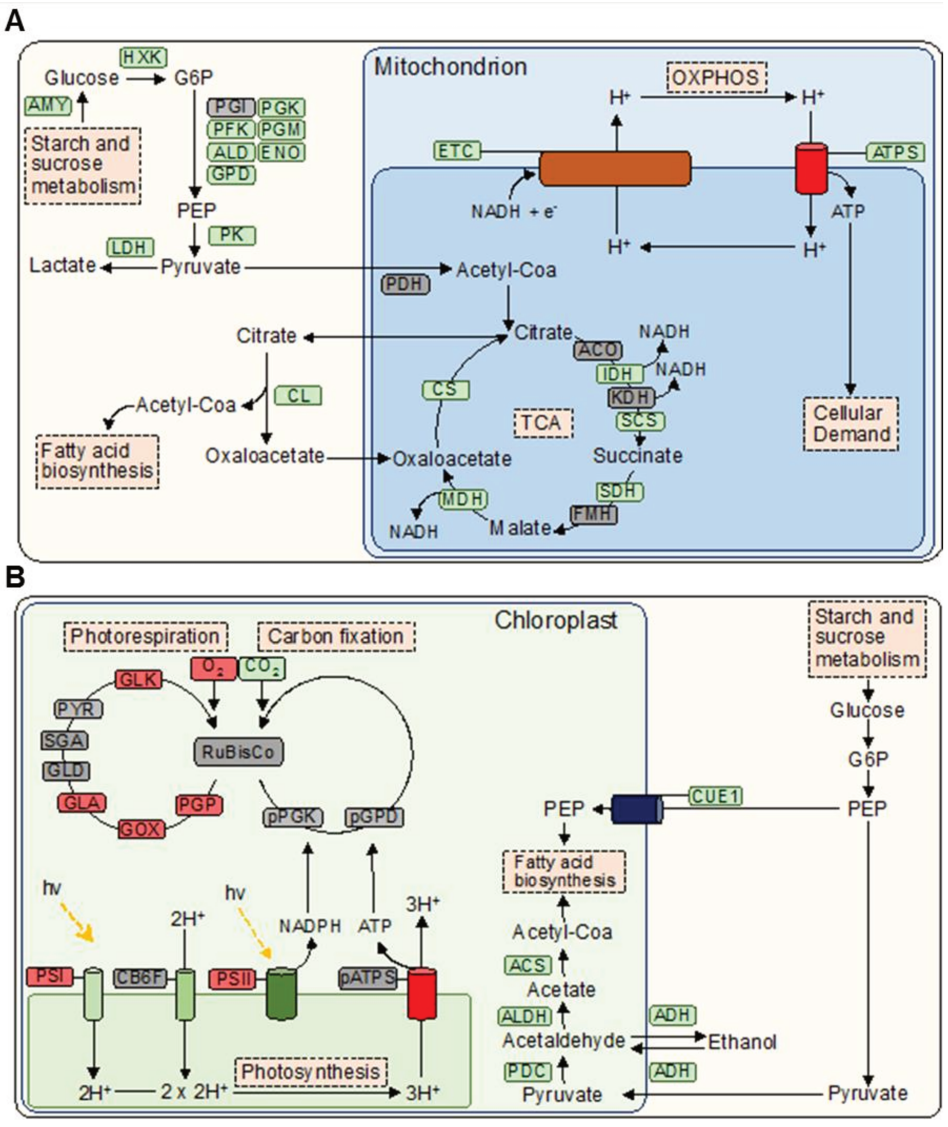

Figure 3. UCP1 affects the expression of metabolic and catabolic pathways in the cytosol, mitochondria and chloroplasts. (A) Pathways affected in the cytosol and mitochondria. **(B)** Pathways affected in the cytosol and chloroplasts. Enzyme abbreviations enclosed by green, red and gray boxes denote increased, decreased and unaltered/unidentified expression of the corresponding genes in transgenic plants in relation to wild type. The full list of abbreviations and fold-changes is presented in Supplemental Table S8-A and B.

We also observed 2.0-fold increased expression of the gene encoding ATP citrate lyase (CL) (Supplemental Table S8-A), which is a cytoplasmic enzyme that links energy metabolism from carbohydrates to the production of fatty acids by converting citrate to acetyl-CoA [69]. An Arabidopsis mutant with moderate reductions in CL activity was reported to have a complex bonsai phenotype with miniaturized organs, smaller cells, aberrant plastid morphologies, reduced cuticular wax deposition and hyperaccumulation of starch, anthocyanins and stress-related mRNAs in its vegetative tissues [70]. Additionally, the gene coding for mitochondrial glycerol-3-phosphate acyltransferase 1 (GPAT1) was shown to be 5-fold up-regulated in P07 [16]. This enzyme mediates the first step of glycerolipid biosynthesis [71,72] and is mandatory for mitochondrial fusion events in humans [71] and for the development of tapetum cells in plants [72].

Glycolysis also provides substrates for chloroplastic metabolism (Fig. 3B). Pyruvate can be transported to chloroplasts, where it is converted to acetaldehyde by the action of pyruvate decarboxylase (PDC). PEP may also be transported to the chloroplast by the PEP translocator (CUE1), where it can be used in several pathways, such as the shikimate pathway for the biosynthesis of aromatic amino acids or for fatty acid biosynthesis [61]. The plastidial PEP pool has been proposed to be the major source of carbon for fatty acid synthesis [58].

Despite the global decrease in transcripts related to PSI and PSII (Fig. 2A and Supplemental Table 5), we did not observe any alterations in the expression of genes that are associated with the other components of the photosynthetic machinery, such as the chloroplastic cytochrome B6/f or the plastidial ATP synthase complex (Fig. 3B and Supplemental Table 8-B). The importance of UCPs to photosynthesis has been previously discussed in a study investigating an *A. thaliana* mutant lacking UCP1 that proposed that UCPs allow for the increased efficiency of photorespiration [32]. In contrast, our transcriptomic data revealed that genes encoding several enzymes that participate in the early steps of photorespiration reactions, such as phosphoglycolate phosphatase (PGP) and glycolate oxidase (GOX), were down-regulated in the transgenic plants (Fig. 3B and Supplemental Table 8-B). No expression alterations were detected for the genes that participate in the light-independent reactions of carbon fixation (Supplemental Fig. 8-B).

Because of the central role of mitochondria in cellular metabolism, all of the processes that occur inside of this organelle must be coordinated with those in other organelles, such as the chloroplasts and cytosol, according to cellular demands [8]. Increased UCP1 activity is able to alter key steps of carbohydrate and fatty acid metabolism in the mitochondria, chloroplasts and cytosol. This coordinated process must involve the transcriptional control of nuclear genes encoding organellar proteins by specific transcription factors.

3.5. Transcription factors up-regulated in P07

The biogenesis of functional mitochondria requires the coordinated expression of genes that are present in both the nuclear and organellar genomes. However, the transcriptional regulators that are capable of fully inducing plant mitochondrial biogenesis remain to be identified [8]. The broad up-regulation of transcripts that are targeted to the mitochondria and chloroplasts and map to important metabolic pathways, such as lipid and carbohydrate metabolism, together with the increased performance of transgenic plants under abiotic stresses, suggests that UCP1-overexpressing plants are good models for the identification of possible regulators that are involved in these processes.

The use of DEGs as queries in a search against the Plant Transcription Factor Database (PlnTFDB 3.0) [43] allowed for the identification of 283 transcription factors that were differentially expressed in P07 (Supplemental Table S9), most of which still have not been functionally characterized in plants. The most representative transcription factor family was AP2-EREBP, which accounted for 21 transcription factors in the assembly (Fig. 4A). This multigenic transcription factor family is unique to plants and has been implicated in hormone, sugar and redox signaling in the context of abiotic stresses, such as cold and drought [73]. The gene encoding the ERF110 transcription factor was the most up-regulated transcription factor in P07 (3.9-fold increased) (Fig. 4B). This transcription factor is involved in ethylene signaling and the regulation of bolting [74]. Bolting has been associated with the cell redox state, in which changes in the NADPH/NADP+ ratio that are caused by alterations in mitochondrial NAD(P)H dehydrogenase interfere with bolting time [75].

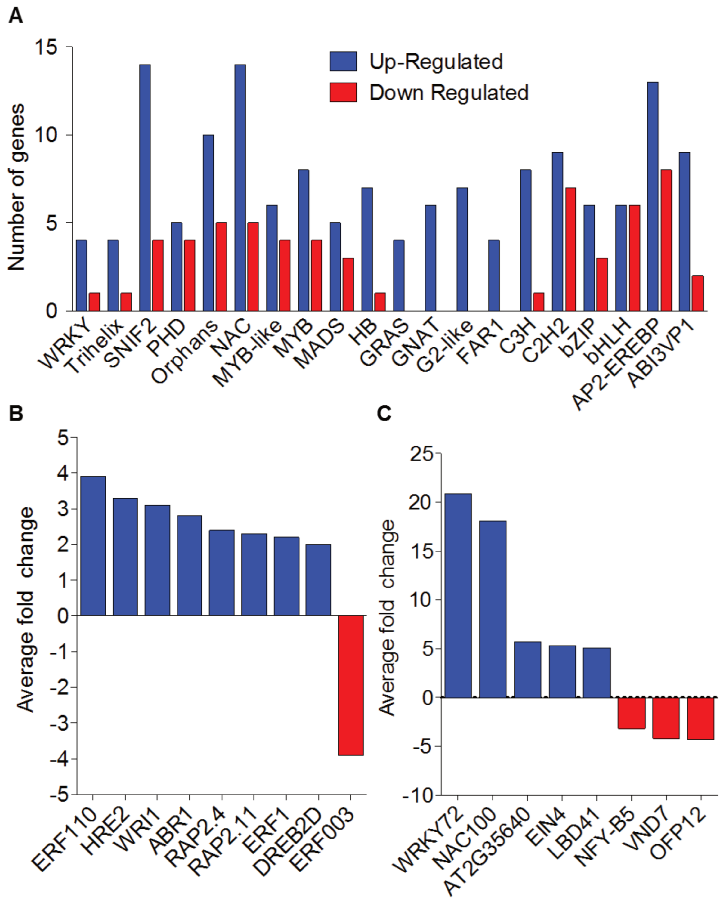

Figure 4. Increased UCP1 activity alters the expression of 283 transcription factors. (A) A total of 283 transcription factors were categorized into families. **(B)** Transcription factors of the AP2-EREBP family with the most greatly altered expression levels between transgenic and wild-type plants. **(C)** Transcription factors with similar expression levels in transgenic and wild-type plants.

Among the differentially expressed AP2-EREBP family members, we also identified the ethylene-responsive regulators HRE2 and WRI1. HRE2 helps regulate the conserved molecular response to low oxygen in plants [76]. Conversely, WRI1 may act in chloroplastic lipid biosynthesis by binding to the promoter sequences of late glycolytic and fatty acid biosynthetic genes [77]. In *wrinkled1* mutant lines, the lack of transcriptional activation of the fatty acid biosynthetic pathway in early maturing embryos is responsible for a severe defect in triglyceride biosynthesis that results in the production of wrinkled seeds that are depleted in lipids [78]. Arabidopsis plants overexpressing WRINKLED1 efficiently stimulate lipid accumulation in seeds [79].

The transcriptional regulators ABR1, RAP2.4, RAP2.11, ERF1 and DREB2D are all involved in abiotic stress responses. The ABR1 gene is highly induced by ABA and abiotic stresses in Arabidopsis, and it was the first transcription factor identified as a repressor of ABA-regulated gene expression [80]. The transcription factors RAP2.4 and RAP2.11 are highly induced by abiotic stresses, such as salt and mannitol, but the constitutive expression of RAP2.4 has been shown to promote leaf senescence [81], while the overexpression of RAP2.11 has been demonstrated to result in enhanced performance under conditions of low potassium and increased ROS production [82]. The DREB2D transcriptional regulator has not yet been characterized, but there are eight members in this subfamily, including DREB2A and DREB2B, that are involved in the response to drought [83]. The down-regulated AP2-EREBP gene ERF003 has not yet been characterized, but computational annotations suggest that it plays roles in the ethylene-signaling pathway and in the end of seed dormancy.

There were also several transcription factors that belong to other families that displayed strong alterations in gene expression in P07 (Fig. 4C). For example, the gene encoding the transcription factor WRKY72 was more than 20-fold up-regulated. This transcription factor is involved in the defense response against pathogens [84], which is in accordance with the observation that the overexpression of UCPs also protects plants against biotic stresses [27]. Another important regulator in this group was EIN4, which is one of the four plant ethylene receptors [85]. The large number of differentially expressed ethylene-responsive transcription factors and the fact that "response to ethylene" was one of the enriched GO categories (Table 3) suggest an important role for the ethylene signaling pathway in the UCP1-overexpressing plant. Genes encoding several of the transcription factors that presented with more than 4-fold up- or down-regulation have not yet been characterized in the literature and may represent interesting candidates that participate in organellar signaling and the stress response.

3.6. Validation of transcriptome expression by qRT-PCR

Transcriptome sequencing analyses must be validated by further biological experiments. Thus, we selected 13 transcripts (Supplemental Table S1) for the validation of

our results using a qRT-PCR analysis. The selected genes represented both up- and down-regulated genes in addition to those whose expression levels were not altered. The fold-change values that were obtained from the transcriptome assembly strongly correlated with the qRT-PCR data with an R-squared value of 0.9693 (Fig. 5).

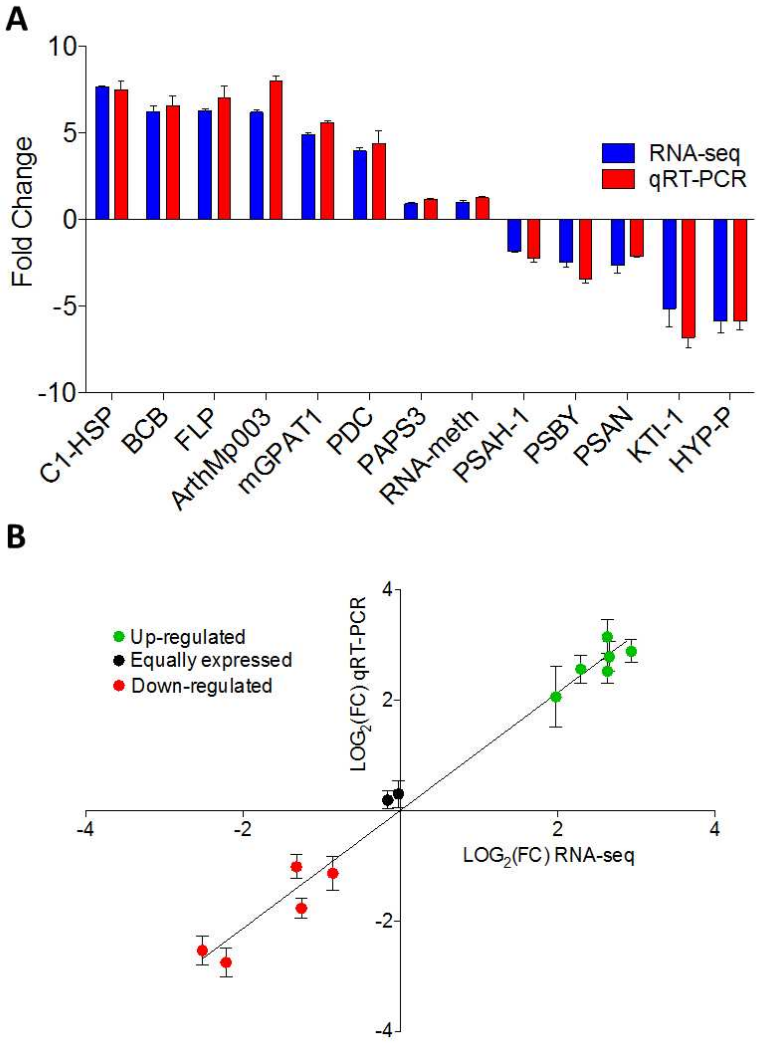

Figure 5 Expression analysis of selected genes from RNA-seq analysis by qRT-PCR. (A) Expression analysis of 13 selected genes assessed through qRT-PCR compared with RNA-seq data. **(B)** Correlation between fold changes reported by the RNA-seq analysis (x-axis) and those obtained using qRT-PCR. Error bars represent SEM (n = 4).

4. Discussion

Recent evidence in non-plant models has suggested that mild mitochondrial stress triggered by a variety of factors results in broad cytosolic and nuclear adaptation. Although varied, these responses appear to induce a wide range of cytoprotective states, resulting in long-lasting metabolic changes [10, 86]. In *Saccharomyces cerevisiae* [87], *C. elegans* [88], *Drosophila melanogaster* [89] and *Mus musculus* [90], for example, dysfunctions in the mitochondrial electron transport complex (ETC) machinery have been shown to result in the reconfiguration of cellular metabolism and increased life spans. This type of retrograde response has been referred to as mitohormesis [10,86]. In plants, we have shown that the overexpression of UCP1 alters mitochondrial-nuclear communication and increases mitochondrial biogenesis, which results in plants that are better adapted to stress [16]. This study investigated the transcriptomic profile of *N. tabacum* plants overexpressing *A. thaliana* UCP1 using Illumina RNA-seq technology to identify the genes and pathways that are altered.

Because the genome of *N. tabacum* has not yet been sequenced, we used the complete genome of *S. lycopersicum* to map the generated transcript contigs, resulting in the identification of 20,045 unique genes. Among them, 2,237 were up-regulated in the transgenic plants, while 991 were down-regulated relative to the wild type. The large number of DEGs suggests the broad adaptation of nuclear gene expression due to increased UCP1 activity. Interestingly, an enrichment analysis showed that the most representative GO term in the group of up-regulated genes was “response to stress” (442 genes) (Table 3). This result clearly indicates that the transgenic cells experienced some type of stressful condition due to the increased UCP1 activity and associated metabolic adaptation. Our previous results suggest that this stress may be metabolic in nature and caused by reduced intracellular ATP levels [16], which is consistent with the function of UCPs [17-19]. Additionally, stress could have been caused by the over-accumulation of UCP1 proteins in the mitochondrial inner membrane, which may have triggered the mitochondrial unfolded protein response (mtUPR). This topic remains poorly understood in plant models, but in *C. elegans,* mtUPR has been demonstrated to contribute to mitochondrial retrograde

regulation, inducing a protective mechanism that increases lifespan and tolerance to heat [10]. Because “starch and sucrose metabolism”, “oxidative phosphorylation”, “lipid metabolism” and “photosynthesis” were among the most represented altered pathways in our data, we suggest that metabolic stress is the main cause of the retrograde response that is induced by UCP1 overexpression, but we do not discard the possibility of a synergistic effect of mtUPR in this mechanism.

The improved photosynthetic capacities of UCP1-overexpressing plants have been demonstrated in both tobacco [26] and tomato plants [27], and in accordance with these results, an insertional knockout of UCP1 in *A. thaliana* decreased photosynthetic capacity[32]. However, we also revealed that genes related to the photosynthesis light reactions were down-regulated in P07 (Table 3, Fig. 2A and Supplemental Table 5). Some of these genes have already been described, such as the PSBX gene, which is not essential for PSII complex assembly or for the photoautotrophic growth of cyanobacteria or Arabidopsis [91]. However, the inhibition of the PSBX and PSBW genes was shown to result in a 30–40% decrease in oxygen production by PSII [92,93]. Regarding the genes that participate in PSI, the inhibition of PSAE-2 did not alter electron flow or photoautotrophic growth in *A. thaliana* [94]. Although we observed alterations in the expressions of PSI- and PSII-associated genes, we did not detect any expression changes in genes encoding other components of the photosynthetic machinery, such as cytochrome B6/f and the plastidic ATP synthase complex (Supplemental Table 8-B). Chloroplasts are the organelles that are most prone to oxidative stress because of their demand for reductive power, excess excitation energy at the photosystems and bivalent oxygen chemistry [50]. Thus, the down-regulation of the photosystem-related genes may represent an attempt to reduce ROS production.

Chloroplastic gene expression was not only negatively affected by UCP1 overexpression because 145 chloroplastic genes were up-regulated in the transgenic plants (Table 2). Among these genes, the one encoding pyruvate decarboxylase (PDC) was of particular interest (Fig. 2B). This chloroplastic enzyme is responsible for the irreversible conversion of pyruvate into acetaldehyde and CO_2, which is an anaerobic pathway for ATP production [60]. The increased expression of the mitochondrial ETC machinery-related

genes [16] that was observed in the P07 plants along with the increased anaerobic respiration suggests a metabolic pattern resembling that during pollen tube development, in which the aerobic and anaerobic pathways are fully active at the same time [63,64]. Not only were the genes associated with the PDC pathway of anaerobic respiration up-regulated, but so was the gene encoding LDH, which anaerobically converts pyruvate into lactate. In fact, UCP1-overexpressing plants have been demonstrated to experience hypoxic stress as shown by mitochondrial morphology [16]. In humans, the donut morphology appears as a consequence of hypoxic stress [95]. The fact that anaerobic pathways were up-regulated in a coordinated manner with transcriptional regulators of hypoxic stress (Fig. 4A) strongly suggests that these plants are indeed suffering from this type of stress. One hypothesis for the increased photosynthesis rate in the transgenic plants is that decreased intracellular oxygen levels benefit RuBisCo activity due to the natural competition that occurs between O_2 and CO_2. In accordance with this hypothesis, genes encoding several enzymes that participate in photorespiration were down-regulated in P07 (Fig. 3 and Supplemental Table 8-B). Additionally, in mammals, hypoxic conditions have been shown to decrease ATP synthesis by the OXPHOS machinery, which causes a burst of ROS production that is necessary for cellular adaptation [20]. If this type of signaling also occurs in plants, plant cells would be expected to attempt to decrease the expression of genes that are related to ROS production at other sites, such as those at PS-I and PS-II. Further molecular and biochemical analyses would be helpful to elucidate the mechanisms that are responsible for the increased carbon assimilation that is concomitant with the decreased PS-I and PS-II gene expression that was observed in the UCP1-overexpressing plants.

The increased carbon assimilation in chloroplasts is in accordance with the higher energy demand shown by the UCP1-overexpressing plants. More substrates must be generated in the form of carbohydrates or fatty acids to sustain the increased aerobic respiration and anaerobic fermentation. The increased flow of intermediary metabolites at the TCA [24] and increased expression of genes that are related to the OXPHOS machinery [16] have already been documented in UCP overexpressors. Additionally, genes encoding enzymes, such as amylases, that provide substrates for glycolysis [68], as well as most of the enzymes that participate in glycolysis reactions in the cytosol and TCA reactions in the

mitochondria, were up-regulated in the transgenic plants (Fig. 3A and Supplemental Table 8-A). Carbohydrate metabolism was induced, as well as genes related to lipid metabolism. The enrichment analysis (Table 3) and KEGG pathway mapping (Supplemental Table S7) suggested alterations in pathways related to "fatty acid beta-oxidation" and "lipid metabolism". Plastidic acetyl-CoA synthase (ACS), which detoxifies the subproducts of fermentation by converting them into fatty acids, and the PEP translocator (CUE1), which transports PEP from the glycolysis pathway to plastids for fatty acid biosynthesis, were both up-regulated in the transgenic plants. Fatty acids are also synthesized inside of the cytosol, where ATP citrate lyase (CL) converts citrate into acetyl-CoA [69]. The up-regulation of this enzyme (Supplemental Table 8-A) is of great importance because it links carbohydrate and fatty acid metabolism and represents a major source of cytoplasmic acetyl-CoA. The increased fatty acid metabolism may represent an attempt to use a more compact means of storing energy. Additionally, these molecules are necessary for membrane biogenesis and organellar development [71,72]. It is necessary to further explore the role of increased lipid metabolism in the transgenic plants.

The broad transcriptomic alterations that were observed in this work suggest that the overexpression of UCP1 provokes metabolic stress that alters cellular energy homeostasis and reconfigures cellular metabolism. This work shows a tight link between UCP1 activity and chloroplastic gene expression, which likely controls ROS production and supplies substrates for energy production. These findings suggest the presence of additional regulators that are difficult to isolate and act solely in mitochondrial-nucleus signaling. Our results provide important insight into the understanding of the molecular events that are induced by UCP1 overexpression in plants. They also show the plasticity of plant energy metabolism and provide a good model for the identification of regulators of organellar retrograde regulation in plants.

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References

[1] W. Martin, M. Mentel, The Origin of Mitochondria, Nature Education 3 (2010) 58.

[2] D.G. Searcy, Metabolic integration during the evolutionary origin of mitochondria, Cell Res. 13 (2003) 229-238.

[3] W. Martin, T. Rujan, E. Richly, A. Hansen, S. Cornelsen, T. Lins, D. Leister, B. Stoebe, M. Hasegawa, D. Penny, Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus, Proc. Natl. Acad. Sci. U S A 99 (2010) 12246-12251.

[4] J.N. Timmis, D. Wang, Endosymbiotic evolution: the totalitarian nucleus is foiled again, Curr. Biol. 23 (2013) R30-32.

[5] M. Unseld, J.R. Marienfeld, P. Brandt, A. Brennicke, The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides, Nat. Genet. 15 (1997) 57-61.

[6] S. Sato, Y. Nakamura, T. Kaneko, E. Asamizu, S. Tabata, Complete structure of the chloroplast genome of *Arabidopsis* thaliana, DNA Res. 6 (1999) 283-90.

[7] I. De Clercq, V. Vermeirssen, O. Van Aken, K. Vandepoele, M.W. Murcha, S.R. Law, A. Inzé, S. Ng, A. Ivanona, D. Rombaut, B. van de Cotte, P. Jaspers, Y. van der Peer, J. Kangasjärvi, J. Whelan, F. Van Breusegem, The membrane-bound NAC transcription factor ANAC013 functions in mitochondrial retrograde regulation of the oxidative stress response in Arabidopsis, Plant Cell. 25 (2013) 3472-3490.

[8] E. Welchen, L. García, N. Mansilla, D.H. Gonzalez, Coordination of plant mitochondrial biogenesis: keeping pace with cellular requirements, Front. Plant Sci. 4 (2013) 551.

[9] O. Yogev, O. Pines, Dual targeting of mitochondrial proteins: mechanism, regulation and function, Biochim. Biophys. Acta 1808 (2011) 1012–1020.

[10] J. Yun, T. Finkel, Mitohormesis, Cell Metab. 19 (2014) 757-766.

[11] J. St-Pierre, S. Drori, M. Uldry, J.M. Silvaggi, J. Rhee, S. Jäger, C. Handschin, K. Zheng, J. Lin, W. Yang, D.K. Simon, R. Bachoo, B.M. Spiegelmam, Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators, Cell 127 (2006) 397-408.

[12] K. Hammani, A. Gobert, K. Hleibieh, L. Choulier, L. Small, P. Giegè, An *Arabidopsis* dual-localized pentatricopeptide repeat protein interacts with nuclear proteins involved in gene expression regulation, Plant Cell 23 (2011) 730-740.

[13] H. Roschzttardtz, I. Fuentes, M. Vásquez, C. Corvalán, G. León, A nuclear gene encoding the iron-sulfur subunit of mitochondrial complex II is regulated by B3 domain transcription factors during seed development in Arabidopsis, Plant Physiol. 150 (2009) 84-95.

[14] O. Van Aken, B. Zhang, S. Law, R. Narsai, J. Whelan, AtWKRY40 and AtWRKY63 modulate the expression of stress-responsive nuclear genes encoding mitochondrial and chloroplast proteins, Plant Physiol. 162 (2013) 254-271.

[15] E. Giraud, S. Ng, C. Carrie, O. Duncan, J. Low, C.P. Lee, O. Van Aken, A.H. Millar, M. Murcha, J. Whelan, TCP transcription factors link the regulation of genes encoding mitochondrial proteins with the circadian clock in *Arabidopsis thaliana*, Plant Cell 22 (2010) 3921-3934.

[16] P. Barreto, I.A.P. Neschich, V. Okura, I.G. Maia, P. Arruda, Overexpression of UCP1 in tobacco induces mitochondrial biogenesis through retrograde signaling and amplifies a broad stress response, BMC Plant Biol. 14 (2014) 1-31.

[17] S. Krauss, C.Y. Zhang, B.B. Lowell, The mitochondrial uncoupling-protein homologues, Nat. Rev. Mol. Cell Biol. 6 (2005) 248-261.

[18] A.E. Vercesi, J. Borecký, I.G. Maia, P. Arruda, I.M. Cuccovia, H. Chaimovich, Plant uncoupling mitochondrial proteins, Annu. Rev. Plant Biol. 57 (2006) 383–404.

[19] D.G. Nicholls, E. Rial, A history of the first uncoupling protein, UCP1, J. Bioenerg. Biomembr. 31 (1999) 399–418.

[20] T.R. Figueira, M.H. Barros, A.A. Camargo, R.F. Castilho, J.C.B. Ferreira, A.J. Kowaltowski, F.E. Sluse, N.C. Souza-Pinto, A.E. Vercesi, Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health, Antioxid. Redox Signal. 18 (2013) 2029-2074.

[21] R.J. Mailloux, C.N. Adjeitey, J.Y. Xuan, M.E. Harper, Crucial yet divergent roles of mitochondrial redox state in skeletal muscle vs. brown adipose tissue energetics, FASEB J. 26 (2012) 363–375.

[22] R.J. Mailloux, E.L. Seifert, F. Bouillaud, C. Aguer, S. Collins, M.E. Harper,Glutathionylation acts as a control switch for uncoupling proteins UCP2 and UCP3, J. Biol. Chem. 286 (2011) 21865–21875.

[23] Y. Ito-Inaba, Y. Hida, H. Mora, T. Inaba, Molecular identity of uncoupling proteins in thermogenic skunk cabbage, Plant Cell Physiol. 49 (2008) 1911-1916.

[24] A.M.O Smith, G. Ratcliffe, L.J. Sweetlove, Activation and function of mitochondrial uncoupling protein in plants, J. Biol. Chem. 279 (2004) 51944-51952.

[25] T.R.S Figueira, P. Arruda, Differential expression of uncoupling mitochondrial protein and alternative oxidase in the plant response to stress, J. Bioenerg. Biomembr. 43 (2011) 67-70.

[26] K. Begcy, E.D. Mariano, L. Mattiello, A.V. Nunes, P. Mazzafera P, I.G. Maia, M. Menossi, An *Arabidopsis* mitochondrial uncoupling protein confers tolerance to draught and salt stress in transgenic tobacco plants, Plos One. 6 (2011) 1-9.

[27] S. Chen, A. Liu, S. Zhang, C. Li, R. Chang, D. Liu, G.J. Ahammed, X. Lin, Overexpression of mitochondrial uncoupling protein conferred resistance to heat stress an *Botrytis cinerea* infection in tomato, Plant Physiol. Biochem. 73 (2013) 245–253.

[28] P. Jezek, J. Borecký, M. Zácková, A.D. Costa, P. Arruda, Possible basic and specific functions of plant mitochondrial uncoupling protein (pUCP), Biosci. Rep. 21 (2001) 237-245.

[29] I.G. Maia, C.E. Benedetti, A. Leite, S.R. Turcinelli, A.E. Vercesi, P. Arruda, AtPUMP: an Arabidopsis gene encoding a plant uncoupling mitochondrial protein, FEBS Lett. 429 (1998) 403-406.

[30] J. Borecky, F.T.S. Nogueira, K.A.P Oliveira, I.G. Maia, A.E. Vercesi, P. Arruda, The plant energy-dissipating mitochondrial systems: depicting the genomic structure and the expression profiles of the gene families of uncoupling protein and alternative oxidase in monocots and dicots, J. Exp. Bot. 57 (2006) 849-864.

[31] M. Brandalise, I.G. Maia, J. Borecký, A.E. Vercesi, P. Arruda, Overexpression of plant mitochondrial uncoupling protein in transgenic tobacco increases tolerance to oxidative stress, J. Bioenerg. Biomembr. 35 (2003) 205-209.

[32] L.J. Sweetlove, A. Lytovchenko, M. Morgan, A. Nunes-Nesi, N.L. Taylor, C.J. Baxter, I. Eickmeier, A.R. Fernie, Mitochondrial uncoupling protein is required for efficient photosynthesis, Proc. Natl. Acad. Sci. U S A. 51 (2006) 19587-19592.

[33] S. Lindgreen, AdapterRemoval: easy cleaning of next-generation sequencing reads, BMC Res. Notes 5 (2012) 1-7.

[34] M.G. Grabherr, B.J. Haas, M. Yassour, J.Z. Levin, D.A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F. di Palma, B.W. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman, A. Regev, Full-length transcriptome assembly from RNA-seq data without reference genome, Nat. Biotechnol. 29 (2011) 644-652.

[35] G.P. Wagner, K. Kin, V.J. Lynch, Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples, Theory Biosci 131 (2012) 281-285.

[36] B. Langmead, C. Trapnell, M. Pop, S.L. Salzberg, Ultrafast and memory-efficient alignment of short DNA sequences to the human genome, Genome Biol 10 (2009) R25.

[37] Tomato Genome Consortium, The tomato genome sequence provides insights into fleshy fruit evolution, Nature 485 (2012) 635-641.

[38] The UniProt Consortium, Activities at the Universal Protein Resource (UniProt) Nucleic Acids Res. 42 (2014) D191-D198.

[39] R.L. Tatusov, N.D. Fedorova, J.D. Jackson, A.R. Jacobs, B. Kiryutin, E.V. Koonin, D.M. Krylov, R. Mazumder, S.L. Mekhedov, A.N. Nikolskaya, B.S. Rao, S. Smirnov, A.V. Sverdlov, S. Vasudevan, Y.I. Wolf, J.J. Yin, D.A. Natale, The COG database: an updated version includes eukaryotes, BMC Bioinformatics 4 (2003) 1-14.

[40] The Gene Ontology Consortium, Gene ontology: tool for the unification of biology, Nat. Genet 25 (2000) 25-29.

[41] M. Kanehisa, S. Goto, KEGG: Kyoto Encyclopedia of Genes and Genomes, Nucleic Acids Res. 28 (2000) 27-30.

[42] A. Conesa, S. Götz, J.M Garcia-Gomez, J. Terol, M. Talon, M. Robles, Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research, Bioinformatics 21 (2005) 3674-3676.

[43] P. Perez-Rodriguez, D.M. Riano-Pachon, L.G.G. Correa, S.A. Rensing, B. Kersten, B. Mueller-Robber, PlnTFDB: updated content and new features of the plant transcription factor database, Nucleic Acids Res. 38 (2009) D822-D827.

[44] C. Dias-Lopes, I.A. Neschich, G. Neshich, J.M. Ortega, C. Granier, C. Chávez-Olortegui, F. Molina, L. Felicori, Identification of new sphingomyelinases D in pathogenic fungi and other pathogenic organisms, PLoS One 8 (2013) 1-12.

[45] P.L. Auer, R.W. Doerge, Statistical design and analisys of RNA sequencing data, Genetics 185 (2010) 405-416.

[46] M.T. Ryan, M.J. Hoogenrad, Mitochondrial-Nuclear communications, Annu. Rev. Biochem. 76 (2007) 701-722.

[47] F. Zhang, T. Pracheil, J. Thornton, Z. Liu, Adenosine Triphosphate (ATP) Is a Candidate Signaling Molecule in the Mitochondria-to-Nucleus Retrograde Response Pathway, Genes 4 (2013) 86-100.

[48] D.G. Nicholls, A history of UCP1, Biochem. Soc. 29 (2001) 751–755.

[49] C. Hourton-Cabassa, A. Mesneau, B. Miroux, J. Roussaux, D. Ricquier, A. Zachowsky, F. Moreau, Alteration of plant mitochondrial proton conductance by free fatty acids. Uncoupling protein involvement, J. Biol. Chem. 277 (2002) 41533-41538.

[50] N. Suzuki, S. Koussevitzky, R. Mittler, G. Miller, ROS and redox signalling in the response of plants to abiotic stress, Plant Cell Environ. 35 (2012) 259-270.

[51] C.H. Foyer, G. Noctor, Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications, Antioxid. Redox Signal. 11 (2009) 861–905.

[52] T. Shikanai, Cyclic Electron Transport Around Photosystem I: Genetic Approaches, Annu. Rev. Plant Biol. 58 (2007) 199-217.

[53] D. Honys, D. Twell, Comparative analysis of the Arabidopsis pollen transcriptome, Plant Physiol 132 (2003) 640–652.

[54] M.C.G. Wheeler, M.A. Tronconi, M.F. Drincovich, C.S. Andreo, U.I. Flügge UI, V.G. Maurino, A Comprehensive Analysis of the NADP-Malic Enzyme Gene Family of Arabidopsis, Plant Physiol. 139 (2005) 39-51.

[55] R. Scheibe, Malate valves to balance cellular energy supply, Physiol. Plant. 120 (2004) 21-26.

[56] I. Hebbelmann, J. Selinski, C. Wehmeyer, T. Goss, I. Voss, P. Mulo, S. Kangasjärvi, E.M. Aro, M.L. Oelze, K.J. Dietz, A. Nunes-Nesi, P.T. Do, A.R. Fernie, S.K. Talla, A.S. Raghavendra, V. Linke, R. Scheibe, Multiple strategies to prevent oxidative stress in Arabidopsis plants lacking the malate valve enzyme NADP-malate dehydrogenase, J. Exp. Bot. 63 (2012) 1445-1459.

[57] E.E. Reid, P. Thompson, C.R. Lyttle, D.T. Dennis, Pyruvate dehydrogenase complex from higher plant mitochondria and proplastids, Plant Physiol 59 (1977) 842–848.

[58] J. Schwender, J.B. Ohlrogge, Y. Shachar-Hill, A flux model of glycolysis and the oxidative pentose phosphate pathway in developing *Brassica napus* embryos, J. Biol. Chem. 278 (2003) 29442–29453.

[59] J. Schwender, Y. Shachar-Hills, J.B. Ohlrogge, Mitochondrial metabolism in developing embryos of *Brassica napus,* J. Biol. Chem. 281 (2006) 34040–34047.

[60] B.P. Smirnov, The biosynthesis of higher acids from acetate in intact chloroplasts of *Spinacea oleracea* leaves, Biokhimii 25 (1960) 419–426.

[61] U.I. Flügge, R.E. Häusler, F. Ludewig, M. Gierth, The role of transporters in supplying energy to plant plastids, J. Exp. Bot. 62 (2011) 2381-2392.

[62] M. Lin, D.J. Oliver, The role of Acetyl-Coenzime A Synthetase in Arabidopsis. Plant. Physiol. 147 (2008) 1822-1829.

[63] N. Gass, T. Glagotskaia, S. Mellema, J. Stuurman, M. Barone, T. Mandel, U. Roessner-Tunali, C. Kuhlemeier, Pyruvate decarboxylase pro- vides growing pollen tubes with a competitive advantage in petunia, Plant Cell 7 (2005) 2355–2368.

[64] C.M. Rounds, L.J. Winship, P.K. Hepler, Pollen tube energetics: respiration, fermentation and the race to the ovule, AoB Plants Plr019 (2011) 1-14.

[65] S.B. Vafai, V.K. Mootha, Mitochondrial disorders as windows into an ancient organelle, Nature 491 (2012) 374-383.

[66] Y. Balmer, A. Koller, G. del Val, W. Manieri, P. Schürmann, B.B. Buchanan, Proteomics gives insight into regulatory function of chloroplast thioredoxins, Proc. Natl. Acad. Sci. U S A, 100 (2003) 370-375.

[67] W. Sakamoto, S.Y. Miyagishima, P. Jarvis, Chloroplast biogenesis: control of plastid development, protein import, division and inheritance, The Arabidopsis Book 6 (2008) 1-30.

[68] F. Wattebled, V. Planchot, Y. Dong, N. Szydlowski, B. Pontoire, A. Devin, S. Ball, C. D'Hulst C, Further evidence for the mandatory nature of polysaccharide debranching for the aggregation of semicrystalline starch and for overlapping functions of debranching enzymes in Arabidopsis leaves, Plant Physiol. 148 (2008) 1309-1323.

[69] T. Sun, K. Hayakawa, K.S. Bateman, M.E. Fraser, Identification of the Citrate-binding site of human ATP-citrate lyase using X-ray crystallography, J. Biol. Chem. 285 (2010) 27418-27428.

[70] B.L. Fatland, B.J. Nikolau, E.S. Wurtele, Reverse genetic characterization of cytosolic acetyl-CoA generation by ATP-citrate lyase in Arabidopsis, Plant Cell 17 (2005) 182-203.

[71] Y. Ohba, T. Sakuragi, E. Kage-Nakadai, N.H. Tomioka, N. Kono, R. Imae, A. Inoue, J. Aoki, N. Ishihara, T. Inoue, S. Mitani, H. Arai, Mitochondria-type GPAT is required for mitochondrial fusion, EMBJO J. 32 (2013) 1265-1279.

[72] Z. Zheng, Q. Xia, M. Dauk, W. Shen, G. Selvaraj, J. Zou, Arabidopsis AtGPAT1, a member of the membrane-bound glycerol-3-phosphate acyltransferase gene family, is essential for tapetum differentiation and male fertility, Plant Cell. 15 (2003) 1872-1887.

[73] K.J. Dietz, M.O. Vogel, A. Viehhauser, AP2/EREBP transcription factors are part of gene regulatory networks and integrate metabolic, hormonal and environmental signals in stress acclimation and retrograde signaling, Protoplasma 245 (2010) 3-14.

[74] L. Zhu, D. Liu, Y. Li, N. Li, Functional phosphoproteomic analisys revels that a serine-62-phosphorylated isoformo of ethylene response factor 110 is involved in Arabidopsis bolting, Plant Physiol.161 (2013) 904-917.

[75] Y.J. Liu, A. Nunes-Nesi, S.V. Wallström, I. Lager, A.M. Michelecka, F.E. Norberg, S. Widell, K.M. Fredlund, A.R. Fernie, A.G. Rasmusson, A redox-mediated modulation of stem bolting in transgenic Nicotiana sylvestris differentially expressing the external mitochondrial NADPH dehydrogenase, Plant Physiol. 150 (2009) 1248-1259.

[76] F. Licausi, J.T. van Dongen, B. Giuntoli, G. Novi, A. Santaniello, P. Geigenberger, P. Perata, HRE1 and HRE2, two hypoxia-inducible ethylene response factors, affect anaerobic responses in Arabidopsis thaliana, Plant J. 62 (2010) 302-315.

[77] S. Baud, M.S. Mendoza, A. To, E. Harscoët, L. Lepiniec, B. Dubreucq, WRINKLED1 specifies the regulatory action of LEAFY COTYLEDON2 towards fatty acid metabolism during seed maturation in *Arabidopsis*, Plant J. 50 (2007) 825–838.

[78] N. Focks, C. Benning, *wrinkled1*: A novel, low-seed-oil mutant of*Arabidopsis* with a deficiency in the seed-specific regulation of carbohydrate metabolism, Plant Physiol. 118 (1998) 91–101.

[79] S. Baud, S. Wuillème, A. To, C. Rochat, L, Role of WRINKLED1 in the transcriptional regulation of glycolytic and fatty acid biosynthetic genes in Arabidopsis, Plant J. 60 (2009) 933-947.

[80] G.K. Pandey, J.J. Grant, Y.H. Cheong, B.G. Kim, L. Li, S. Luan, ABR1, an APETALA2-domain transcription fator that function as a repressor of ABA response in Arabidopsis, Plant Physiol. 139 (2005) 1185-1193.

[81] H. Xu, X. Wang, J. Chen, Overexpression of the Rap2.4f transcriptional factor in Arabidopsis promotes leaf senescence, Sci. China Life Sci. 53 (2010) 1221-1226.

[82] M.J. Kim, D. Ruzicka, R. Shin, D.P. Schachtman, The Arabidopsis AP2/ERF transcription factor RAP2.11 modulates plant response to low-potassium conditions, Mol. Plant 5 (2012) 1042-1057.

[83] Y. Sakuma, K. Maruyama, Y. Osakabe, F. Qin, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki, Functional analysis of an Arabidopsis transcription factor, DREB2A, involved in drought-responsive gene expression, Plant Cell 18 (2006) 1292-1309.

[84] K.K. Bhattarai, H.S. Atamian, I. Kaloshian, T. Eulgem, WRKY72-type transcription factors contribute to basal immunity in tomato and Arabidopsis as well as gene-for-gene resistance mediated by the tomato R gene Mi-1, Plant J. 63 (2010) 229-240.

[85] J. Hua, E.M. Meyerrowitz, Ethyelene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana, Cell 94 (1998) 261-71.

[86] M. Ristow, K. Zarse, How increased oxidative stress promotes logevity and metabolic health: The concept of mitochondrial hormesis (mitohormesis), Exp Gerontol. 45 (2010) 410-418.

[87] P.A. Kirchman, S. Kim, C.Y. Lai, S.M. Jazwinski, Interorganelle signaling is a determinant of longevity in *Saccharomyces cerevisiae,* Genetics 152 (1999) 179–190.

[88] S.S. Lee, R.Y. Lee, A.G. Fraser, R.S. Kamath, J. Ahringer, G. Ruvkun, A systematic RNAi screen identifies a critical role for mitochondria in *C.elegans* longevity, Nat. Genet. 33 (2003) 40–48.

[89] J.M. Copeland, J. Cho, T. Lo Jr., J.H. Hur, S. Bahadorani, T. Arabyan, J. Rabie, J. Soh, D.W. Walker, Extension of *Drosophila* life span by RNAi of the mitochondrial respiratory chain, Curr. Biol. 19 (2009) 1591–1598.

[90] J. Lapointe, Z. Stepanyan, E. Bigras, S. Hekimi, Reversal of the mitochondrial phenotype and slow development of oxidative biomarkers of aging in long-lived Mclk1+/- mice, J. Biol. Chem. 284 (2009) 20364–20374.

[91] H. Katoh, M. Ikeuchi, Targeted disruption of psbX and biochemical characterization of photosystem II complex in the thermophilic cyanobacterium Synechococcus elongatus, Plant Cell Physiol. 42 (2001) 179-188.

[92] J.G. García-Cerdán, D. Sveshnikov, D. Dewez, S. Jansson, C. Funk, W.P. Schröder, Antisense inhibition of the PsbX protein affects PSII integrity in the higher plant Arabidopsis thaliana, Plant Cell Physiol. 50 (2009) 191-202.

[93] J.G. García-Cerdán, L. Kovács, T. Tóth, S. Kereïche, E. Aseeva, E.J. Boekema, F. Mamedov, C. Funk, W.P. Schröder, The PsbW protein stabilizes the supramolecular organization of photosystem II in higher plants, Plant J. 65 (2011) 368-381.

[94] A. Ihnatowicz, P. Pesaresi, D. Leister, The E subunit of photosystem I is not essential for linear electron flow and photoautotrophic growth in Arabidopsis thaliana, Planta 226 (2007) 889-95.

[95] X. Liu, G. Hajnóczky, Altered fusion dynamics underlie unique morphological changes in mitochondria during hypoxia-reoxygenation stress, Cell Death Differ. 18 (2011) 1561-1572.

Supplemental Files

Supplemental Table S1. Genes selected for experimental expression validation and the corresponding primers used for qRT-PCR reactions

Supplemental Table S2. Output of *N. tabacum* wild type and P07 plant transcriptome sequencing

Supplemental Table S3. COG classifications of differentially expressed genes in P07 compared with wild type

Supplemental Table S4. Gene ontology assignments for DEGs

Supplemental Table S5. Representative groups of differentially expressed chloroplastic genes

Supplemental Table S6. Differentially expressed chloroplastic ribosomal subunits

Supplemental Table S7. (A) KEGG mapping of up-regulated genes (B) and down-regulated genes

Supplemental Table S8. (A) List of enzymes and genes cited in Figure 3A **(B)** and the list of enzymes and genes cited in Figure 3B

Supplemental Table S9. Family classifications of differentially expressed transcription factors

Supplemental Table S1. Genes selected for experimental expression validation and the corresponding primers used for qRT-PCR reactions.

Tomato Annotation	Tair Annotation	Gene symbol	Uniprot Annotation	RNA-Seq Average FC	**Primers (5' - 3')**
Solyc03g019690.1.1	AT1G73260	KTI-1	Q3S477_SOLTU	-6.6	Forward: gagcggttcgccatctatg Reverse: cccattgtccttcccacca
Solyc06g063340.2.1	AT3G20260	HYP-P	B7EKP5_ORYSJ	-5.8	Forward: agcatgcaggcggtcataaa Reverse: gggacgttgaagcagaagg
Solyc08g013670.2.1	AT5G64040	PSAN	D2K7Z2_TOBAC	-2.4	Forward: tccaactcctcagccaatgc Reverse: gcgaagtttgcaccacttgt
Solyc10g077120.1.1	AT1G67740	PSBY	B6SR26_MAIZE	-1.7	Forward: agagggagacaacaggggtt Reverse: ggttcagggctggttgaaga
Solyc03g120640.2.1	AT3G16140	PSAH-1	PSAH1_ARATH	-1.7	Forward: aatcaagggcctagcgggaa Reverse: cagcaccagccctggattta
Solyc10g086680.1.1	AT1G07400	C1-HSP	Q69B17_CARPA	7.7	Forward: acccaggtcctcttccttg Reverse: gatcgccgaacactcttgg
Solyc06g065850.2.1	AT5G25250	FLP	B6Q909_PENMQ	6.4	Forward: gaagaaagcagggtgggct Reverse: cttccctctgcaactcagc
Solyc07g008130.2.1	AT5G20230	BCB	O82576_MAIZE	6.2	Forward: aggagacaaattgccgcca Reverse: ccagggccaaaaattagcg
Solyc10g048060.1.1	ArthMp003	UMP	M030_ARATH	6.2	Forward: aaccgtatccaagctccgtg Reverse: cggggtctcggttgatttc
Solyc07g056320.2.1	AT1G06520	mGPAT1	B9T753_RICCO	5.0	Forward: aatcgaacggtcggtatcgg Reverse: actggatccgggtgtcctat
Solyc09g005110.2.1	AT4G33070	PDC	Q84V95_LOTCO	4.0	Forward: tgcaatttgccagccattcc Reverse: gcagcttccaatcccctctt
Solyc12g096330.1.1	AT5G10620	RNA-meth	A3UBP0_CROAH	1.0	Forward: gtggttccgctcacttctc Reverse: ttcaggatgaccttggccc
Solyc12g099180.1.1	AT3G06560	PAPS3	Q7XJ92_ARATH	1.0	Forward: cctccagcatggcttagtg Reverse: tcaaggcgcaactggaaaga
Internal reference					
ABI58612.1	NA	ACT1	Nicotiana tabacum mRNA for actin		Forward: ggaggacattcaacccctt Reverse: ctaggaaaaacagccctcg

Supplemental Table S2. Output of N. tabacum wild type and P07 plant transcriptome sequencing

Sample	WT	P07
Number of total cleaned reads	302.685.541	240.109.208
Biological Replicate 1	113.267.169	27.905.560
Biological Replicate 2	55.603.556	90.289.253
Biological Replicate 3	83.066.501	65.760.027
Biological Replicate 4	50.748.225	56.154.368
Total nucleotide (Gbp) of cleaned reads	22.7	18.0
Bases ≥ Q20 (%)	98.1	98.0
GC content (%)	44.2	43.6

Supplemental Table S3. COG classifications of differentially expressed genes in P07 compared with wild type

Major Groups	Functional Category	Up-regulated	Down-regulated
Celular Processes and Signaling	Cell wall/membrane/envelope biogenesis	18	4
	Cell motility	1	0
	Posttranslational modification, protein modification and chaperones	162	53
	Signal transduction mechanisms	192	48
	Intracellular traficking, secretion and vesicular transport	59	9
	Defense mechanisms	15	6
	Extracellular structures	7	2
	Nuclear structures	2	0
	Cytoeskeleton	33	17
	Total	**489**	**139**
Information Storage and Processing	RNA processing and modification	56	10
	Chromatin structure and dynamics	15	4
	Translation, ribossomal structure and biogenesis	46	13
	Transcription	78	35
	Replication, recombination and repair	25	20
	Total	**220**	**82**
Metabolism	Energy production and conversion	78	22
	Cell cycle control, cell division, chromossome partitioning	36	19
	Aminoacid transport and metabolism	62	26
	Nucleotide transport and metabolism	20	4
	Carbohydrate transport and metabolism	97	43
	Coenzime transport and metabolism	20	4
	Lipid transport and metabolism	56	29
	Inorganic ion transport and metabolism	55	27
	Secondary metaboliste biosynthesis, transport and metabolism	82	53
	Total	**506**	**227**
Poorly Characterized	General function (prediction only)	376	89
	Function unknown	88	13
	Total	**464**	**102**

Supplemental Table S4. Gene ontology assignments for DEGs

Ontologies	Up-regulated	Down-Regulated
0	375	171
1	248	105
2	289	108
3	221	102
4	202	106
5	173	70
6	160	59
7	119	56
8	96	36
9	55	24
10	51	19
11	59	13
12	22	12
13	33	19
14	24	8
15	14	14
16	15	12
17	11	3
18	11	9
19	5	1
20	5	4
21	10	2
22	3	2
23	3	1
24	5	1
25	3	1
26	9	3
27	1	0
28	1	2
29	1	2
30	5	0
31	1	2
32	2	0
33	0	1
34	1	1
35	1	0
36	0	1
37	0	0
38	0	0
39	3	0
40	0	1
41	0	0
42	0	0
43	0	1

Supplemental Table S5. Representative groups of differentially expressed chloroplastic genes

Group/Alias	Tomato Annotation	TAIR annotation	Fold Change	UNIPROT annotation
Photosystem				
PSBI	Solyc02g038710.1.1	ArthCp006	1.6	D3WCU5_RHOSS
YCFIII	Solyc09g015320.1.1	ArthCp023	1.5	D3WBH6_9AQUA
PSAD-2	Solyc06g054260.1.1	AT1G03130	-1.6	B4FAW3_MAIZE
PSBX	Solyc05g025600.1.1	AT2G06520	-1.5	B1PPX5_9MYRT
PSBW	Solyc06g084050.2.1	AT2G30570	-1.5	B6TMB3_MAIZE
PSAE-2	Solyc09g063130.2.1	AT2G20260	-1.6	B6TH55_MAIZE
PSAF	Solyc02g069450.2.1	AT1G31330	-1.6	Q9XQB4_PHAAU
NPQ4	Solyc06g060340.2.1	AT1G44575	-1.6	A8HPM2_CHLRE
PSBTN	Solyc12g099650.1.1	AT3G21055	-1.6	PST2_GOSHI
PSAK	Solyc08g006930.2.1	AT1G30380	-1.7	Q84QE6_TOBAC)
PSAH-1	Solyc03g120640.2.1	AT3G16140	-1.7	PSAH1_ARATH
PSBY	Solyc10g077120.1.1	AT1G67740	-1.7	B6SR26_MAIZE
PSAO	Solyc06g074200.2.1	AT1G08380	-1.8	Q949Q5_ARATH
PSAH-2	Solyc06g066640.2.1	AT1G52230	-1.8	PSAH2_ARATH
PSBP-1	Solyc07g044860.2.1	AT1G06680	-1.8	PSBP_SOLLC
PSAG	Solyc07g066150.1.1	AT1G55670	-1.8	Q9XQB4_PHAAU
PSAN	Solyc08g013670.2.1	AT5G64040	-2.4	D2K7Z2_TOBAC
Lipid, Aminoacid and Carbon Metabolism				
PLA2A	Solyc02g065090.2.1	AT2G26560	3.8	Q9FZ08_TOBAC
phospholipase A1-Iigamma	Solyc02g077000.2.1	AT4G18550	2.7	Q8RZ40_ORYSJ
Lipoxygenase 6	Solyc05g014790.2.1	AT1G67560	2.4	B9RMJ4_RICCO
IPP2	Solyc04g056390.2.1	AT3G02780	2.3	Q84RZ8_9GENT
LCB1	Solyc08g066020.1.1	AT4G36480	2.1	Q94IB8_ARATH
LACS9	Solyc07g045290.2.1	AT1G77590	2.0	ACSL3_HUMAN
GDSL esterase/lipase	Solyc02g071720.2.1	AT1G29670	2.0	GDL15_ARATH
ACS	Solyc07g017860.2.1	AT5G36880	2.0	Q2J3D0_RHOP2
Aspartate-glutamate racemase	Solyc03g116460.2.1	AT1G15410	4.2	D4XDK3_9BURK
ASE2	Solyc01g110520.2.1	AT4G34740	2.0	GLMS_METKA
D-3-phosphoglycerate dehydrogenase	Solyc03g123830.2.1	AT1G17745	2.0	D2D316_GOSHI
Pyruvate decarboxylase 1	Solyc09g005110.2.1	AT4G33070	3.9	Q84V95_LOTCO
pMDH	Solyc03g115990.1.1	AT3G47520	2.3	O48906_MEDSA
NADP-ME3	Solyc05g050120.2.1	AT5G25880	2.2	O04936_SOLLC
CT-BMY	Solyc08g077530.2.1	AT4G17090	2.0	Q5F305_SOYBN
CUE1	Solyc03g112870.2.1	AT5G33320	2.0	Q9MSB4_MESCR
G6PD1	Solyc01g100950.2.1	AT5G35790	1.5	B9RFN8_RICCO
PFK5	Solyc04g014270.2.1	AT2G22480	1.5	D7LE10_ARALY
Phosphoglycerate mutase 1	Solyc07g044840.2.1	AT1G09780	1.5	B9S1V6_RICCO
ENO1	Solyc03g114500.2.1	AT1G74030	1.5	Enolase (A9P745_HELAN)
ISA3	Solyc06g009220.2.1	AT4G09020	1.5	B1R1G0_CLOBU
fructose-bisphosphate aldolase 3	Solyc05g008600.2.1	AT2G01140	1.5	B9SJY9_RICCO

Supplemental Table S6. Differentially expressed chloroplastic ribosomal subunits

Tomato Annotation	TAIR annotation	Fold Change	UNIPROT annotation
Solyc02g069840.2.1	AT1G16740 (NP_173118.1)	4,1	50S ribosomal protein L20 (B9GNB5_POPTR)
Solyc01g007280.2.1	RPS4(NP_051061.1)	2,2	30S ribosomal protein S4 chloroplastic (B5MF80_PETIN)
Solyc07g045390.1.1	AT5G27820 (NP_198134.1)	2,2	50S ribosomal protein L18 (B6SMG7_MAIZE)
Solyc05g009570.2.1	AT1G68590 (NP_564934.1)	2,1	Ribosomal protein PSRP-3/Ycf65 (B5W261_SPIMA)
Solyc05g007910.2.1	AT1G48350 (NP_175268.1)	1,9	50S ribosomal protein L18 (B6WB57_9FIRM)
Solyc01g007590.2.1	RPL14 (NP_051094.1)	1,7	50S ribosomal protein L14 chloroplastic (D3WBB8_9BORA)
Solyc11g056360.1.1	AT2G07675 (NP_178773.1)	1,7	30S ribosomal protein S12 (C8PSU8_9SPIO)
Solyc01g017530.1.1	RPS2 (NP_051048.1)	1,7	30S ribosomal protein S2 (RS2_NOSP7)
Solyc08g068220.2.1	AT5G15220 (NP_568310.1	1,6	50S ribosomal protein L27 (B6T4C5_MAIZE)
Solyc09g064410.1.1	RPL33 (NP_051080.1)	1,6	50S ribosomal protein L33 chloroplastic (D3WBK4_9AQUA)
Solyc01g007460.2.1	RPS18 (NP_051081.1)	1,5	30S ribosomal protein S18 chloroplastic (D3WEG6_HEUSA)
Solyc06g073090.2.1	AT5G24490 (NP_568447.1)	-1,5	Ribosomal subunit interface protein (Q2JIS4_SYNJB)
Solyc00g009100.2.1	AT1G71720 (NP_177317.2)	-1,7	30S ribosomal protein S1-like (Q5ZC75_ORYSJ)
Solyc04g079790.2.1	RPS9(NP_177635.1)	-1,7	30S ribosomal protein S9 (B6SZ34_MAIZE)
Solyc01g110550.2.1	AT4G34730 (NP_195199.2)	-1,8	Ribosome-binding factor A (B6TDG2_MAIZE)

Supplemental Table S7-A. KEGG mapping of up-regulated genes

Pathway	Seqs in Pathway
Purine metabolism	38
Starch and sucrose metabolism	29
Phenylpropanoid biosynthesis	20
Glycolysis / Gluconeogenesis	18
Phenylalanine metabolism	18
Oxidative phosphorylation	17
Pentose and glucuronate interconversions	15
Amino sugar and nucleotide sugar metabolism	13
Lipid biosynthesis	13
Drug metabolism - cytochrome P450	12
TCA Cycle	12
Glutathione metabolism	12
Phosphatidylinositol signaling system	12
Glycerophospholipid metabolism	11
Inositol phosphate metabolism	11
Glycerolipid metabolism	11

Supplemental Table S7-B. KEGG mapping of down-regulated genes

Pathway	Seqs in Pathway
Starch and sucrose metabolism	25
Pentose and glucuronate interconversions	24
Porphyrin and chlorophyll metabolism	11
Phenylalanine metabolism	9
Phenylpropanoid biosynthesis	7
Amino sugar and nucleotide sugar metabolism	6
Sphingolipid metabolism	6
Glycerolipid metabolism	6
Purine metabolism	5
Aminobenzoate degradation	5
Carbon fixation in photosynthetic organisms	5
Other glycan degradation	5
Glycosphingolipid biosynthesis - ganglio series	5
Galactose metabolism	5
Glycosaminoglycan degradation	5
Glycolysis / Gluconeogenesis	4
Glyoxylate and dicarboxylate metabolism	4

Supplemental Table S8-A. List of enzymes and genes cited in Figure 3A

Enzyme / Tomato Annotation	TAIR Annotation	Fold Change	UNIPROT Annotation
Amylase (AMY)			
Solyc08g077530.2.1	AT4G17090	2.0	Beta-amylase (Q5F305_SOYBN)
Solyc03g095710.2.1	AT4G25000	1.7	Alpha-amylase (Q8LP27_IPONI)
Solyc04g082090.2.1	AT1G76130	1.6	Alpha-amylase (D1MEA8_SOLTU)
Solyc08g007130.2.1	AT4G17090	1.6	Beta-amylase 8 (D7MC27_ARALY)
Hexokinase (HXK)			
Solyc06g066440.2.1	AT4G29130	1.5	Hexokinase (Q9FR27_SOLLC)
Phosphoglucose-isomerase (PGI)			
Not identified	-	-	-
Phosphofructokinase (PFK)			
Solyc08g066100.2.1	AT2G22480	2.3	Phosphofructokinase family protein (D7LE10_ARALY)
Solyc04g014270.2.1	AT4G26270	1.7	Phosphofructokinase family protein (D7MLU4_ARALY)
Solyc07g045160.2.1	AT4G26270	1.5	Phosphofructokinase family protein (D7MLU4_ARALY)
Aldolase (ALD)			
Solyc05g008600.2.1	AT2G01140	1.5	Fructose-bisphosphate aldolase (B9SJY9_RICCO)
Glyceraldehyde 3-phosphate dehydrogenase (GPD)			
Solyc05g014470.2.1	AT1G13440	1.7	Glyceraldehyde 3-phosphate dehydrogenase (Q8LK04_SOLTU)
Phosphoglycerate kinase (PGK)			
Solyc07g066600.2.1	AT1G79550	1.5	Phosphoglycerate kinase (Q2VCK2_SOLTU)
Phosphoglycerate mutase (PGM)			
Solyc04g005010.2.1	AT3G50520	2.1	Phosphoglycerate mutase (A4TBN9_MYCGI)
Solyc05g006090.2.1	AT3G50520	1.5	Phosphoglycerate mutase (A4TBN9_MYCGI)
Enolase (ENO)			
Solyc03g114500.2.1	AT1G74030	1.5	Enolase (A9P745_HELAN)
Pyruvate Kinase (PK)			
Solyc04g008740.2.1	AT5G08570	1.8	Pyruvate kinase (Q3S1N4_SOLTU)
Solyc10g083720.1.1	AT3G52990	1.6	Pyruvate kinase (Q94KE3_ARATH)
Solyc09g008840.2.1	AT3G52990	1.6	Pyruvate kinase (A9P7U5_POPTR)
Pyruvate Dehydrogenase Complex (PDH)			
Solyc05g006520.2.1	AT1G59900	1.3	Pyruvate dehydrogenase alpha subunit (B6TMS5_MAIZE)
Solyc03g097680.2.1	AT5G50850	1.3	Pyruvate dehydrogenase beta subunit (B6T6H3_MAIZE)
Aconitase (ACO)			
Solyc03g005730.2.1	AT4G35830	1.1	Aconitate hydratase (ACO1_ARATH)
Isocitrate Dehydrogenase (IDH)			
Solyc02g082860.2.1	AT4G35260	1.9	Isocitrate dehydrogenase (A3LYS8_PICST)
Ketoglutarate dehydrogenase (KDH)			
Solyc07g064800.2.1	AT5G55070	1.3	2-oxoglutarate dehydrogenase complex (B6TRW8_MAIZE)
Solyc12g005080.1.1	AT4G26910	1.3	2-oxoglutarate dehydrogenase complex (B6TRW8_MAIZE)
Succinyl Co-A Synthethase (SCS)			
Solyc06g083790.2.1	AT2G20420	1.6	Succinyl-CoA synthase (B4D5D9_9BACT)
Succinate dehydrogenase (SDH)			
Solyc02g085350.2.1	AT5G66760	1.7	Succinate dehydrogenase (B8CCT7_THAPS)
Fumarase (FMH)			
Solyc09g075450.2.1	AT5G50950	1.2	Fumarate hydratase class II (C5GAL1_AJEDR)
Malate Dehydrogenase (MDH)			
Solyc01g090710.2.1	AT5G43330	1.8	Malate dehydrogenase (MDHC_ORYSJ)
Citrate Synthase (CS)			
Solyc01g073740.2.1	AT2G44350	1.5	Citrate synthase (O24135_TOBAC)
ATP Citrate Lyase (CL)			
Solyc01g059880.2.1	AT1G10670	2.0	ATP citrate lyase a-subunit (Q93YH4_LUPAL)
Solyc12g099260.1.1	AT3G06650	1.9	ATP citrate lyase a-subunit (Q93YH4_LUPAL)
Lactate Dehydrogenase (LDH)			
Solyc08g078850.2.1	AT4G17260	1.7	L-lactate dehydrogenase (Q96569_SOLLC))

Supplemental Table S8-B. List of enzymes and genes cited in Figure 3B

Enzyme / Tomato Annotation	TAIR Annotation	Average Fold Change	InterPro Annotation
Phosphoenolpyruvate translocator (CUE1)			
Solyc03g112870.2.1	AT5G33320	2.0	PEP translocator (Q9MSB4_MESCR)
Pyruvate decarboxylase (PDC)			
Solyc09g005110.2.1	AT4G33070	3.9	Pyruvate decarboxylase 1 (Q84V95_LOTCO)
Solyc10g076510.1.1	AT4G33070	3.9	Pyruvate decarboxylase (B0ZS79_PRUAR)
Aldehyde dehydrogenase (ALDH)			
Solyc05g005700.2.1	AT1G23800	1.8	Aldehyde dehydrogenase 1 (Q84V96_LOTCO)
Acetyl-CoA synthethase (ACS)			
Solyc07g017860.2.1	AT5G36880	2.2	Acetyl-coenzyme A synthetase (Q2J3D0_RHOP2)
Alcohol Dehydrogenase (ADH)			
Solyc08g083280.2.1	AT1G64710	2.7	Alcohol dehydrogenase 2 (B6TDN5_MAIZE)
Solyc06g059740.2.1	AT1G77120	1.9	Alcohol dehydrogenase 2 (Q84UY3_PETHY)
Photosystem I (PSI)			
Solyc09g015320.1.1	ArthCp023	1.5	Photosystem I chlorophyll a apoprotein A1 (D3WBH6_9AQUA)
Solyc06g054260.1.1	AT1G03130	-1.6	Photosystem I reaction center subunit II (B4FAW3_MAIZE)
Solyc09g063130.2.1	AT2G20260	-1.6	Photosystem I reaction center subunit IV A (B6TH55_MAIZE)
Solyc02g069450.2.1	AT1G31330	-1.6	Photosystem I reaction center subunit III (Q9XQB4_PHAAU)
Solyc08g006930.2.1	AT1G30380	-1.7	Photosystem I subunit X psaK (Q84QE6_TOBAC)
Solyc03g120640.2.1	AT3G16140	-1.7	Photosystem I subunit VI-1 chloroplastic (PSAH1_ARATH)
Solyc06g074200.2.1	AT1G08380	-1.8	Photosystem I reaction center subunit O (Q949Q5_ARATH)
Solyc06g066640.2.1	AT1G52230	-1.8	Photosystem I subunit VI-2 chloroplastic (PSAH2_ARATH)
Solyc07g066150.1.1	AT1G55670	-1.8	Photosystem I reaction center subunit III (Q9XQB4_PHAAU)
Solyc08g013670.2.1	AT5G64040	-2.4	Photosystem I reaction center subunit (D2K7Z2_TOBAC)
Photosystem II (PSII)			
Solyc02g038710.1.1	ArthCp006	1.6	Photosystem II reaction center protein I (D3WCU5_RHOSS)
Solyc05g025600.1.1	AT2G06520	-1.5	Chloroplast photosystem II subunit X (B1PPX5_9MYRT)
Solyc06g084050.2.1	AT2G30570	-1.5	Photosystem II reaction center W protein (B6TMB3_MAIZE)
Solyc06g060340.2.1	AT1G44575	-1.6	Photosystem II-associated protein (A8HPM2_CHLRE)
Solyc12g099650.1.1	AT3G21055	-1.6	Photosystem II 5 kDa protein chloroplastic (PST2_GOSHI)
Solyc10g077120.1.1	AT1G67740	-1.7	Photosystem II subunit psbY (AHRD V1 *-*- B6SR26_MAIZE)
Solyc07g044860.2.1	AT1G06680	-1.8	Oxygen-evolving enhancer protein 2 (PSBP_SOLLC)
Plastidic ATP synthase (pATPs)			
Solyc02g080540.1.1	AT4G04640	1.0	ATP synthase gamma chain (B7FIR4_MEDTR)
Solyc05g050500.1.1	AT4G09650	-1.1	ATP synthase F1 delta subunit (B4B4T7_9CHRO)
Cytochrome b6/f (CB6F)			
Solyc01g103190.2.1	AT4G21192	1.2	COX assembly mitochondrial protein (COXAM_SCHPO)
Solyc05g012440.2.1	AT1G02410	1.1	Cytochrome c oxidase assembly protein ctaG (B6T5S8_MAIZE)
Solyc02g068930.2.1	AT5G45040	-1.1	Cytochrome c6 (D7MKY9_ARALY)
Solyc10g051200.1.1	AT1G49380	-1.2	Cytochrome c biogenesis protein family (D7KE35_ARALY)
RuBisCo			
Solyc01g007330.2.1	ArthCp030	2.3	Ribulose bisphosphate carboxylase large chain (RBL_SOLLC)
Solyc03g034220.2.1	AT5G38410	-1.3	Ribulose bisphosphate carboxylase small chain (A9YTZ7_SOLTU)
Plastidic Phosphoglycerate Kinase (pPGK)			
Solyc07g066610.2.1	AT1G56190	-1.2	Phosphoglycerate kinase (AHRD V1 ***- O81394_SOLTU)
Plastidic Glyceraldehyde 3-phosphate dehydrogenase			
Solyc04g009030.2.1	AT3G26650	-1.3	Glyceraldehyde-3-phosphate dehydrogenase (D7UNZ5_BRARC)
Phosphoglycolate phosphatase (PGP)			
Solyc11g008620.1.1	AT5G36700	-2.1	Phosphoglycolate phosphatase (C1FHY2_9CHLO)
Glycolate Oxidase (GOX)			
Solyc07g056540.2.1	AT3G14420	-1.5	Glycolate oxidase (AHRD V1 ***- C0S8Q7_PARBP)
Glyoxylate aminotransferase (GLA)			
Solyc04g054310.2.1	AT4G39660	-1.5	Alanine-glyoxylate aminotransferase (D2KZ10_WHEAT)
Glycine dehydrogenase (GLD)			
Solyc08g065220.2.1	AT4G33010	-1.4	Glycine dehydrogenase P (AHRD V1 **** Q6V9T1_ORYSJ)
Serine-Glyoxylate Aminotransferase (SGA)			
Not identified	-	-	-
Pyruvate reductase (PYR)			
Not identified	-	-	-
Glycerate Kinase			
Solyc03g120430.2.1	AT1G80380	-1.8	Glycerate kinase (C1DYY5_9CHLO)

Supplemental Table S9. Family classification of the differentially expressed transcription factors

	Up-Regulated		Down-Regulated			Up-Regulated		Down-Regulated	
Family	**Total**	**Literature**	**Total**	**Literature**	**Family**	**Total**	**Literature**	**Total**	**Literature**
ABI3VP1	9	3	2	2	**HMG**	1	1	0	0
Alfin-like	0	0	1	1	**HSF**	3	2	1	1
AP2-EREBP	13	7	8	2	**Jumonji**	2	1	1	1
ARF	3	2	0	0	**LIM**	0	0	1	0
ARID	1	0	0	0	**LOB**	2	1	3	2
ARR-B	1	0	1	1	**LUG**	0	0	0	0
AUX/IAA	2	1	0	0	**MADS**	5	2	1	1
BES1	2	1	0	0	**mTERF**	3	0	0	0
bHLH	6	2	6	0	**MYB**	8	5	3	3
BSD	1	0	0	0	**MYB-related**	6	2	4	3
bZIP	6	4	3	1	**NAC**	14	7	4	3
C2C2-CO-like	1	0	0	0	**OFP**	1	0	1	0
C2C2-DOF	3	1	3	3	**Orphans**	10	6	5	4
C2C2-GATA	2	0	0	0	**PHD**	5	2	4	3
C2H2	9	3	7	1	**PLATZ**	2	0	0	0
C3H	8	1	1	0	**Pseudo ARRB**	1	1	1	1
CAMTA	1	1	0	0	**RWP-RK**	3	2	0	0
CCAAT	2	1	2	2	**SBP**	3	1	0	0
CSD	1	1	0	0	**SET**	3	1	1	0
DBP	1	0	0	0	**SNF2**	14	6	4	2
DDT	0	0	1	0	**SWI-SNF-SWI3**	2	2	0	0
FAR1	4	0	0	0	**TAZ**	1	1	3	0
FHA	1	1	1	1	**TCP**	2	1	0	0
G2-like	7	4	0	0	**TRAF**	2	0	1	0
GNAT	5	2	0	0	**TRIHELIX**	4	2	1	0
GRAS	4	1	0	0	**WRKY**	4	0	1	0
GRF	1	1	1	1	**zf-HD**	2	1	0	0
HB	7	4	2	1	**TOTAL**	**204**	**88**	**79**	**40**

DISCUSSÃO GERAL

Neste trabalho foi proposto como objetivo principal identificar os mecanismos moleculares desencadeados pela superexpressao da UCP1 de Arabidopsis thaliana em *Nicotiana tabacum* e seus efeitos na resposta a condições de estresse abiotico. As UCPs são reguladas transcricionalmente para, sob determinadas condições desfavoráveis, funcionar como uma válvula de escape, diminuindo a força protonmotiva e a formação de ROS pela ETC [5,17,26-28]. Estas condições incluem estresses causados pelo frio e situações onde a demanda celular por ATP diminui. No entanto, os efeitos positivos resultantes da sua superexpressão em plantas não são simplesmente provocados por uma diminuição na produção de ROS. Mostramos que a superexpressão de uma única proteína mitocondrial é capaz de desencadear um amplo mecanismo de sinalização retrógrada, alterar a morfologia mitocondrial e aumentar o processo de biogênese mitocondrial em conjunto com a regulação de genes relacionados à maquinaria antioxidante e responsivos a estresses. Este mecanismo de sinalização é provavelmente desencadeado por uma disfunção metabólica causada pelo desacoplamento da respiração, e consequente diminuição da síntese de ATP.

A ampla alteração na expressão gênica nos levou a explorar mais profundamente pela primeira vez os efeitos globais, em nível de transcrição, desencadeados pela superexpressão da UCP1. Essa análise nos levou a confirmar de fato que a superexpressão da UCP1 tem um efeito estressante a nível celular, visto que genes responsivos a estresses correspondem à categoria mais representada entre os genes com expressão aumentada. Essa afirmação pode soar contraditória, visto que a superexpressão da UCP1 tem um efeito benéfico. De fato, estudos recentes em *Saccharomyces cereviseae* [54]*, C. elegans* [55]*, Drosophila melanogaster* [56] em *Mus musculus*[57] mostram que disfunções no metabolismo mitocondrial são capazes de induzir adaptações no metabolismo celular levando a um aumentao no tempo de vida e na resistência a estresses abióticos tanto no curto como no longo prazo. Estas adaptações em plantas transgênicas superexpressando UCP1 não estão apenas localizadas na mitocôndria, mas também em vias metabólicas localizadas no citoplasma e nos cloroplastos. Entre as alterações mais interessantes se encontram a de vias relacionadas à respiração anaeróbica. Em plantas durante o

desenvolvimento do grão de pólem, as células são capazes de induzir a respiração aeróbica em conjunto com a anaeróbica a fim de promover energia para a alta demanda celular por ATP [58,59]. Hipotetizamos que o aumento em vias anaeróbicas nas plantas transgênicas faz parte do mecanismo compensatório causado pelo aumento da respiração desacoplada, e contribui para a síntese de ATP. Este resultado também corrobora as alterações morfológicas que foram observadas em experimentos de microscopia eletrônica, visto que em mamíferos elas são provocadas por um estresse causado por hipóxia [14]. Outra das alterações mais interessantes se relaciona a genes que participam dos fotossistemas, cuja expressão se encontra diminuída em plantas transgênicas. Esse fato de certa maneira vai de encontro às afirmações feitas por estudos anteriores, que em constraste mostram um aumento na capacidade de assimilação de carbono em plantas transgênicas superexpresando UCP1 [34,35], e uma diminuição da assimilação de carbono em mutantes para UCP1 de *Arabidopsis thaliana* [36]. Nos trabalhos anteriores não foram realizadas análises em nível de transcrição de genes relacionados ao fotossistema. A nossa hipótese é de que estas alterações podem não provocar consequências para as reações dependentes de luz da fotossíntese, mas sim estar relacionadas à diminuição dos principais sítios de produção de ROS no cloroplasto: os fotossistemas 1 e 2 [60]. Outra hipótese é de que a alteração do estado redox celular provocado pela superexpressão da UCP1 observada tanto em estudos anteriores [35], como neste trabalho, poderiam interferir na eficiência dos fotossistemas. A inferência de hipóteses baseadas apenas em dados de expressão deve ser feita com cuidado quando não acompanhadas de outras análises. Desta maneira, ainda permanece elusiva a relação entre a diminuição na expressão de genes relacionados ao fotossistema e o aumento na assimilação de carbono em plantas transgênicas.

A ampla gama de processos alterados nas plantas transgênicas dificulta a identificação de reguladores de um processo específico, como o de biogênese mitocondrial. De fato, a principal dificuldade em se encontrar reguladores específicos de genes mitocôndrias se dá pelo fato que interferências nesta organela geralmente desencadeiam alterações em outros compartimentos celulares [7]. Dentre os 283 fatores de transcrição identificados neste trabalho existem vários que já foram caracterizados e cujas funções corroboram os resultados apresentados nesta tese. Apesar disso, grande parte se refere a

reguladores cuja função ainda é desconhecida e que podem ser explorados para o estudo do processo de biogênese mitocondrial e resposta a estresses.

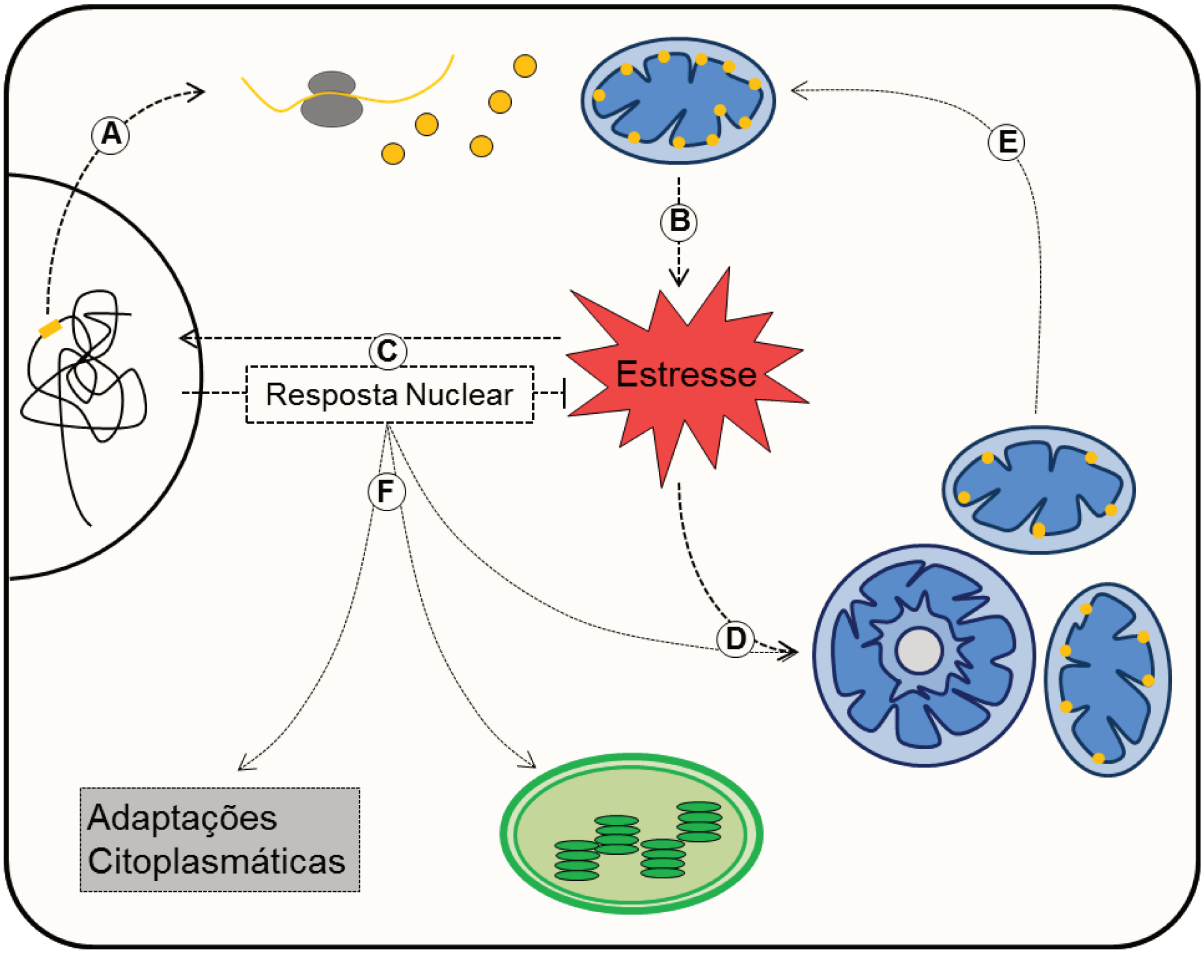

Figura 5. Modelo proposto para o mecanismo de resposta celular à superexpressão da UCP1. **(A)** A superexpressão da UCP1 leva a um aumento na respiração desacoplada e provoca um **(B)** estresse a nível celular. Este estresse provovalmente é causado pela diminuição nos níveis intracelulares de ATP, mas outro mecanismo possível seria desencadeado pelo acúmulo excessivo de proteínas na membrana interna mitocondrial. Não é descartada a hipótese de um efeito sinergístíco entre estes dois tipos de estresse. **(C)** Um mecanismo de sinalização retrógrada é induzido e aumenta a expressão de genes responsivos a estresse em conjunto com **(D)** genes mitocondriais codificados pelo genoma nuclear e mitocondrial. Em resposta temos alterações morfológicas na mitocôndria e um aumento no número destas organelas, que **(E)** reentram no ciclo. **(F)** Em resposta às alterações no metabolismo celular, não apenas genes mitocôndrias têm expressão diferencial em plantas transgênicas, mas também genes mapeados em importantes vias metabólicas do cloroplasto e do citoplasma. Em conjunto, estes mecanismos mostram uma ampla e diversa adaptação celular em resposta a superexpressão da UCP1.

CONCLUSÕES

Os resultados apresentados nesta tese trazem grandes avanços para o entendimento do mecanismo molecular que confere resistência a estresses em plantas transgênicas superexpressando UCP1. Enquanto anteriormente se acreditava que o efeito da superexpressão da UCP1 seria simplesmente diminuir a produção de ROS, o que se vê é que os altos níveis intracelulares desta proteína provocam um estresse a nível celular. Um mecanismo de sinalização retrógrada é ativado e resulta em uma reconfiguração do metabolismo e melhor desempenho sob condições estressantes. O estudo deste mecanismo, definido como mitohermese, tem sido realizado em diversos organismos-modelo exceto plantas. A partir de agora este mecanismo pode ser explorado em plantas e pode contribuir para o desenvolvimento de variedades mais resistentes a estresses abióticos.

REFERÊNCIAS BIBLIOGRÁFICAS

1. Mazzarello, P: **A unifying concept: the history of cell theory.** *Nat. Cell Biol.* 1999*,* **1**, E13-E15.

2. Searcy DG: **Metabolic integration during the evolutionary origin of mitochondria**. *Cell Res* 2003, **13**:229-238.

3. McBride HM, Neuspiel M, Wasjak S: **Mitochondria: more than just a powerhouse.** *Cell Curr. Biol.* 2006, **16**:R551-R560.

4. Poirier Y, Antonenkov VD, Glumoff T, Hiltunen JK: **Peroxisomal beta-oxidation--a metabolic pathway with multiple functions.** Biochim. Biophys. Acta 2006, **12:**1413-1426.

5. Krauss S, Zhang CY, Lowell BB: **The mitochondrial uncoupling-protein homologues.** Nat Rev Mol Cell Biol. 2005, **6**:248-61.

6. Vafai SB, Mootha VK: **Mitochondrial disorders as windows into an ancient organelle.** *Nature* 2012, **491**:374-383.

7. Welchen E, Garcia L, Mansilla N, Gonzalez DH: **Coordination of plant mitochondrial biogenesis: keeping pace with cellular requirements.** *Front Plant Sci* 2013, **4:**551.

8. Zabaleta E, Heiser V, Grohmann L, Brennicke A: **Promoters of nuclear-encoded respiratory chain complex I genes from Arabidopsis thaliana contain a region essential for anther/pollen-specific expression.** *Plant J* 1998, **156**:49-59.

9. Binder S, Brennicke A: **Gene expression in plant mitochondria: transcriptional and post-transcriptional control.** *Philos Trans R Soc Lond B Biol Sci* 2003, **3586**:181-188.

10. Welchen E, Chan RL, Gonzalez D H: **The promoter of the *Arabidopsis* nuclear gene COX5b-1, encoding subunit 5b of the mitochondrial cytochrome c oxidase, directs tissue-specific expression by a combination of positive and negative regulatory elements.** *J. Exp. Bot.* 2004, **55**:1997–2004.

11. Nicholls DG, Locke RM: **Thermogenic mechanisms in brown fat**. *Physiol Rev*. 1984, **64:**1–64.

12. Kajimura S, Seale P, Spiegelman BM: **Transcriptional control of brown fat development.** *Cell Metab* 2010, 11:257-262.

13. Liesa M, Shirihai OS: **Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure.** *Cell Metab* 2013, 17:491-506.

14. Liu X, Hajnóczky G: **Altered fusion dynamics underlie unique morphological changes in mitochondria during hypoxia-reoxygenation stress.** Cell Death Differ 2011, **18**:1561–1572.

15. Giegè P, Sweetlove LJ, Congat V, Leaver CJ: **Coordination of nuclear and mitochondrial genome expression during mitochondrial biogenesis in Arabidopsis.** *Plant Cell* 2005, **17:**1497–1512.

16. Turrens JF: **Mitochondrial formation of reactive oxygen species**. *J Physiol* 2003, **552**: 335–344.

17. Figueira TR, Barros MH, Camargo AA, Castilho RF, Ferreira JCB, Kowaltowski AJ, Sluse FE, Souza-Pinto NC, Vercesi AE: **Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health**. *Antioxid Redox Signal* 2013, **18**: 2029-2074.

18. Nicholls DG, Rial E: **A history of the first uncoupling protein, UCP1.** *J. Bioenerg. Biomembr.* 1999, **31:**399–418.

19. Kozak LP, Anunciado-Koza R: **UCP1: its involvement and utility in obesity**. *Int. J. Obes* 2008, **32:**S32-S38.

20. Klaus S: **Adipose tissue as a regulator of energy balance**. *Curr Drug Targets.* 2004, 5:241-250.

21. Klaus S, Rudolph B, Dohrmann C, Wehr R: **Expression of uncoupling protein 1 in skeletal muscle decreases muscle energy efficiency and affects thermoregulation and substrate oxidation.** *Physiol. Genomics* 2005, **21:**193-200.

22. Vercesi AE, Martins LS, Silva MAP, Leite HMF, Cuccovia IM, Chaimovich H: **PUMPing plants**. *Nature* 1995, **375**:24.

23. Stuart JA, Harper JA, Brindle KM, Brand MD: **Uncoupling protein 2 from carp and zebrafish, ectothermic vertebrates.** *Biochim. Biophys. Acta* 1999, **1413**:50–54.

24. Raimbauld S, Dridi S, Denjean F, Lachuer J, Couplan E, Bouilaud F, Bordas A, Duchamp C, Taouis M, Ricquier D*:* **An uncoupling protein homologue putatively involved in facultative muscle thermogenesis in birds**. *Biochem. J. 2001, 353:441–44*

25. Fridell YWC, Sanchez-Blanco A, Silvia BA, Helfand SL: **Functional characterization of a Drosophila mitochondrial uncoupling protein**. J. Bioenerg. Biomembr. 2004, **36:**219–28.

26. Vercesi AE, Borecký J, Maia IG, Arruda P, Cuccovia IM, Chaimovich H: **Plant uncoupling mitochondrial protein**s. *Annu Rev Plant Biol* 2006, **57**:383-404.

27. Maia IG, Benedetti CE, Leite A, Turcinelli SR, Vercesi AE, Arruda P: **AtPUMP: an Arabidopsis gene encoding a plant uncoupling mitochondrial protein.** *FEBS Lett* 1998, **429:**403–406.

28. Borecky J, Nogueira FTS, Oliveira KAP, Maia IG, Vercesi AE, Arruda P: **The plant energy-dissipating mitochondrial systems: depicting the genomic structure and the expression profiles of the gene families of uncoupling protein and alternative oxidase in monocots and dicots.** *J Exp Bot* 2006, 57:849-864.

29. Ito-Inaba Y, Hida Y, Mora H, Inaba T: **Molecular identity of uncoupling proteins in thermogenic skunk cabbage.** *Plant Cell Physiol* 2008, **49:**1911–1916.

30. Boss O, Samec S, Paoloni-Giacobino A, Rossier C, Dulloo A, Seydoux J, Muzzin P, Giacobino JP: **Uncoupling protein-3: a new member of the mitochondrial carrier Family with tissue-specific expression.** *FEBS Lett.* 1997, **408**:39-42.

31. Fleury C, Neverova M, Collins S, Raimbault S, Champigny O, Levi-Meyrueis C, Bouillaud F, Seldin MF, Surwit RS, Ricquier D, Warden CH: **Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia.** *Nat. Genet.* 1997, **15**:269–72.

32. Mao WG, Yu XX, Zhong A, Li WL, Brush J, Sherwood SW, Adams SH, Pan G: **UCP4, a novel brain-specific mitochondrial protein that reduces membrane potential in mammalian cells.** *FEBS Lett.* 1999, **443**:326–30.

33. Brandalise M, Maia IG, Borecký J, Vercesi AE, Arruda P: **Overexpression of plant mitochondrial uncoupling protein in transgenic tobacco increases tolerance to oxidative stress.** *J Bioenerg Biomembr* 2003, **35:**205–209.

34. Begcy K, Mariano ED, Mattiello L, Nunes AV, Mazzafera P, Maia IG, Menossi M: **An *Arabidopsis* mitochondrial uncoupling protein confers tolerance to draught and salt stress in transgenic tobacco plants.** *Plos One* 2011, **6:**e23776.

35. Chen S, Liu A, Zhang S, Li C, Chang R, Liu D, Ahammed GJ, Lin X: **Overexpression of mitochondrial uncoupling protein conferred resistance to heat stress an *Botrytis cinerea* infection in tomato.** *Plant Physiol Biochem* 2013, **73:**245–253.

36. Sweetlove LJ, Lytovchenko A, Morgan M, Nunes-Nesi A, Taylor NL, Baxter CJ, Eickmeier A, Fernie AR: **Mitochondrial uncoupling protein is required for efficient photosynthesis.** *Proc Natl Acad Sci U S A* 2006, **103:**19587–19592.

37. Smith AMO, Ratcliffe G, Sweetlove LJ: **Activation and function of mitochondrial uncoupling protein in plants.** *J Biol Chem* 2004, **279:**51944–51952.

38. Unseld M, Marienfeld JR, Brandt P, Brennicke A: **The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotide.** *Nat Genet* 1997, **15:**57–61.

39. Woodson JD, Chory J: **Coordination of gene expression between organellar and nuclear genomes.** *Nat Rev Genet* 2008, **9:**383–395.

40. Ryan MT, Hoogenrad MJ: **Mitochondrial-nuclear communications.** *Annu Rev Biochem* 2007, **76:**701–722.

41. Pesaresi P, Schneider A, Kleine T, Leister D: **Interorganellar communication.** *Curr Opin Plant Biol* 2007, **10:**600–606.

42. Howell KA, Millar AH, Whelan J: **Ordered assembly of mitochondria during rice germination begins with pro-mitochondrial structures rich in components of the protein import apparatus.** *Plant Mol. Biol.* 2006, **60**:201–223.

43. Taylor NL, Howell KA, Heazlewood JL, Tan TY, Narsai R, Huang S, Whelan J, Millar AH: **Analysis of the rice mitochondrial carrier family reveals anaerobic accumulation of a basic amino acid carrier involved in arginine metabolism during seed germination**. *Plant Physiol.* 2010, **154:**691-704.

44. Cvetkovska M, Vanlerberghe GC: **Alternative oxidase impacts the plant response to biotic stress by influencing the mitochondrial generation of reactive oxygen species.** *Plant Cell Environ.* 2013, **36**:721–732.

45. Roschzttardtz H, Fuentes I, Vásquez M, Corvalán C, León G, Gómez I, Araya A, Holuique L, Vicente-Carbajosa J, Jordana X: **A nuclear gene encoding the iron-sulfur subunit of mitochondrial complex II is regulated by B3 domain transcription factors during seed development in *Arabidopsis thaliana*.** *Plant Physiol* 2009, **150:**84–95.

46. Van Aken O, Zhang B, Law S, Narsai R, Whelan J: **AtWKRY40 and AtWRKY63 modulate the expression of stress-responsive nuclear genes encoding mitochondrial and chloroplast proteins.** *Plant Physiol* 2013, **162:**254–271.

47. Giraud E, Ng S, Carrie C, Duncan O, Low J, Lee CP, Van Aken O, Millar AH, Murcha M, Whelan J: **TCP transcription factors link the regulation of genes encoding mitochondrial proteins with the circadian clock in *Arabidopsis thaliana*.** *Plant Cell* 2010, **22:**3921–3934.

48. De Clercq I, Vermeirssen V, Van Aken O, Vandepoele K, Murcha MW, Law SR, Inzé A, Ng S, Ivanova A, Rombaut D, Van de Cotte B, Jaspers P, Van de Peer Y, Kangasjärvi J, Whelan J, Van Breusegem F: **The membrane-bound NAC transcription factor ANAC013 functions in mitochondrial retrograde regulation of the oxidative stress response in Arabidopsis.** *Plant Cell.* 2013, **25:**3472–3490.

49. Parikh VS, Morgan MM, Scott R, Clements LS, Butow RA: **The mitochondrial genotype can influence nuclear gene expression in yeast.** *Sciente* 1987, **235**:576-580.

50. Jazwinski SM, Kriete A: **The yeast retrograde response as a model of intracellular signaling of mitochondrial dysfunction.** *Front. Physiol.* 2012, **3:**1-12.

51. Nargund AM, Pellegrino MW, Fiorese CJ, Baker BM, Haynes CM: **Mitochondrial import efficiency of ATFS-1 regulates mitochondrial UPR activation.** *Science* 2012, **337:587-590.**

52. Yun J, Finkel T: **Mitohormesis.** *Cell Metab.* 2014, **19:757-766.**

53. St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jäger S, Handschin C, Zheng K, Lin J, Yang W, Simon DK, Bachoo R, Spiegelmal BM: **Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators.** *Cell* 2006, **127:**397-408.

54. Kirchman PA, Kim S, Lai CY, Jazwinski SM: **Interorganelle signaling is a determinant of longevity in *Saccharomyces cerevisiae***. *Genetics* 1999, **152**:179–190.

55. Lee SS, Lee RY, Fraser AG, Kamath RS, Ahringer J, Ruvkun G: **A systematic RNAi screen identifies a critical role for mitochondria in *C.elegans* longevity**. *Nat. Genet*. 2003, **33**:40–48.

56. Copeland JM, Cho J, Lo Jr. T, Hur JH, Bahadorani S, Arabyan T, Rabie J, Soh J, Walker DW: **Extension of *Drosophila* life span by RNAi of the mitochondrial respiratory chain**. *Curr. Biol.* 2009, **19**:1591–1598.

57. Lapointe J, Stepanyan Z, Bigras E, Hekimi S: **Reversal of the mitochondrial phenotype and slow development of oxidative biomarkers of aging in long-lived Mclk1+/- mice.** *J. Biol. Chem.* 2009, 284:20364–20374.

58. Gass N, Glagotskaia T, Mellema S, Stuurman J, Barone M, Mandel T, Roessner-Tunali U, Kuhlemeier C: **Pyruvate decarboxylase pro- vides growing pollen tubes with a competitive advantage in petunia.** *Plant Cell* 2005, **7**: 2355–2368.

59. Rounds CM, Winship LJ, Hepler PK: **Pollen tube energetics: respiration, fermentation and the race to the ovule.** *AoB Plants* 2011, **19**:1-14.

60. Suzuki N, Koussevitzky S, Mittler R, Miller G: **ROS and redox signalling in the response of plants to abiotic stress.** *Plant Cell Environ.* 2012, **35**:259-270.

ANEXOS

Anexo I

Pedro Barreto <pedropbarretto@gmail.com>

Your recent submission to PSL

1 message

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Corresponding Author: Paulo Arruda
Co-Authors: Pedro Barreto, PhD; Vagner Okura, MsSci; Izabella A Neschich, PhD; Ivan G Maia, PhD;
Title: UCP1 overexpression in tobacco alters organellar signaling and reconfigures cellular energy metabolism

If you did not co-author this submission, please contact the Corresponding Author of this submission at parruda@unicamp.br; do not follow the link below.

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Anexo II

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18 de junho de 2014.

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Identificação:
Doutorado: Pedro Paulo A. F. Arantes Pereira Barreto, CPG-GBM UNICAMP
Projeto: Alterações no metabolismo energético provocadas pela superexpressão da proteína desacopladora mitocondrial 1 (UCP1) em tabaco induzem biogênese mitocondrial e resposta global a estresses

Parecer:
Projeto aprovado pela CIBio/CBMEG sob número 02/2013.
Coordenador: Prof. Dr. Paulo Arruda

Profa. Dra. Edi Lúcia Sartorato
Presidente da CIBio/CBMEG - UNICAMP

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