JOÃO HENRIQUE FROTA CAVALCANTI

ENERGY METABOLISM IN *Arabidopsis thaliana*: TCA CYCLE EVOLUTION, AMINO ACIDS DEGRADATION AND ALTERNATIVE PATHWAYS

Thesis presented to the Universidade Federal de Viçosa as part of the requirement of the Plant Physiology Graduate Program for obtention of the degree of *Doctor Scientiae.*

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BIOGRAPHY

João Henrique Frota Cavalcanti, son of Francisco Juscelino Maciel Cavalcanti and Mirna Maria Frota Cavalcanti, was born in Fortaleza, Ceará State, Brazil, in February 18th, 1985.

In 2004, he started the undergraduate course in Biology at the Universidade Federal do Ceará (UFC), Fortaleza, Ceará State, Brazil and achieved the bachelor degree in December of 2008. He started his Master course at the same university where he achieved the Master degree in Master in Biochemistry in 2011.

In the same year(2011), he started his PhD studies at the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil. He spent 11 months as a guest PhD student at the Leibniz Universität Hannover, Hannover, Germany, under the supervision of Prof. Dr. Hans-Peter Braun before finish his studies in Plant Physiology at UFV under the supervision of Prof. Wagner L. Araújo.

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ABSTRACT

CAVALCANTI, João Henrique Frota, D.Sc., Universidade Federal de Viçosa, July 2015.**Amino acids degradation and its association with energy metabolism in *Arabidopsis thaliana*.**Advisor: Wagner L. Araújo. Co-advisor: Adriano Nunes-Nesi.

Plant mitochondrion are involved in several key cellular processes that goesbeyond energy production being also associated with programmed cell death, fruit ripening and even light-associate process including phorespiration and photosynthesis. In this context, mitochondria acquisition by host cell brought evolutionary advances for the existing plant cell by the preservation of diverse metabolic pathways including both those related to energy metabolism as well as those associated with lipids, nucleotides and vitamin biosynthesis. The most notorious heritage is related to the tricarboxylic acid (TCA) cycle. The TCA cycle is an essential pathway which is related to reducing power (NADH and FADH2) generation, nitrogen assimilation and photosynthesis optimization. It has been suggested that the TCA cycle operated as isolated steps prior endosymbiosis events and that only after mitochondria acquisition it was possible for it to be organized and function as a cycle. The TCA cycle is composed by a set of eight enzymes. However, each enzyme is encoded by several genes which are targeting not just to mitochondria, but that are also imported into others subcellular compartments. These TCA enzymes located in other subcellular compartiments result in likely a broader connection between mitochondria and other organelles (e.g. peroxissome and chloroplast) allowing a bypass of the intermediates of the cycle switching his operation to an unusual in non-cyclic modes flux. It is also currently accepted that under stress conditions, which leads to decreases in carbohydrate levels, the TCA cycle can function in non-cyclic flux mode due to diminishing of carbon skeleton the enter it making required that be fed by anauplerotic reactions. Therefore, amino acids become essential to support respiration and ATP synthesis under such situations. Compelling evidence have demonstrated that branched chain amino acids (BCAA) and lysine can supply electrons to the mitochondrial electron transport chain (mETC) by the action of the electron transfer flavoprotein (ETF)-ETF: ubiquinone oxidoreductase (ETF/ETFQO) system and associated dehydrogenases. In plants, only isovaleryl-CoA dehydrogenase (IVDH) and (*D*)-2-hydroxyglutarate dehydrogenase (D2HGDH) have been characterized as electron donnor to the ubiquinol pool via this system so far by the degradation of BCAA and lysine, respectively. In fact, BCAA catabolism is of pivotal importance to provide intermediates to TCA cycle, particularly under stress situations, whereas lysine shows a strict association with the TCA cycle being required to couple amino acid degradation and energy generation. The electron transfer through the mETC is tightly coupled to ATP synthesis and use electron donates by NADH and $FADH_2$ to phosphorylate ADP to ATP. However, our knowledged regarding the organization of the mitochondrial oxidative phosphorylation (OXPHOS) system and its alternatives pathways under energy limitation remains elusive. Thus, this thesis, which is focused on the function of respiration within the context of the role of the TCA cycle as well as the

function of alternative electron donors to the mETC, iscomprised by three independent stand-alone chapters focusing on energy metabolism and alternative respiration in *Arabidopsis thaliana*. Hence to obtain a compreenhesive picture of how the TCA cycle evolved and to which extend its alternative pathways interact to adjust to different cellular and metabolic requirements, three experimental approaches were used: (*i*) by using bioinformatic approaches we investigated the evolutionary history of TCA cycle genes allowing the generation of a model for the origin of the TCA cycle genes in plants and connected its evolution with TCA cycle behavior under a range of stress; (*ii*) the importance of lysine deficiency were investigated by using an Arabidopsis mutant with reduced activity of the lysine biosynthesis enzyme *L*,*L*-diaminopimelate aminotransferase (dapat), and (*iii*) the metabolic reprograming associated with the OXPHOS system were investigated following carbon limitation.. In brief, the results presented here provided several novel findings and allowed, at least preliminarly, mechanistic interpretation thereof. First, it facilitate the elucidation of the evolutionary origem of the TCA cycle in land plants providing support to the contention that the origin of isoforms present in different subcellular compartments might be associated either with gene-transfer events which did not result in correct targeting or with new gene copys generated by genome duplication and horizontal transfer gene. Additionally, coexpression analyses of TCA cycle genes following different stress conditions in both shoot and root tissues demonstrated the presence of a large molecular plasticity and provided an explanation for the modular operation of the TCA cycle in land plants. Secondly, by using an Arabidopsis mutant with reduced activity of the Lys biosynthesis enzyme *L*,*L*-diaminopimelate aminotransferase (dapat) it was demonstrated that lysine biosynthesis deficiency mimics stress situation and impacts both plant growth and leaf metabolism.Thirdly, by evaluating OXPHOS system behavior following carbon starvation and how a range of amino acids can impact respiratory complexes it was possible to further demonstrate that OXPHOS is affected in function of the carbon source and that alternative pathways are induced under this condition.In addition, immunoblotting assays revealed that OXPHOS system is most likely regulated by posttranslational modification. When considered together these results highlight the complexity and specificity of plant respiration during evolution and that it is differently affected following energy limitation by the usage of alternative substrates. The results discussed here support the contention that ETF/ETFQO is an essential pathway able to donate electrons to the mETC and that amino acids are alternative substrates maintaining respiration under carbon starvation.The results obtained are discussed in the context of current models of metabolic evolution showing the strict association of energy metabolism with amino acids metabolism, and where possible, mechanistic insights are properly discussed.

Key-words: alternative substrate respiration; energy deprivation; mitochondria evolution; mitochondria metabolism; neofunctionalization; OXPHOS; paralogous genes; stress response; TCA cycle;

RESUMO

CAVALCANTI, João Henrique Frota, D.Sc., Universidade Federal de Viçosa, Julho 2015. **Degradação de aminoácidos e sua associação com o metabolismo energético em *Arabidopsis thaliana*.** Orientador: Prof. Wagner L. Araújo. Co-Orientador: Prof. Adriano Nunes-Nesi.

Mitocondrias vegetais estão envolvidas em vários processos chaves da célula, vão além da produção de energica, tais como morte celular programada, amadurecimento de frutos, ou mesmo aqueles processos dependente de luz como fotossíntese e fotorrespiração. Dessa forma, aquisição mitocondrial pela célula hospedeira trouxe avanços para as atuais células vegetais: desde a manutenção de diversas vias metabólicas que incluem o metabolismo energético bem como processos de bissíntese de lipidios, nucleotidios e vitaminas. No tocante ao metabolismo energ-etico, destaca-se a herança do ciclo do ácido tricarboxílico. Este ciclo é uma via essencial relacionada com a produção de poder redutor (NADH e $FADH_2$), assimilação de nitrogênio e otimização da fotssíntese. Acredita-se que o ciclo do ácido tricarboxílico oprerasse como passos isoladados antes do processo endossimbiótico e somente após a aquisição da mitocôndria resultou que aquele organizar-se e atuasse como uma via cíclica. O cíclo do ácido tricarboxílico é composto por oito enzimas. Contudo, cada enzima é codificada por vários genes os quais são endereçados para diversos compartimetos celulares e, não somente, mitocôndrias. Essas enziimas locallizadas em diferentes comparimentos subcelulares acarretaram em uma possível ampla conecção entre mitocôndrias e outras organelas (peroxissomos e cloroplastos) permitindo fluxos alternativos dos intermediários do ciclo cujo resultado alterou seu funcionamento para um não convencional modo não cíclico. É bastante aceito que sob estresses, no quais reduzem os níveis de carboidratos. O ciclo do ácido tricarboxílico pode funcionar no modo não cíclico, devido a perda de esquelos carbônico que entram se fazendo necessário ser alimentado por reações anapleuróticas. Portanto, aminoácidos tornam-se fundamentais para suprir a respiração e síntese de ATP sob tais situações. Fortes evidencias demonstraram que aminoácidos de cadeia ramificada (BCAA) e lisina podem fornecer elétrons para o sistema a cadeia de transporte de elétrons mitocondrial pela ação do sistema flavoproteína de transferência de elétrons (ETF)- ETF: ubiquinona oxidorredutase (ETF/ETFQO). Em plantas, duas enzimas: Isovaleril-CoA desidorgenase (IVDH) e (D)-2-hidroxidoglutarato desidrogenase (D2HGDH) foram caracterizadas como doadores de elétrons para o pool de ubiquinone através do sistema ETF/ETFQO a partir da degradação de BCAA e lisina, respectivamente. Na verdade, o catabolismo de BCAA mostra-se de uma importância fundamental para nutrir o ciclo do ácido tricarboxílico, principalmente, em situações de estresse enquanto lisina mostra uma estreia associação com o ciclo do ácido tricarboxílico sendo importante para fazer um elo da degradação de aminoácido com a geração de energia. A transferência de elétrons através da

cadeia transportadora de elétrons mitocondrial acopla a síntese de ATP a partir da regeneração de NADH e $FADH_2$ para fosforilar ADP a ATP. Contudo, o conhecimento com relação a organização do sistema de fosforilação oxidativa (OXPHOS) e sua via alternativa sob limitação energética permanece escasso. Assim, essa tese, a qual se concentra no funcionamento da respiração em um contexto que o ciclo do ácido tricarboxílico e via alternativa como doador de elétrons para cadeia transportadora de elétrons mitocondrial, é composta por três independentes capítulos centrados no metabolismo energético e respiração alternativa em *Arabidopsis thaliana*. Por isso, para se obter uma visão global de como ocorre o envolvimento e interação do ciclo do ácido tricarboxílico juntamente da via alternativa para coordenar o ajustamento das necessidades metabólicas e celulares, três abordagens experimentais foram usadas (*i*) uma abordagem *in silico*, nós investigamos a história evolucionária dos genes do ciclo do ácido tricarboxílico gerando um modelo para a origem dos genes do ciclo em plantas bem como seu comportamento submetido a uma série de estresse; (*ii*) a importância da biossíntese de lisina foi investigado usando mutante de Arabidopsis com reduzida atividade da enzima *L*,*L*-diaminopimelato aminotransferase (dapat) da via biossintética de lisina; (*iii*) reprogramação metabólica do sistema OXPHOS associado a limitação de carbono foi investigado. Rapidamente, os resultados apresentados aqui forneceram resultados que permitiu, no mínimo um prévio, a elaboração de mecanismo do metabolismo energético junto a vias alternativas. Primeiramente, permitiu a elucidação da origem evolutiva dos constituintes do ciclo do ácido tricarboxílico em plantas fornecendo elemento para a origem das isoformas presentes nos diferentes compartimentos subcelulares os quais que devem ser associados com eventos de transferência gênica ou com novas cópias geradas por duplicação genômica. Ademais, análises de co-expressão dos genes do ciclo em diferentes condições estressantes em ambos tecidos parte aérea e raiz demonstrou a presença de plasticidade molecular e forneceu uma explicação para o funcionamento do ciclo do ácido tricarboxílico em plantas. Após isso, o uso de Arabidopsis mutante com reduzida atividade para biossíntesi de lisina *L*,*L*-diaminopimelato aminotransferase (dapat) foi demonstrada que biossíntese de lisina simula condições de estresse e impacta no crescimento e metabolismo foliar. Por fim, uma avaliação de como o comportamento do sistema OXPHOS sob limitação de carbono e como vários aminoácidos podem impactar os complexos respiratórios foi possível demonstrar que o sistema OXPHOS tem sua função afetada por diferentes fontes de carbono e que vias alternativas são induzidas sob essas condições. Ademais, imunoensaios revelaram que é mais provável ser regulado por modificações pós traducionais. Juntos, esses resultados realçam a complexidade e especificidade da respiração vegetal durante evolução e que é differentemente afetado por linitações energéticas e pelo uso de substratos alternativos. Os resultados discutidos aqui suportam que ETF/ETFQo é uma via essencial capaz de

doa elétrons para a cadeia transportadora de elétrons e que amioácidos são substratos alternativos para manter a respiração sob limitação de carbono. Os resultados obtidos são discutidos em um contexto de evolução metabólica mostrando estreia associação da metabolismo energético com metabolismo de aminoácidos e onde possível mcanísticos são devidamente discutidos.

Palavras chaves: ciclo do ácido tricarboxílico; escassez de energia; evolução mitochondrial; fosforilação oxidativa; genes parálogos, metabolismo mitocondrial; neofuncionalização; respiração; resposta a estresse; substratos alternativos

1. OVERVIEW

Energy metabolism in plant is operated by both chloroplast and mitochondria through their electron transfer chains during the processescalled photophosphorylation and oxidative phosphorylation, respectively, allowing the generation of chemical energy as adenosine-5'-triphosphate (ATP). The primary function of mitochondria is to synthesize the majority of ATP in plant cells. In addition to their major role as the primary site of energy transduction and ATP synthesis in plant cells, it is becoming evident that mitochondria participate in several other key cellular processes such as programmed cell death (Valerberghe et al., 2009), seed germination (Pracharoenwattana et al., 2007), fruit maturation (Perotti et al., 2014), carbon-nitrogen interaction (Rocha et al., 2010), and plant cell redox and signaling (Daloso et al., 2015). This fact notwithstanding, mitochondrial function also support several light-associated processes including photorespiration, nitrogen metabolism, reductant transport, and the redox balance (Millar et al., 2011).Furthermore, evidences have been accumulated over the last decade indicating that mitochondrial metabolism is necessary to sustain photosynthesis (Padmasree et al., 2002; Noguchi and Yoshida, 2008; Nunes-Nesi et al., 2008; Araújo et al., 2012). Accordingly, recent studies have revealed that the photosynthetic performance can in fact be improved by modifications in either the activity of the mitochondrial electron transport chain (mETC) (Sweetlove et al., 2006; Millar et al., 2011) or of the tricarboxylic acid (TCA) cycle (Nunes-Nesi et al., 2008).In this context, the TCA cycle, a central mitochondrial pathway, have emerged as an essential player involved in photosynthesis optimization as showed by several studies (Carrari et al., 2003; Nunes-Nesi et al., 2005; Lemaitre et al., 2007; Nunes-Nesi et al., 2007; Studart-Guimarães et al., 2008; Araújo et al., 2011a). Bearing this in mind, it seems clear that mitochondria is not only a powerhouse but also a key organelle to carry out several reactions in the cell and as such being an organelle of central importance for a wide range of metabolic processes. By harboring multiple metabolic pathwaysrecent years have witnessed great efforts in establishing the importance of the mitochondrial metabolism and the roles of the TCA cycle in fueling plant respirationproviding new insights in plant biology research with far-reaching implications in agriculture and biotechnology. Thus, mitochondria acquisition as free-living organism by early host cell brought evolutionary advances by the preservation of diverse metabolic pathways including those related to energy metabolism as well as those associated with lipids, nucleotides and vitamin biosynthesis (Gabaldón and Huynen, 2003).

It is assumed that eukaryotic cells arose through the capture of free-living bacteria by endosymbiosis (Koonin, 2010) and their gradual conversion into organelles (plastid and mitochondria). However, how exactly the mitochondrial symbiosis took place is still unclear and

two main endosymbiotic models are normally presented, namely the archezoan scenario (Margulis, 1970) and the symbiogenesis scenario (Martin and Müller, 1998). The essential differences between these models are related to what kind of host cell "captured" the mitochondria ancestor. While the archezoan scenario suggest that the eukaryotic cell was a host, the symbiogenesis scenario provide evidences that host cell did not belong to the Eukarya domain, but rather that the Archeabacteria was the host and that only after the mitochondrial symbiosis the subcellular compartments and nucleus appeared as the eukaryotic cell (see reviews: Martin, 2001, Koonin, 2010, Gray, 2012).

Another fundamental issue regarding to mitochondrial evolution is concerning to its origin (Gray, 2012). It is worth mentioning that the vast majority of evidences suggest a monophyletic cladeto mitochondria origin due to two main findings: (*i*) genes encoded by the mitochondrial genome are a subset found in *Reclinomonas americana* and (*ii*) rRNA is arranged as a bacterial operon (Lang et al., 1997). Furthermore, the lack of genes belonging to bacterial operons, that should be on the mitochondrial genome, and that are located on nuclear genomesare suggestive of an endosymbiosis gene transfer process (Gray et al., 2012). In fact, this transfer process resulted in a smallernumber of functional genes on the mitochondrial genome, in general, and thus the remaining genes are only constituents of the mETC, ribossomal and transfer RNA (Anderson et al., 1981; Kurland and Andersson, 2000; Sloan et al., 2012). Additionally, while gene transfer process seems to not be observed in animals anymore, in land plants and green algae this process still seems to be ongoing (Adam and Palmers, 2003; Bonen and Calixte, 2006; Liu et al., 2009). Endosymbiosis gene transfer process normally led to gene loss during evolution. Thus, comparison between alphaproteobacteria and existing mitochondrion genomes demonstrated that only a portion of orthologous genes is clustered together meaning that a high number of genes from mitochondria ancestor have been lost (Adam and Palmers, 2003, Liu et al., 2007; Gray, 2012). It is important to mention, however, that gene transfer from mitochondria genome to the nucleus is not a guarantee that their respective proteins will be effectively exported into mitochondria, and therefore it is also possible that this protein go into another subcellular compartment (Heazlewood et al., 2003).

As stated above, plant mitochondria is a highly unusual and complex organelle which is clearly different from those of other eukaryotes in a number of aspects (reviewed in details in Mackenzie and McIntosh, 1999) and therefore plant energy metabolism is of particular importance in the context of evolution since most of genes (and protein) seems to have an eubacterial (alphaproteobacteria) origin (Rivera et al., 1998: Horiike et al., 2001; Schnarrenberg and Martin, 2002). Accordingly, TCA cycle enzymes are challenging to study in the context of mitochondrial evolution because approximately half of the proteins seemsto be present before the mitochondria ancestor acquisition while the other half are thought to be branched from the eubacteria homologous (Schnarrenberg and Martin, 2002). In short, the endosymbiosis gene transfer allowed

the TCA enzymes to function as a cycle in the existing mitochondrion (Schnarrenberg and Martin, 2002) at the same time that other pathways continued associated with the TCA cycle and added even more functions (and complexity) to this highly important and specialized pathway (Gabaldón and Huynen, 2003; Araújo et al., 2012; Nunes-Nesi et al., 2013).

The TCA cycle,an universal feature of the metabolism of any aerobic organisms, is composed by a set of eight enzymes presented in the mitochondrial matrix coupling the product of the oxidation of pyruvate to CO_2 with the production of NADH by the respiratory chain (Fernie et al., 2004; Millar et al., 2011). Notwithstanding, cyanobacteria are characterized by an apparently incomplete TCA cycle due to the lack of 2-oxoglutarate dehydrogenase (OGDH) complex. However, it was recently found that in *Synechococcus* sp the action of the 2-oxoglutarate decarboxylase (OGDC) coupled to the succinic semialdehyde dehydrogenase (SSADH) is able to functionally complete the cycle allowing succinate synthesis (Zhang and Brian, 2011). Remarkably, in cyanobacteria the primary role of the TCA cycle is most likely the production of precursors to other biosynthetic pathways albeit it might also generate energy particularly during darkness (Zhang and Brian, 2011). It refers normally to an unusual TCA cycle in cyanobacteria (Steinhauser et al., 2012). Accordingly this different TCA cycle behavior is not an exclusivity of cyanobacteria, and thus plants also display an incomplete TCA cycle under some situations (Sweetlove et al., 2010). The conclusive implication for this behavior is that different steps in the TCA cycle have functions others than maintain a cyclic flux, and that a fine metabolic balancing between these functions is likely to depend on the physiological context in which the pathway is operating and that non-cyclic flux mode are more active in some tissue than others (Sweetlove et al., 2010; Araújo et al., 2012; Nunes-Nesi et al., 2013). For instance, shoot tissues present both flux behavior along the diurnal cycle since at night a cyclic flux mode is required to sustain ATP demand and at day it seems that the non-cyclic flux act providing organic acids to integrate other pathways such as providing 2-oxoglutarate for nitrogen assimilation (Lee, 2010; Araújo et al., 2014). As previously highlighted by Sweetlove et al. (2010) to enhance our understanding of non-cyclic modes within the TCA cycle is technically a challenge due to several bypass carried out by the TCA cycle isoforms which are located in other subcellular compartments (Millar et al., 2011) which explain, at least partially, the extensive subcellular compartmentation in plant cell. The origin of the isoforms present in different cell compartments might be associated either with gene transfer events which did not result in proper targeting of the protein to mitochondrion or with duplication events (Schnarrenberg and Martin, 2002; Blanc et al., 2003, Freeling, 2009; Palmieri et al., 2011). Consequently, these paralogous genes have allowed to the TCA cycle genes to accumulate more functions within a cell through a process of neofunctionalization or subfunctionalization (Force et al., 1999).

In situations in which high ATP synthesis is required by plant cell, the TCA cycle operates in the normal flux mode to support plant respiration, which is mostly dependent of carbohydrate oxidation (Plaxton and Podesta, 2006; Sweetlove et al., 2010; Araújo et al., 2012). Plants can also use alternative substrates such as lipids and amino acids to generate energy; however, unlike the situation observed in mammals, the usage of these substrates occurs only in conditions which carbohydrates level are decreased (e.g; under stress situations) (Buchanan-Wollaston et al., 2005; Araújo et al., 2011b). Among these alternative substrates, it has been demonstrated that amino acids can act as electron donor to support respiration (Ishizaki et al., 2005; Ishizaki et al., 2006; Araújo et al., 2010; Engqvist et al., 2011; Kochevenko et al., 2012). However, amino acids degradation associated to energy metabolism occurs through a distinct route that differs from the classical pathway of respiration which begins with glycolysis in cytosol, supporting organic acid oxidation in the mitochondria by the TCA cycle (Ishizaki et al., 2005). Notably, this alternative pathway of respiration is performed by the action of the electron transfer flavoprotein (ETF)-ETF: ubiquinone oxidoreductase (ETF/ETFQO) system and associated dehydrogenases which already is very well characterized in mammals (Watmough and Frerman, 2010). Further information about the occurrence of this alternative pathway of respiration in land plants had been recently obtained by using loss-of-function Arabidopsis mutants (Ishizaki et al., 2005; Ishizaki et al., 2006; Araújo et al., 2010; Engqvist et al., 2011; Peng et al., 2015). Perhaps more importantly, our understanding of the functional importance of this pathway under stress conditions (Araújo et al., 2011b) and along a diurnal cycle (Lee et a., 2010; Peng et al., 2015) has been substantially increased over the last years.

In mammals the presence of 11 dehydrogenases able to donate electrons to the ETF/ETFQO pathway has been demonstrated (Beckmann and Frerman, 1985). In plants, however, only two dehydrogenases-isovaleryl-CoA dehydrogenase (IVDH) and (*D*)-2-hydroxyglutarate dehydrogenase (D2HGDH) have been described as electron donor so far (Araújo et al., 2010). IVDH and D2HGDH are involved in the catabolism of branched-chain amino acids (BCAA, isoleucine, leucine and valine) and lysine, respectively (Araújo et al., 2010; Engqvist et al., 2011). Additionally, by performing a screening of mutants involved in the BCCA catabolism under extended darkness it was observed an early senescence in basically all these mutant; however,*ivdh1-2* mutant displayed always the strongest yellow phenotype and lost the capacity to recovery the green phenotype after 15 days of treatment after returning to light (Peng et al., 2015). That being said, this finding add further evidence to support the contentionthat IVDH might use other substrates as aromatic amino acids and that it likely exerts a key role on the electron provisionto the alternative respiration mediated by the ETF/ETFQO complex (Araújo et al., 2010, Araújo et al., 2011b). Interestingly, recent studies have provided evidence that the function of BCAA catabolism is dependent on the circadian rhythm in which both transcripts and protein levels

of enzymes related to BCAA catabolism are increased at the night (Lee et al., 2010; Peng et al., 2015). It should be mentioned, however, that several mutantsof genes involved in the BCAA catabolism growing in different photoperiods displayedno changes in either vegetative or reproductive phenotype (Peng et al., 2015). Taken together with the fact thatunder extended darkness the phenotype observed is highly distinct, these results clearly suggest that BCAA catabolism is of pivotal importance to provide intermediates to TCA cycle under stress situations, but it presents rather a minor importance during a normal diurnal cycle.

Approaches aiming to improve lysine content in crop plants have been largely unsuccessful.The reason for the lack of success was associated with: (*i*) limited availability of genetic resources for high lysine content; and (*ii*) thegenetic traits for high lysine content are strongly associated with other deleterious traits causing abnormal vegetative development. This fact apart, enhancement of lysine levels in seeds also causes major problems associated with delayed germination and defective vegetative development. Further studies have also demonstrated that these phenotypes were most likely associated with the metabolic connection between the cellular energy metabolism and both the synthesis and catabolism of lysine (Jader and Joshi, 2009; Araújo et al., 2010; Kirma et al., 2012; Boex-Fontvieille et al., 2013). In good agreement, attempts to improve nutritional quality of seeds by the inhibition of lysine catabolism have reported diminishment of TCA cycle intermediates (Zhu ang Galili, 2003; Ufaz and Galili, 2008; Angelovici et al., 2009; Angelovici et al., 2011). Collectively, these data indicated that lysine catabolism is likely required to couple amino acid degradation and energy generation by maintaining cellular homeostasis (Galili et al., 2014). Furthermore, compelling evidences had also demonstrated that lysine metabolism is necessary in others key functions within the plant cell such as to support alternative respiration (Engqvist et al., 2009; Araújo et al., 2010; Engqvist et al., 2011) and pathogen defense (Rate and Greenberg, 2001; Song et al., 2004). Moreover, 2-hydroxyglutarate (2HG), a metabolite generated by lysine catabolism, has recently emerged as a potential metabolite involved in photorespiration and nitrogen assimilation (Boex-Fontvieille et al., 2013; Kuhn et al., 2013).

As stated and discussed above mitochondrial metabolism is required for several functions within plant cell where the function of the TCA cycle and its connections seems to be a key for the maintenance of an adequate balance in plant cells. Furthermore, amino acids metabolism is of critical significance in land plants to support the TCA cycle by supplying organic acids or to donate electron to respiration. Thus, this thesis is largely focused on the function of respiration within the context of the role of the TCA cycle as well as the function of alternative electron donors to the mETC. That being said, the main aim of this work was to obtain a comprehensive view of how and to which extent plant respiration and its alternative pathways interact to fulfill different cellular and

metabolic requirements. To this end, a range of complementary experimental approaches were used and therefore the thesis is organized as a compilation of three independent stand-alone chapters which discuss about energy metabolism focusing on alternative respiration in *Arabidopsis thaliana*.

CHAPTER 1. Evolution and function of the TCA cycle

Although a comprehensive analysis of the TCA cyclefunction has been recently performed our understanding of the evolutionary history of the TCA cycle in land plants remains limited. Byinvestigating the evolutionary history of all TCA cycle genes was possible to validate a model for the origin of the TCA cycle genes in plants. A framework for co-expression analysis of TCA cycle genes in a range of treatments in both shoot and root tissues was created, providing an evolutionary explanation for the modular operation of the TCA cycle. The main results indicate that prior to endosymbiotic events the TCA cycle seemed to operate only as isolated steps in both the host cell and mitochondria ancestor (alphaproteobacteria). Furthermore, in silico analyses allow us to suggest new insights into the possible roles of TCA cycle enzymes in different plant tissues.

CHAPTER 2. Lys biosynthesis deficiency alters growth and metabolism

Stress situations result in physiological and molecular changes concomitantly to the suppression of energy production in plant cell and thus alternative electrons donors to the mETC are required. Recent work has demonstrated that lysine degradation allows the maintenance of the mETC by providing electrons to ETF/ETFQOvia the action of D-2HGDH.The importance of lysine deficiency and its connections with whole metabolism and growth were investigated by using an Arabidopsis mutant with reduced activity of the lysine biosynthesis enzyme *L,L*-diaminopimelate aminotransferase (dapat) and the results are discussed in the context of current models of amino acids and protein mobilization in situations of limited energy supply.

CHAPTER 3. Arabidopsis cell culture under carbon starvation

Reductant power (NADH and $FADH_2$) generated by the TCA cycle might be recycling by oxidative phosphorylation (OXPHOS) system which includesmETC and ATP synthase. Changes associated with the OXPHOS system were investigated following carbon limitation. The preliminary results showed an interesting behavior among members of OXPHOS system andthat its behavior is differently affected by different amino acids. In addition, immunoblotting assays revealedthat OXPHOS system is most likely regulated by posttranslational modification (PTM). The results obtained are discussed in the context of our current understanding of the transient reconfiguration of metabolism following stress situations and possible avenues for future research are provided.

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Evolution and Functional Implications of the Tricarboxylic Acid Cycle as Revealed by Phylogenetic Analysis

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Abstract

The tricarboxylic acid (TCA) cycle, a crucial component of respiratory metabolism, is composed of a set of eight enzymes present in the mitochondrial matrix. However, most of the TCA cycle enzymes are encoded in the nucleus in higher eukaryotes. In addition, evidence has accumulated demonstrating that nuclear genes were acquired from the mitochondrial genome during the course of evolution. For this reason, we here analyzed the evolutionary history of all TCA cycle enzymes in attempt to better understand the origin of these nuclear-encoded proteins. Our results indicate that prior to endosymbiotic events the TCA cycle seemed to operate only as isolated steps in both the host (eubacterial cell) and mitochondria (alphaproteobacteria). The origin of isoforms present in different cell compartments might be associated either with gene-transfer events which did not result in proper targeting of the protein to mitochondrion or with duplication events. Further in silico analyses allow us to suggest new insights into the possible roles of TCA cycle enzymes in different tissues. Finally, we performed coexpression analysis using mitochondrial TCA cycle genes revealing close connections among these genes most likely related to the higher efficiency of oxidative phosphorylation in this specialized organelle. Moreover, these analyses allowed us to identify further candidate genes which might be used for metabolic engineering purposes given the importance of the TCA cycle during development and/or stress situations.

Key words: mitochondria, pathway evolution, plant respiration, phylogeny, TCA cycle.

Introduction

Mitochondria, vital cytoplasmic organelles of eukaryotic cells, were identified over 50 years ago as being responsible for oxidative energy metabolism and the synthesis of the majority of respiratory adenosine-5′-triphosphate (ATP) in plants, animals, and fungi (Logan 2006; Araújo, Nunes-Nesi, et al. 2012). Although mitochondria contain their own genome and the machinery for its replication (Gray et al. 2001; Burger et al. 2003; Gray 2012), they are only semiautonomous. Indeed the majority of mitochondrial polypeptides is encoded in the nuclear genome, synthesized in the cytosol, and imported into the mitochondria posttranscriptionally (Unseld et al. 1997; Whelan and Glaser 1997; Tanudji et al. 2001; Duby and Boutry 2002; Eckers et al. 2012). Thus, it is assumed that eukaryotic cells arose through the capture of free-living bacteria by endosymbiosis and their gradual conversion into organelles (plastid and mitochondria).

Today, it is essentially beyond question that eukaryotes originated from an ancient unicellular bacterial-like cell by the engulfment of other free-living bacteria. In doing so, they acquired new functionalities through a cooperative relationship—a process termed endosymbiosis or endocytobiosis (Gray et al. 2001; Martin et al. 2002). Heterotrophic eukaryotes have, furthermore, evolved from an archaea-like ancestor through engagement of an alphaproteobacteria (related to Rickettsiales) in an event that marks the origin of mitochondria (Gray et al. 2001; Thrash et al. 2011). The evolution of phototrophic eukaryotes began with the acquisition of oxygenic photosynthesis by primary endosymbiosis in which a heterotrophic unicellular eukaryote acquired a cyanobacterium-like

cell as a plastid (Hohmann-Marriott and Blankenship 2011). Phylogenetic studies have indicated that the primary endosymbiont was likely a close relative to filamentous heterocystous N_2-fixing cyanobacteria (related to Nostocales; Deusch et al. 2008). Although some evidence suggests that primary endosymbiosis occurred only once in evolutionary history (McFadden and van Dooren 2004; Tirichine and Bowler 2011), ultrastructural, biochemical, and genetic analyses have revealed that secondary endosymbiosis arose multiple times (Kutschera and Niklas 2008; Nakayama and Ishida 2009). Given its extraordinary metabolic plasticity, it is not surprising that some of these characteristics of cyanobacteria seem to have been transferred into the heterotrophic eukaryote during this process leading to the evolution of algae and higher plants (Margulis 1970; Reyes-Prieto et al. 2007). Hence, many plant genes have originated from the cyanobacterial endosymbiont, including those coding for proteins involved in photosynthesis, respiration, and many other metabolic as well as regulatory functions (Martin et al. 2002; Kern et al. 2011).

Over the last decades, our exponentially increasing capacity for genome sequencing has generated vast amounts of sequence data of prokaryotic and eukaryotic providence. As such, novel opportunities to study the molecular evolution not only of individual enzymes but also of specific pathways consisting of several enzymatic reactions have now become possible. As mitochondria are organellar descendents of an alphaproteobacterial ancestor merged with a eukaryotic cell, it would be interesting to understand how mitochondrial pathways evolved and assembled during the course of the evolution. Although biochemical studies in the late 1960s have contributed much to our knowledge about the function of metabolic pathways and the regulation of metabolism, it was believed that cyanobacteria, like some other prokaryotes, harbored an incomplete tricarboxylic acid (TCA) cycle given that they lack the enzyme 2-oxoglutarate dehydrogenase (OGDH) (Pearce and Carr 1967; Smith et al. 1967). It was thought that the operation of the glyoxylate shunt or, alternatively, aspartate transaminase reactions functioned to complete this pathway (Steinhauser et al. 2012). Consequently, the presence of an incomplete TCA cycle had been used to explain various aspects of our current understanding of the physiology of cyanobacteria. However, compelling evidence has recently demonstrated that two other enzymes, namely 2-oxoglutarate decarboxylase (OGDC) and succinic semialdehyde dehydrogenase, are able to functionally substitute OGDH and succinyl-CoA ligase to generate reducing equivalents, thereby closing the cycle (Zhang and Bryant 2011). Remarkably, the presence of the OGDH complex in eukaryotic cells indicates that these genes were acquired by transfer events independent of the endosymbiotic process (Schnarrenberger and Martin 2002).

Recent advances have demonstrated that the TCA cycle intermediates play several key roles in cell physiology (for a review, see Araújo, Nunes-Nesi, et al. 2012) highlighting the plasticity of carbon metabolism in plants. Interestingly, many of the reactions in the TCA cycle can be bypassed by reactions resident in other subcellular compartments with only those catalyzed by succinyl-CoA ligase and succinate dehydrogenase (SDH) being unique to mitochondrion (Millar et al. 2011; Nunes-Nesi et al. 2013). Thus, all other enzymes are most likely encoded by paralogous genes and thus it is reasonable to assume that to accumulate so many functions within a cell, the number of TCA cycle genes increased in higher plants most likely due to multiple horizontal gene transfer and polyploidization events (Schnarrenberger and Martin 2002; Blanc et al. 2003). Accordingly, it has been assumed that genome reduction plays an important role during the endosymbiosis so that the mitochondrial genome shrinks relative to its bacteria ancestor (Gray et al. 2001). Furthermore, several genes belonging to the ancestral genome seem to have been transferred to the host genome by horizontal gene transfer (Dyall et al. 2004), whereas some of the remaining ones have been lost with their function replaced by host processes (Gabaldón and Huynen 2003). Thus, a selective set of metabolism pathways encoded by both mitochondrial genome and nuclear genome are most likely maintained in mitochondria to sustain its current functions.

We here investigated the evolutionary history of the TCA cycle based on phylogenetic analyses of all enzymes of the cycle and compared the trees obtained for individual enzymes searching for general patterns of phylogenetic similarities or discordance among them. We additionally analyzed the sequences of paralogous genes encoding TCA cycle enzymes and created a framework for coexpression analysis showing quantitative, temporal, and spatial differences among mitochondrial genes in both shoot and root tissues. Here, we present a comparative genomic study using robust phylogenetic analyses, including a vast number of taxa to validate an evolutionary model for the origin of TCA cycle genes in plants and to add novel insights into the TCA cycle relationship. Our results revealed tight connections among TCA cycle genes and that most of those genes originated after duplication events that occurred in plants. Additionally, we demonstrated that the emergence of different roles for TCA cycle genes, especially during suboptimal conditions in vascular plants, occurred through a process of neofunctionalization and/or subfunctionalization. These combined results are discussed in the context of the current models of the metabolic evolution and its connections providing clues to the understanding of the organization principles of mitochondria.

Materials and Methods

Data Mining for Nucleotides and Deduced Putative Protein Sequences of TCA Cycle Enzymes

Protein sequences were retrieved from GenBank through the pBLAST algorithm using all mitochondrial TCA cycle

enzymes from *Arabidopsis thaliana* as query. Basic Local Alignment Search Tool (BLAST) searches were performed at National Center for Biotechnology Information nucleotide and protein database to search for sequences of TCA cycle enzymes in plants, mammals, and yeast. Additionally, data mining was performed in the cyanobacteria (CyanoBASE; http://genome.microbedb.jp/cyanobase, last accessed October 13, 2014) and *Escherichia coli* (http://genprotec.mbl.edu, last accessed October 13, 2014) genome databases in order to establish TCA cycle gene orthology to aid in understanding molecular evolution. When selecting the sequences we tried to include sequences from plants, animals, cyanobacteria, and fungi in addition to a representative sample of gene diversity and ancient family from eubacteria and archaebacteria. In some cases, homologs were not available from all sources. Sequences were aligned using the ClustalW software package (Higgins and Sharp 1988) using default parameters. Neighbor-joining trees (Saitou and Nei 1987) were constructed with MEGA5 software (Tamura et al. 2011) using midpoint rooting. Distances were calculated using pairwise deletion and Poisson correction for multiple hits, and bootstrap values were obtained with 1,000 pseudoreplicates.

Sequence data from this article can be found in the GenBank/EMBL databases under the accession numbers shown in supplementary table S1 and data sets S1–S4, Supplementary Material online.

In *Silico Coexpression and Correlation Analysis of TCA Cycle Genes*

For gene expression analyses, the online tools of Genevestigator (http://www.genevestigator.com, last accessed October 13, 2014; Zimmermann et al. 2004) and e-Northens w.Expression browser (http://bar.utoronto.ca/affydb/cgi-bin/affy_db_exprss_browser_in.cgi, last accessed October 13, 2014; Toufighi et al. 2005) were used. The heat map was constructed using the obtained gene expression data sets with the software package TMEV (Saeed et al. 2003). Coexpression network analyses were performed using coefficient values calculated using the PRIMe coexpression database (Akiyama et al. 2008; http://prime.psc.riken.jp/?action=coexpression_index, last accessed October 13, 2014) calculated from publicly available microarray data. Connections between each gene were prepared by "interconection of sets" and "union of sets" searches. The criteria for interactome frameworks were performed at the cut-off value of 0.5 and 0.7, whereas criteria for correlation candidates were correlation values of 0.7, and P values < 0.05. Correlation networks were determined using Pearson's correlation ($P < 0.01$). The output files which were formatted with ".net" file from PRIMe database were later used to drawn the networks using Pajek software (Batagelj and Mrvar 1998) (http://vlado.fmf.uni-lj.si/pub/networks/pajek/, last accessed October 13, 2014).

Results and Discussion

Due to the intrinsic complex structure of some TCA cycle enzymes consisting of multiple subunits (e.g., OGDH complex, succinyl-CoA ligase, and SDH), we analyzed each enzyme of the cycle individually by creating their respective phylogenetic trees attempting to infer the evolutionary history on an enzyme-by-enzyme basis. The only exception to this was the simultaneous phylogenetic analysis we conducted for the enzymes OGDH, pyruvate dehydrogenase (PDH), and OGDC (fig. 1). This construction was designed to facilitate the understanding of the evolutionary history of these enzymes of relatively similar function—indeed they share a common subunit. It has long been known that plant OGDH requires TPP, NAD^+, and ADP (Bowman et al. 1976) and that the enzyme competes with PDH for intramitochondrial NAD^+ and CoA (Dry and Wiskich 1987), the latter fact being of particular importance given that OGDH and PDH share a common subunit (E3). It is important to mention that range of studies have revealed that although OGDH is a key control point involved in the regulation of fluxes through the TCA cycle (Araújo, Nunes-Nesi, et al. 2012) the inhibition of PDH by light also reduces the TCA cycle flux (Randall et al. 1990; Tcherkez et al. 2011) allowing the elucidation of the precise physiological role of this enzymes. Although the evolution of these enzymes is somewhat complicated given that some organism used here present particular changes on the cycle structure associated with the absence of an OGDH and the presence of an alternative OGDC (Zhang and Bryant 2011), our results show that OGDC is phylogenetic closer to OGDH than to PDH clustering in the same branch as the former (fig. 1). Interestingly, in organisms as cyanobacteria there is no molecular evidence showing the presence of OGDH suggesting that TCA cycle was incomplete within this organism. However, an alternative to the lack of OGDH was recently found in cyanobacteria *Sycnechoccocus* sp which is able to synthesis succinate from 2-oxoglutarate (Zhang and Bryant 2011). Remarkable this alternative enzyme is the so-called OGDC which in *Sycnechoccocus* sp is encoded by SynPCC7002_A2770 gene requiring a second enzyme, SSADH, to synthesize succinate and complete the cycle (Zhang and Bryant 2011; Steinhauser et al. 2012). It should be mentioned that this OGDC reported in *Sycnechoccocus* sp is actually the acetolactate synthase gene which is phylogenetically distinct from both OGDH and OGDC from *Euglena gracilis* and *Mycobacterium* spp. (this group is branched between OGDH and PDH groups and it is highlighted by green background in fig. 1) even though this gene also encodes a TPP-dependent enzyme (fig. 1). Remarkably, the highly regulated production of 2-oxoglutarate by the action of the OGDH is involved in glucose oxidation through the TCA cycle occupying an amphibolic branch point in the cycle, where the energy-producing reaction of the 2-oxoglutarate degradation competes with glutamate synthesis through nitrogen

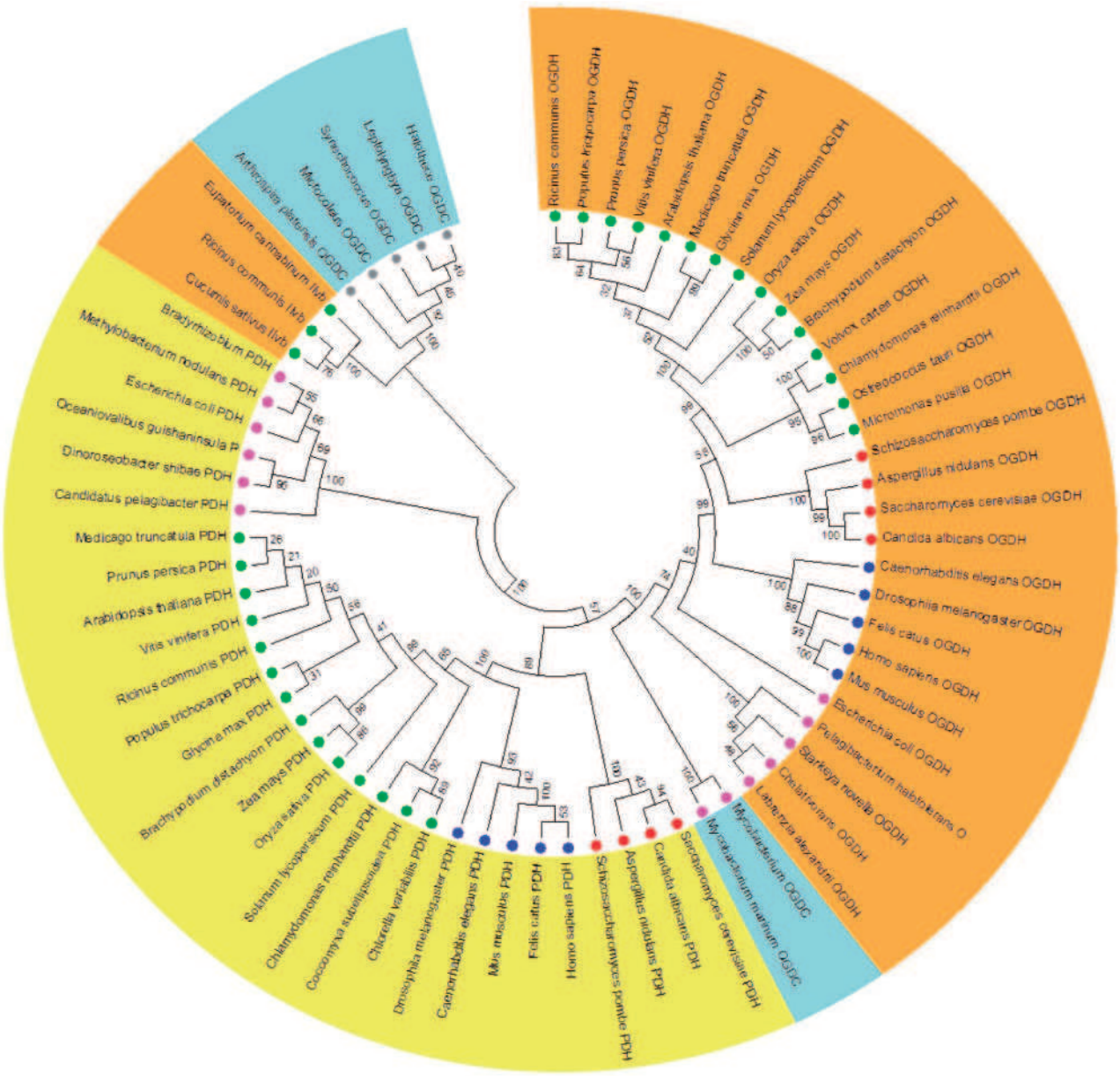


Fig. 1.—The distinct phylogeny of OGDH, PDH, OGDC, and OGDC. Phylogenetic tree was built using putative amino acids sequences from OGDH (orange background), PDH (yellow background), OGDC (blue background), and OGDC from cyanobacteria (green background) and acetolactate synthase. Sequences of putative proteins from plants are highlighted by green circle, yeast by red circle, animals by blue circle, algae by dark green circle, bacteria by pink circle, and cyanobacteria by gray cycle.

incorporation into 2-oxoglutarate. As such, the importance of this step within the cycle is manifold: 1) OGDH is a key regulatory point allowing the flux of 2-oxoglutarate through TCA cycle (Zhang and Bryant 2011; Nunes-Nesi et al. 2013), 2) the synthesis of the essential amino acid lysine through the α-aminoadipate pathway requires 2-oxoglutarate (Kirma et al. 2012) and the synthesis of 2-oxoglutarate generates a branch linking this metabolite to other pathways such as amino acid biosynthesis (Araújo, Tohge, et al. 2012), and finally 3) the 2-oxoglutarate seems to be a connection between classical and alternative pathways of respiration in feeding electrons to the mitochondrial electron transport chain (Araújo et al. 2010).

Interestingly, we also observed the presence of one putative OGDC, most likely derived from cyanobacteria, in the chloroplasts of land plants but being demarcated as acetolactate synthase. It should be noted, however, that compelling evidence has shown that the endosymbiotic event of

cyanobacteria by host cells culminated in plastid evolution in those plants (Keeling 2013). Notably, acetolactate synthase catalyzes the first enzymatic step in the synthesis of branched-chain amino acids (valine, leucine, and isoleucine) in plants (Chipman et al. 2005). Altogether this idea reinforces the hypothesis that the cyanobacterial TCA cycle performs a crucial function in producing precursor metabolites for biosynthetic reactions (Zhang and Bryant 2011) and that the usage of alternative pathways for respiration requires the presence of 2-oxoglutarate completing the cycle (Araújo et al. 2010). In addition, it is known that SSADH is active in mitochondrion of plant cells where it functions as an alternate succinate source, particularly under conditions of stress (Rocha et al. 2010; Sweetlove et al. 2010). Taken together, these results demonstrated a tight coordination of cell metabolism and indicate that during evolution the correct targeting of specific proteins allowed their subcellular compartmentation and specific functions in eukaryotes organisms.

Citrate Synthase

Citrate synthase (CS), which is often regarded as the first enzyme of the TCA cycle (Fernie et al. 2004), catalyzes the combination of oxaloacetate and acetyl CoA to produce citrate. This enzyme has been focus of many studies in plant–soil interactions suggesting that it is an important determinant of root citrate exudation (Kochian et al. 2004) and an important mediator of both phosphate uptake and aluminum tolerance (de la Fuente et al. 1997). The mitochondrial CS has also been suggested to play important roles in floral development (Landschutze et al. 1995) and as a source of carbon skeletons for nitrogen assimilation (Sienkiewicz-Porzucek et al. 2008). Although these studies have greatly enhanced our understanding of the role of CS in specific developmental and environmental interactions, they provide little information concerning the evolutionary history of isoforms of this enzyme. To enlighten this issue, a phylogenetic tree was constructed for CS isoenzymes (fig. 2*A*) and the sequences used clustered into two distinct groups related to mitochondrial or cytosolic isoforms. Interestingly, cytosolic isoforms of eukaryotic organisms are closer to CS of *E. coli*, which can be assumed as a sort of alpha proteobacteria related to be the mitochondrial ancestor, whereas mitochondrial isoforms were isolated in a distinct clade. This finding suggests that before the endosymbiotic event had occurred the eukaryotic ancestor already owned a CS gene and that CS family gene is clustered by horizontal gene transfer to mitochondria ancestor to host (eukaryotic cell) events.

Aconitase

Aconitase catalyzes the reversible conversion of citrate into isocitrate through the formation of the intermediate product *cis*-aconitate. Two isoforms of this enzyme have been detected in land plants with the mitochondrial isoform being involved in the TCA cycle (Carrari et al. 2003), whereas the cytosolic participates in a range of processes such as citrate metabolism in the cytosol and the glyoxylate cycle (Hayashi et al. 1995). Interestingly in wild species tomato (*Solanum penelli*), the aconitase mutant allele (which corresponds to SlAco3b) is deficient in both cytosolic and mitochondrial aconitase proteins (Carrari et al. 2003), suggesting that at least in tomato this gene product is dual targeted. Therefore, we decided to analyze the evolutionary history of mitochondrial and cytosolic isoforms of a range of organisms (fig. 2*B*). As would perhaps be expected, regardless of the isoform plant aconitase was clustered together in our analyses. In contrast, the situation was quite different for animal aconitase, in which we could observe that the mitochondrial isoforms were quite different than the cytosolic isoforms, the latter being more similar to plant mitochondrial isoforms (fig. 2*B*). Noteworthy, although cyanobacterial aconitase formed a completely distinct and isolated cluster, yeast aconitase grouped with aconitase from animals. One possible explanation for this pattern is that cyanobacteria presents one aconitase type B (AcnB), which is functionally distinct from the others, whereas eukaryotic organisms and *Eschrichia coli* share cytosolic and mitochondrial aconitase type A (AcnA). Simultaneously, our results demonstrated that segregation between cytosolic and mitochondrial isoforms in eukaryotic organisms seems to have occurred during the course of the evolution and that it might be explained by independent horizontal gene transfer events.

Following the identification of the genes encoding aconitase, it has become apparent that although the number of genes encoding aconitase varies, particularly between plant species, with some having two and other three genes, the gene products are often dual targeted to both cytosol and mitochondria (Carrari et al. 2003; Arnaud et al. 2007; Morgan et al. 2013). It is important to mention that although this enzyme is highly sensitive to oxidative stress (Verniquet et al. 1991; Lehmann et al. 2009), the aconitase isoforms seem to differ in their relative sensitivity in which mitochondrial isoforms are likely more sensitive to oxidative stress than cytosolic isoforms in eukaryotic cells (Walden 2002). This fact coupled with the central point of this enzyme in the regulation of organic acid metabolism, aconitase seems to be a potential and suitable target for metabolic engineering applications. For instance, the recent combination of genetic and molecular approaches has demonstrated the crucial role of this enzyme in controlling organic acid content in ripe tomato fruit (Morgan et al. 2013), whereas in citrus fruit it was possible to manipulate the fruit acidity by changes in amino acid metabolism (Degu et al. 2011).

Isocitrate Dehydrogenase

Isocitrate is oxidatively decarboxylated to 2-oxoglutarate by either NAD+ or $NADP^+$-dependent isocitrate dehydrogenase (IDH), generating CO_2 and NADH or NADPH, respectively

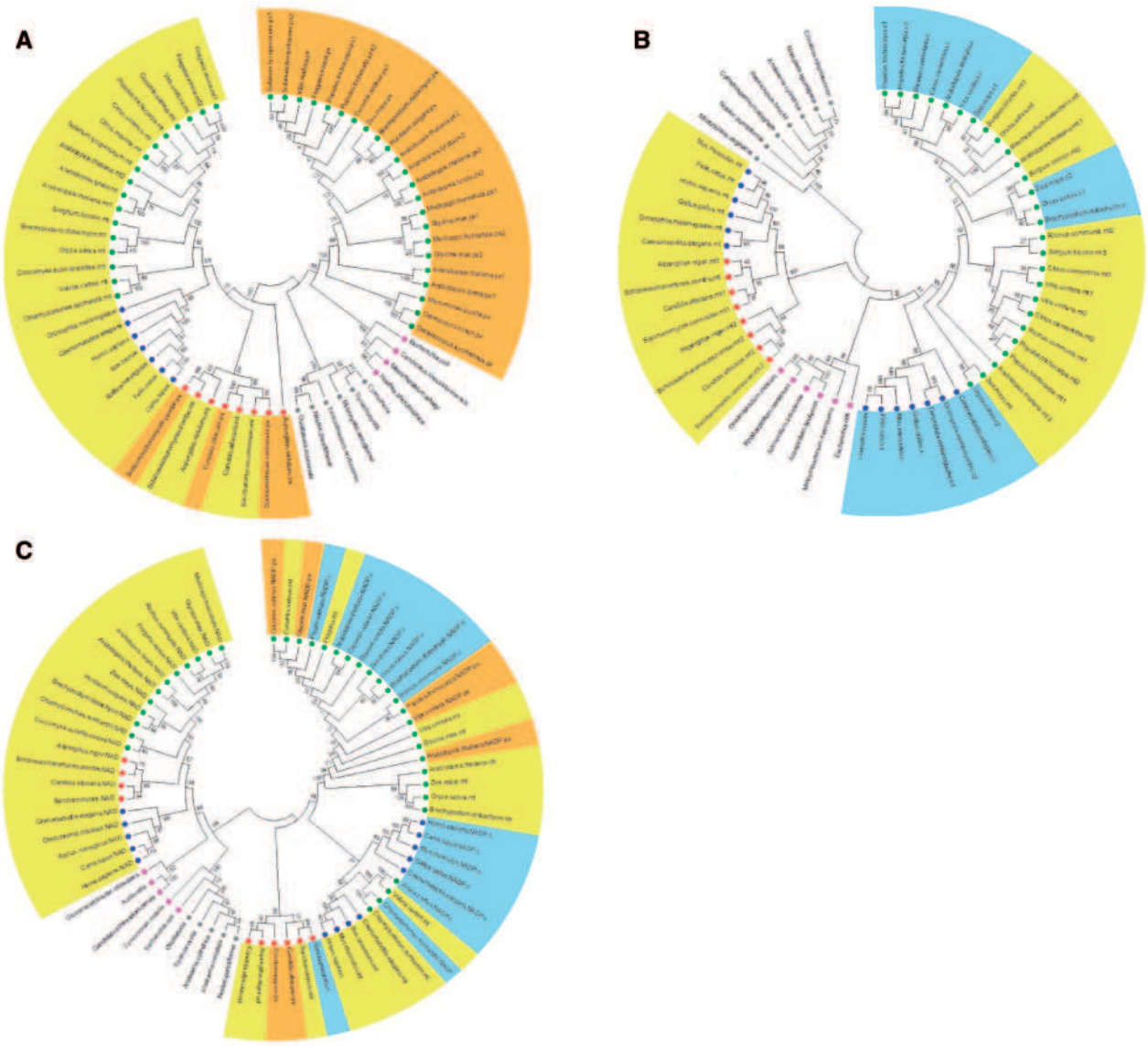


Fig. 2.—The phylogenetic tree of (*A*) CS, (*B*) aconitase, and (*C*) IDH. Trees were built using putative amino acids sequences from CS, aconitase, and IDH. Subcellular compartments are highlighted by background colors and in all cases figures follow the same pattern: Mitochondrial isoforms are highlighted by yellow background, peroxisomal isoforms by orange background, and cytosolic isoforms by blue background. Sequences of putative proteins from plants are highlighted by green circle, yeast by red circle, animals by blue circle, algae by dark green circle, bacteria by pink circle, and cyanobacteria by gray circle.

(Lemaitre et al. 2007; Sienkiewicz-Porzucek et al. 2010; Sulpice et al. 2010). The function of this enzyme has been associated with the maintenance of the 2-oxoglutarate level and therefore the regulation of nitrogen assimilation (Stitt and Fernie 2003; Nunes-Nesi et al. 2010), as well as with the recycling of lysine during alternative respiratory pathways (Boex-Fontvieille et al. 2013) and in tolerance to biotic stress (Leterrier et al. 2012; Dghim et al. 2013). However, there is controversy concerning the evolutionary history of IDH regarding whether eukaryotic cells arisen of either a single progenitor gene or through independent duplications of an ancestor IDH gene within each kingdom (Schnarrenberger and Martin 2002; Hodges et al. 2003). Our results demonstrate that NAD^+-dependent IDH is closer to alphaproteobacteria and cyanobacteria whereas $NADP^+$-dependent IDH isoforms (e.g., peroxisome and cytosolic) cluster in other branches of our phylogenetic tree (fig. 2*C*). Thus our findings seem to be more related to the first hypothesis regarding IDH evolution (Schnarrenberger and Martin 2002) suggesting that the presence of at least one IDH gene ancestor for each kingdom is the most reasonable explanation for the different subcellular localization observed within this enzyme. This suggestion is

further supported by the findings that for instance, IDH isoforms of plants and animals were grouped in isolated cluster branches of tree differently than what is shown here where NAD^+-dependent IDH of animals and plants cluster in the same branch. On the other hand, peroxisome and cytosolic isoforms of IDH clustered in other branch and are much closer between them. One conspicuous feature concerning the role of IDH in human is a recurrent association with brain cancers and leukemia (Kranendijk et al. 2010; Tonjes et al. 2013) which has been associated with the accumulation of 2-hydroxyglutarate. The accumulation of this compound in dark-induced senescent mutant plants involved with alternative pathways of respiration (Araújo et al. 2010; Araújo, Ishizaki, et al. 2011; Engqvist et al. 2011) coupled with the recent association of this enzyme with lysine metabolism (Boex-Fontvieille et al. 2013) suggests that this enzyme might have yet further functions. Thus, returning to a more evolutionary perspective, altogether these data indicate that most likely IDH arises in two independent manners in eukaryotic cells: 1) Horizontal gene transfer of mitochondrion ancestor (NAD^+-dependent) to host cells and 2) before endosymbiotic events, host cells have already obtained $NADP^+$-dependent gene which further segregated in several isoforms. This dual idea is in good agreement with the several novel functions that have been associated with both cytosolic and mitochondrial isoforms of this surprisingly enzyme and highlights that further examination of this enzyme should provide significant insights into an integrated overview of the metabolic connections of this enzyme.

Succinyl-CoA Ligase

Succinyl-CoA ligase catalyzes the reversible interconversion of succinyl-CoA to succinate using inorganic phosphate and dinucleotide to produce trinucleotide and CoA. It has been demonstrated that the inhibition of this enzyme in tomato plants leads to only minor changes in both respiratory and photosynthetic metabolism, most likely due to a compensatory upregulation of the GABA shunt (Studart-Guimarães et al. 2007). Characterization of the regulatory properties of this enzyme suggests that allosteric control regulating the flux through the TCA cycle would allow high cyclic flux in carbon rich times and reduced flux in times of carbon deficiency (Studart-Guimarães et al. 2007) suggesting that the succinyl-CoA enzyme may represent an adaptive mechanism in the attempt to maintain the rate of respiration under suboptimal conditions.

In yeast, the heterodimeric protein succinyl-CoA is encoded by two single-copy genes (Studart-Guimarães et al. 2005). Thus in an attempt to identify the pattern of evolution of this enzyme, we constructed phylogenetic trees using sequences ranging from yeast to mammalian proteins of both alpha and beta subunits encoded by different genes (fig. 3*A*) expecting that they had their own evolutionary history. One can easily note 1) a clear separation of alpha and beta subunits (different colors shown in fig. 3*A*) and 2) a wide segregation among kingdoms when analyzing succinyl-CoA ligase sequences which clearly suggest that eubacterial cells emerged from mitochondria ancestor and that succinyl-CoA ligase genes were most likely transferred to eubacterial genome. Another point to reinforce these suggestions is that although in higher plants there are two single copy genes encoding cytosolic and mitochondrial isoforms, these genes present high similarity between each other clustering in the same branch (e.g., *Solanum lycopersicum*) or in very close branches of sisters-species (e.g., alpha gene of *A. thaliana* and *Arabidopsis lyrata* are in the same cluster and the same happening with beta genes but in a different cluster) (fig. 3*A*).

Succinate Dehydrogenase

SDH, also often referred to as complex II, has a dual function, being important in both the TCA cycle and the aerobic respiratory chain, through the catalysis of the oxidation of succinate to fumarate and the reduction of ubiquinone to ubiquinol, respectively (Affourtit et al. 2001; Araújo, Nunes-Nesi, et al. 2011). The conserved elements of this complex, a mere four polypeptides, comprise two peripheral membrane proteins—a flavoprotein (SDH1), and an iron-sulfur binding protein (SDH2)—as well as two integral membrane proteins (SDH3 and SDH4) (Rasmusson et al. 2008). However, the evolutionary history of these subunits is as yet unknown. For the sake of simplicity, multiple alignments of SDH sequences were carried out using only the flavoprotein and iron-sulfur subunits and the results obtained are shown in figure 3*B*. Although responsible for SDH function these subunits were relatively different and clustered in independent groups regardless the organisms evaluated here (fig. 3*B*). As it can be deduced by the data presented in figure 3*B* there are more similarity between plant and animal for each subunit than among cyanobacteria SDH subunits, indicating a mitochondrial origin for the eukaryotic gene. Although the proteins branch from the alpha proteobacterial homologs, it seems that the genes for SDH were most likely acquired from the mitochondrion ancestor. Altogether our data revealed a short divergence in terms of evolution and proximity of eukaryotic cells with the mitochondria ancestor. In good agreement with the view concerning the evolutionary history of SDH from mitochondria to nuclear genome, it has been demonstrated that at least some SDH genes were lost during horizontal gene transfer (Adams et al. 2002; Choi et al. 2006). Taken as a whole this feature might, at least partially, explain cross kingdom differences in the structural architecture of SDH (e.g., *A. thaliana* has 12 subunits; supplementary table S1, Supplementary Material online).

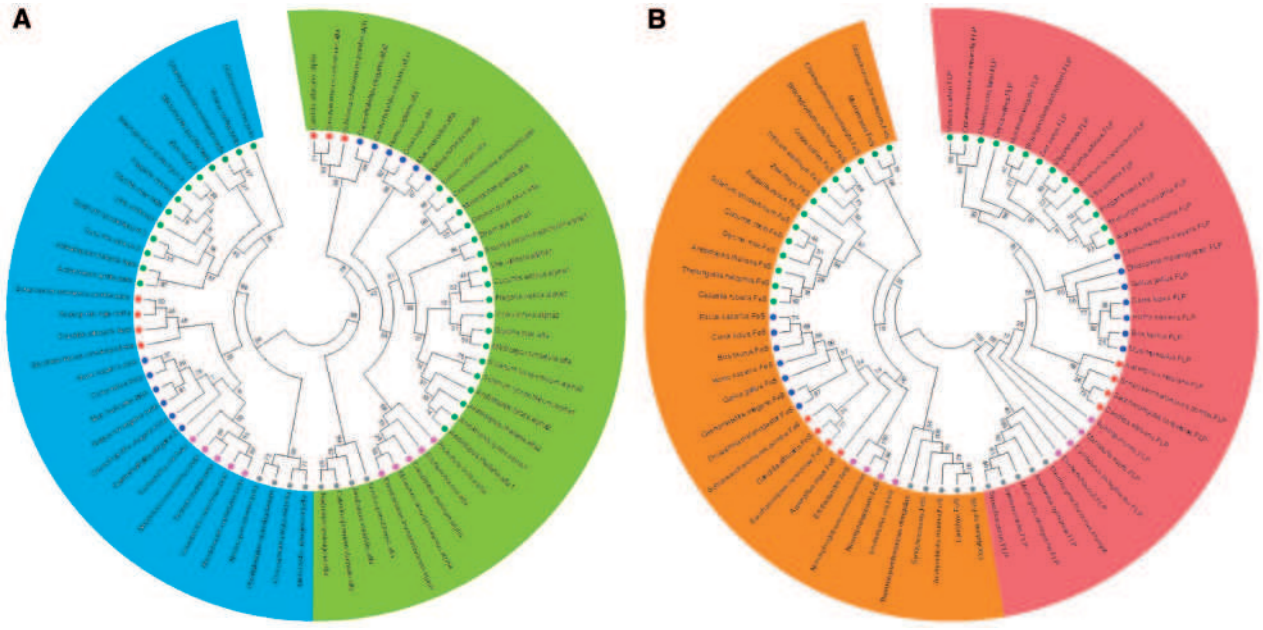


Fig. 3.—The phylogenetic tree of (*A*) succinl-CoA ligase and (*B*) SDH. Trees were built using putative amino acids sequences from (*A*) succinyl-CoA ligase highlighting the two subunits: Alpha (green background) and beta (blue background); and (*B*) SDH highlighting two subunits: Flavoprotein (red background) and iron-sulfur (orange background). Sequences of putative proteins from plants are highlighted by green circle, yeast by red circle, animals by blue circle, algae by dark green circle, bacteria by pink circle, and cyanobacteria by gray circle.

Fumarase

Fumarase (or fumarate hydratase) catalyses the reversible hydration of fumarate to malate (Nunes-Nesi et al. 2007). Interestingly, although the mitochondrial fumarase seems to be an essential enzyme (Pracharoenwattana et al. 2010) characterization of a cytosolic fumarase mutant suggests that fumarate accumulation in the light is linked to nitrogen assimilation and increased starch in leaves of *A. thaliana* (Pracharoenwattana et al. 2010). Accordingly, fumarase activity has been shown to be high in guard cells of *Vicia faba* and *Pisum sativum* (Hampp et al. 1982; Outlaw 2003). Moreover and consistent with this observation, transgenic tomato plants with a reduced expression and activity of fumarase of up to 75% were characterized to have marginally elevated fumarate contents and that the reduced growth phenotype observed on a whole plant basis plants could be linked to impaired stomatal functioning (Nunes-Nesi et al. 2007), rather than a direct metabolic effect, resulting in CO_2 limitation of photosynthesis. It was also observed that these transgenic plants had altered shoot to root resource partitioning (van der Merwe et al. 2009).

Although the presence of a cytosolic fumarase has not yet been reported in Solanaceous species, the genome of both potato and tomato seems to encode these genes. In good agreement, data mining reveals that unlike most of other kingdoms, plants species harbor genes encoding both cytosolic and mitochondrial isoforms (fig. 4*A*). It should be mention that in some vertebrates, such as rat, the cytosolic enzyme is encoded by the same gene as the mitochondrial isoform which is generated by an alternative translation site (Suzuki et al. 1992). Additionally *Saccharomyces cerevisae* also harbors two enzymes located in the cytosol and mitochondrial due to different cleavage sites (Wu and Tzagoloff 1987). Nevertheless, fumarase gene of plants clustered in the Eudicots group suggesting the occurrence of a recent duplication event. In support to this point of view, paralogous genes are more similar to each other than to orthologous genes (e.g., *A. thaliana* mitochondrial and cytosolic genes cluster closer than the mitochondrial genes from *A. thaliana* and *Vitis vinifera*). Similar observations in terms of evolution of other mitochondrial genes families such as the *Arabidopsis* S-adenosylmethionine carrier SAMC1 (At4g39460) and SAMC2 (At1g34065) were also recently described (Palmieri et al. 2011) supporting our hypothesis that intraspecies paralogous were originate through recent duplication events within the Eudicot clade.

Malate Dehydrogenase

The last step of the TCA cycle is catalyzed by malate dehydrogenase (MDH) and constitutes the reversible oxidation of malate to produce oxaloacetate (Nunes-Nesi et al. 2005). The mitochondrial isoform of this enzyme is important not only for NADH oxidation within the TCA cycle but is also responsible for the exchange of reducing equivalents between

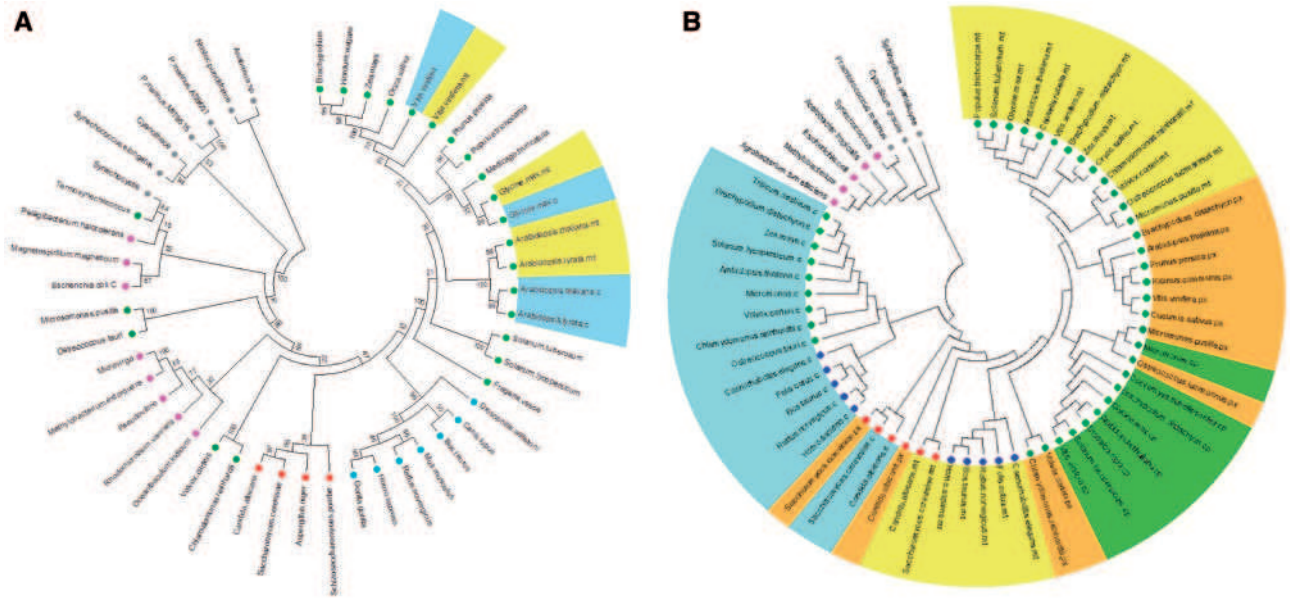


Fig. 4.—The phylogenetic tree of (*A*) fumarase and (*B*) MDH. Trees were built using putative amino acids sequences from (*A*) fumarase and (*B*) MDH. Subcellular compartments are highlighted as in figure 2: Mitochondrial isoforms are highlighted by yellow background, peroxissomal isoforms by orange background, and cytosolic isoforms by blue background. Sequences of putative proteins from plants are highlighted by green circle, yeast by red circle, animals by blue circle, algae by dark green circle, bacteria by pink circle, and cyanobacteria by gray circle.

metabolic pathways in different cell compartments (Scheibe et al. 2005; Pracharoenwattana et al. 2007; Tomaz et al. 2010). Moreover, in *Arabidopsis* the number of nuclear genes that encode polypeptide components is 1, 3, 2, and 2 with localization in plastid, cytosol, mitochondria, and peroxisomes, respectively. Biochemical characterization of these subunits has revealed a broad connection of the MDH not only with respiration (Nunes-Nesi et al. 2005; Tomaz et al. 2010) but also with several other processes such as photorespiration, ß-oxidation of fatty acids, seed germination, and stress tolerance (Tesfaye et al. 2001; Pracharoenwattana et al. 2007; Cousins et al. 2008; Wang et al. 2010). Moreover, metabolic control coefficients of dark respiration have indicated that the control of leaf respiration is largely dominated by the enzyme MDH and that much of the control through the TCA cycle is shared between this enzyme, fumarase, and OGDH (Araújo, Nunes-Nesi, et al. 2012). Briefly, a central and complex role for this enzyme has been demonstrated suggesting its importance in the partitioning of carbon and energy in higher plants, providing new directions for bioengineering of plant growth rate and new insights into the molecular mechanisms linking respiration, photosynthesis, and photorespiration in plants (Cousins et al. 2008; van der Merwe et al. 2009; Tomaz et al. 2010).

Our data demonstrated that in order to play a range of role as described above it must be required that MDH has been spread across several subcellular compartments. Accordingly, we also had shown that MDH from cyanobacteria and bacteria clusters closer (fig. 4*B*). Moreover, our results indicate that cytosolic MDH most likely rises from horizontal gene transfer (fig. 4*B*). These findings are also in agreement with previous observation (Schnarrenberger and Martin 2002). Additionally, we also believe that other MDH isoforms had originated from cytosolic isoform (fig. 4*B*). This idea is highly interesting given that plastid isoforms clustered together in plants and green algae species suggesting that the plastid isoform of MDH arises after segregation between autotrophic and heterotrophic organisms (fig. 4*B*). Additionally, it seems highly possible that this plastid isoform ($NADP^+$-dependent) was created through cytosolic (NAD^+-dependent) isoform.

Collectively, our data indicated that the evolution of the TCA cycle was consistent with one step-by-step acquisition of each gene especially in free-living organisms which are the ancestor of mitochondrion. Accordingly, it is well known that paralogous genes often belong to the same species and cope with different evolutionary pressure arising either novel function of an existing gene (neofunctionalization) or in case of recent gene duplication similar function may still remain (subfunctionalization) (Force et al. 1999; Dani et al. 2014). Furthermore, paralogous sequence might provide useful insights into the way genome sequences evolve. For instance, although peroxisomal MDH is essential for ß-oxidation and seed germination (Pracharoenwattana et al. 2005) and has only a limited impact on photorespiration (Cousins et al.

2008), the mitochondrial MDH is important for both plant respiration and plays a key role on photorespiration regulating plant growth in *Arabidopsis* (Tomaz et al. 2010). In addition, overexpression of cytosolic MDH in alfalfa (*Medicago sativa*) increased aluminum tolerance through metal chelation in the soil (Tesfaye et al. 2001). These studies highlight a complex form of functional specialization between isoforms in different compartments and clearly show that changes in the amount of one specific isoform can have far reaching effects on plant growth and development. Surprisingly, although several studies have targeted on mitochondrial TCA cycle gene through reverse genetic approach or specific inhibitor (reviewed in (Araújo, Nunes-Nesi, et al. 2012; Nunes-Nesi et al. 2013) with a few exceptions our understanding of the role of other subcellular compartments isoforms has been mostly hampered (Pracharoenwattana et al. 2005, 2010; Sulpice et al. 2010) limiting our understanding of the complex evolution of this pathway. When considered together these results coupled with the ones we presented here strongly suggest that the TCA cycle most likely occurred in isolated steps in free-living organisms ancestor of mitochondrion (Schnarrenberger and Martin 2002; Gabaldón and Huynen 2003) and indicate that some enzymes (e.g., CS, MDH, and aconitase—discussed above) were acquired for eukaryotic cell by lateral gene transfer from several independent events using eubacteria donors.

Interactions among Mitochondrial TCA Cycle Genes

The TCA cycle is composed by a set of eight enzymes presented in the mitochondrial matrix cycle coupling the product of the oxidation of pyruvate and malate (generated in the cytosol) to CO_2 with the production of NADH by the respiratory chain (Fernie et al. 2004; Millar et al. 2011). It is known that genes encoding proteins that are involved in the same process, here meaning the TCA cycle, should be simultaneously expressed in time and space therefore here we choose coexpression data to aid in the discovery of patterns and novel players involved in this important process. Coexpression network analysis is commonly used to identify genes that have similar expression patterns and therefore represents an important tool to predict gene functionality using public transcriptome data sets. The availability of large amounts of gene expression data and the growing power of bioinformatics, coupled with availability of computational resources, opens new avenues to discover proteins involved in important processes, such as plant respiration.

Our first in silico coexpression method was performed using all genes or a large group of genes that are computed by coexpression responses for different sets of microarrays data (Akiyama et al. 2008) using separate data sets of hormone (fig. 5*A* and *B*), developmental changes (fig. 5*C* and *D*), and stress responses (fig. 5*G* and *H*). Interestingly, both hormone treatment and developmental change data sets demonstrated a high and intense regulation within the TCA cycle genes when compared with stress experiment (fig. 5). This observation can be expected in the light of, for instance, recent results showing a strong association between hormone and energy metabolism (Araújo, Tohge, et al. 2012; Ribeiro et al. 2012a, 2012b) as well as evidence concerning that GA might be able to modify primary metabolism at the entry point of TCA cycle (Yazaki et al. 2003; Jan et al. 2006). Although the results highlighted above provide clear support to the role of energetic metabolism and particularly the TCA cycle as a central through in supplying ATP and other fundamental metabolites to support growth and development, our current understanding of the general role of hormones in the regulation of plant metabolism and growth is still limited and deeply deserving further investigation.

In order to further develop our analysis, we conducted an expanded guide-gene approach that used interconnections between sets of specific guide genes of related biological processes (fig. 6). For this end, we used three sets of "bait genes" known to be involved in plant TCA cycle (40 genes), mitochondrial carrier (24), and stress responses and photorespiratory genes (22) aiming to identify further candidate genes involved in the mechanism of regulation of plant respiration (supplementary table S2, Supplementary Material online). As would perhaps be expected, this coexpression revealed close connections between these candidate genes. Furthermore, we were able to identify two connected cluster of coexpressed genes that could be clearly separated. First, we identified genes in Candidate Cluster I (20 genes) which coexpressed with all processes making them logical candidates for further investigations. It should be mention that the some genes in the Candidate Cluster II (17 genes) correlated better with the TCA cycle and mitochondrial stress-related genes, whereas some specific genes within the Candidate Cluster I seem to highly coexpressed with mitochondrial carriers. One interesting feature of the network that has been generated is that among the genes present in the Candidate Cluster I we found several genes involved in photosynthesis or photorespiration which are located in other subcellular compartments suggesting a close association of mitochondrial related processes with other processes within the plant cell—as has been noted in many experimental studies (Carrari et al. 2003; Nunes-Nesi et al. 2005, 2007; Tcherkez et al. 2005; Sweetlove et al. 2006; Tomaz et al. 2010; Araújo, Nunes-Nesi, et al. 2011; Foyer et al. 2011; Timm et al. 2012; Boex-Fontvieille et al. 2013). Thus although mitochondrial genes such as NAD(P)H dehydrogenase (At5g58260) and genes involved in redox balance control (At5g04140; ferredoxin-dependent glutamate synthase 1) were present among our candidate genes, other uncharacterized genes classified as unknown or hypothetical were coexpressed suggesting that further investigation of mitochondrial metabolism might identify other as yet unknown connections within the TCA cycle. Further functional characterization of these genes will likely help us in identifying genes

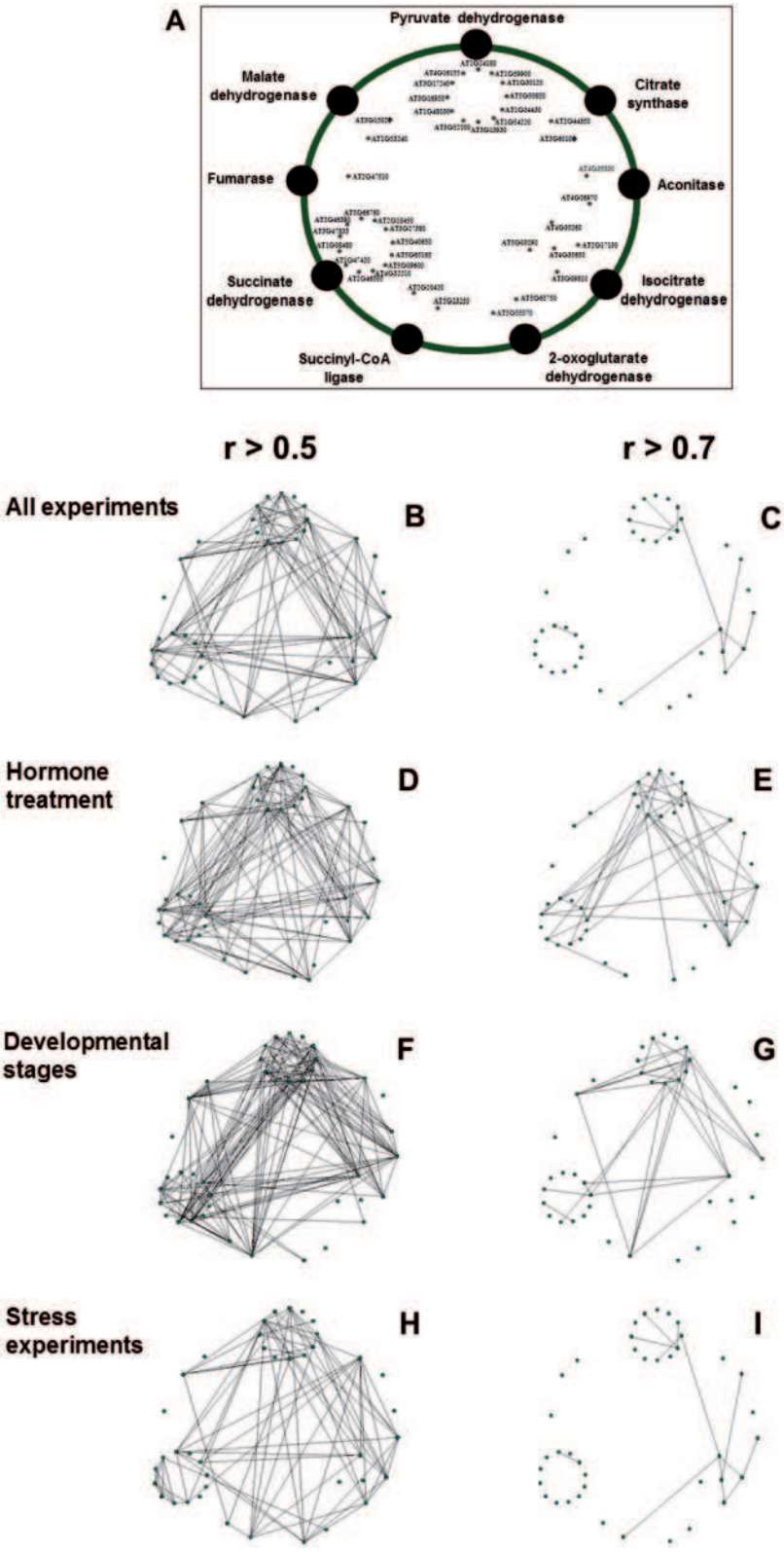


Fig. 5.—Coexpression analysis using mitochondrial genes of the TCA cycle from plant. (*A*) Framework for coregulation network analysis of mitochondrial isoforms of TCA cycle-related genes using the Pajek software. Transcriptional data mining was performed using coexpression PRIMe database (http://prime.psc.riken.jp/?action=coexpression_index, last accessed October 13, 2014) using as trap the genes listed in supplementary table S1, Supplementary Material

(continued)

that control and regulate plant respiration as well as facilitating the discovery of novel gene functions with potential biotechnological applications.

Physiological Importance of Mitochondrial TCA Cycle Genes under Suboptimal Conditions

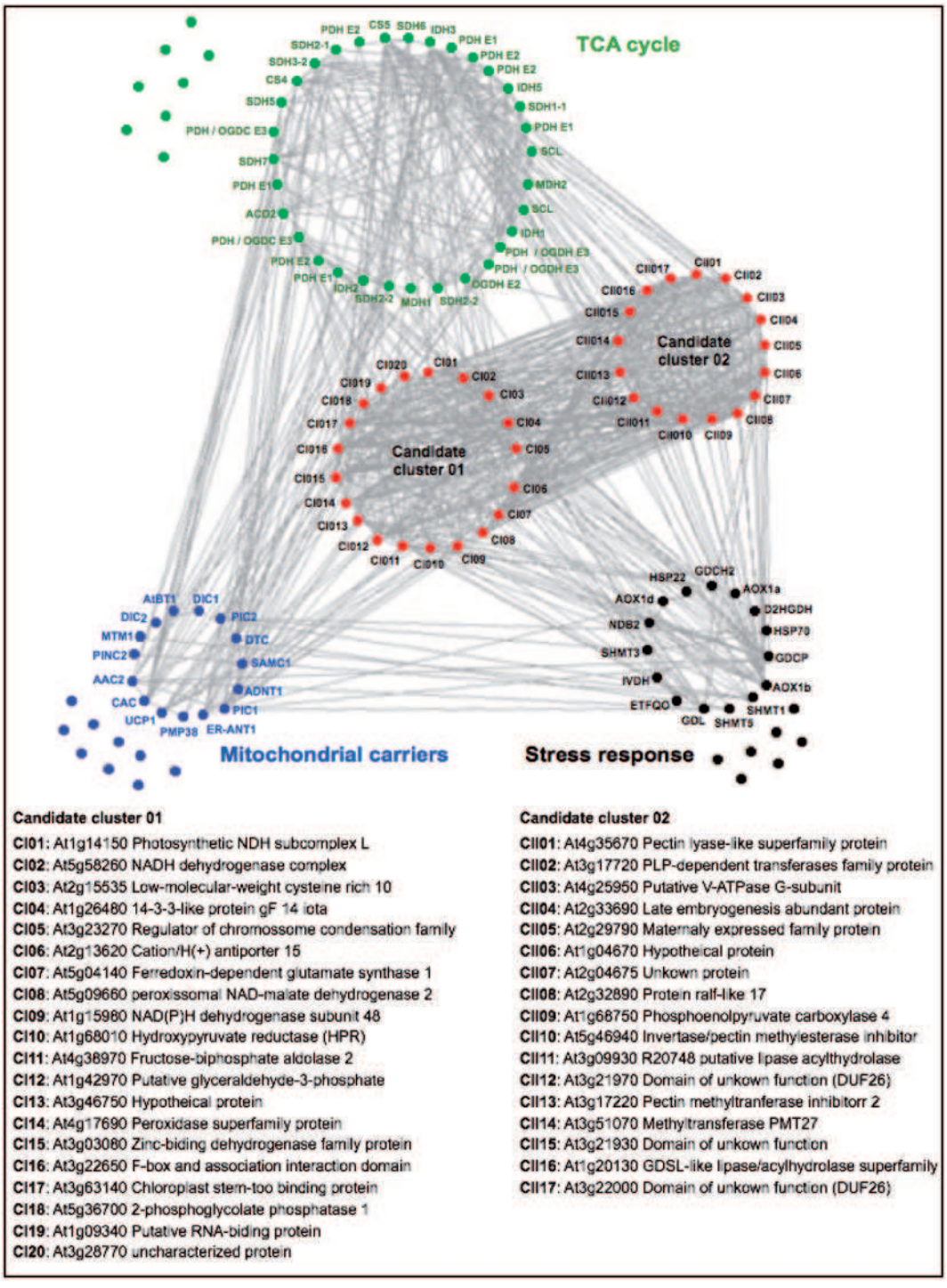
Although plant respiration is mainly dependent on carbohydrate oxidation (Plaxton and Podesta 2006; Araújo, Nunes-Nesi, et al. 2012), it has been recently demonstrated that the oxidation of alternative substrates becomes considerably important during various stress conditions, which affects carbohydrates supply (Ishizaki et al. 2005, 2006; Engqvist et al. 2009; Araújo et al. 2010). Additionally, plant metabolism is highly reorganized under a range of different stress conditions including salt, cold, drought, and oxidative stress (Kaplan et al. 2004, 2007; Gong et al. 2005; Sanchez et al. 2008, 2012; Alet et al. 2012; Siahpoosh et al. 2012), allowing plants to continue to produce indispensable metabolites while preventing the accumulation of reactive oxygen species. Thus, we here postulate that the functionally translated portion of the genome plays an essential role in plant stress, and therefore extended bioinformatic studies will likely provide a finer picture of protein networks involved in metabolic pathways that are important for cellular detoxification and tolerance mechanisms. As such, a prominent topic in plant mitochondria research involves linking mitochondrial composition and function to environmental stress responses. In order to understand transcriptional changes of genes encoding TCA cycle proteins, we evaluated data from AtGenExpress concerning a wide range of stress including exposure to cold, osmotic, salt, drought, genotoxic, oxidative, UV-B, wounding, and heat stress displayed in a heat map documentation of the relative expression of 40 TCA cycle-related genes in both shoot and roots (fig. 7). Interestingly, the majority of the genes whose function has been experimentally investigated showed strong transcriptional regulation following stress situation, either in root or in shoot tissue. Notably, the expression profile observed in shoots under different stress conditions seems to be independent of the changes observed in roots (fig. 7*A* and *B*, respectively). For instance, when the expression profiles of all gene copies of IDH (with the exception of idh3, discussed below) were compared distinct patterns are apparent in roots and shoots. Although in roots only a moderate decrease in expression was observed after 1 and 3-h exposure to osmotic and salt stresses, respectively, in shoots gene expression is highly upregulated by cold, osmotic, salt, wounding, and heat. Differential patterns of expression between shoot and root were also observed for MDH 2, fumarase, and the majority of the SDH subunits under osmotic and UV-B stresses at least 1 h after stress application (fig. 7). Thus, as would be expected that given the profile previously observed for genes encoding mitochondrial carriers (Palmieri et al. 2011), the pattern of expression of the TCA cycle-related genes under the stress conditions appears to be more affected by environmental changes in shoots than in roots.

Our analysis also revealed a molecular plasticity in transcripts level of the TCA cycle genes between the tissues analyzed (fig. 7). As such, although most of the TCA cycle-related genes are upregulated in shoots (fig. 7*A*) these genes are normally downregulated in roots under a range of stresses (fig. 7*B*). Thus, in roots a small group of genes seem to be stress-induced involving six TCA cycle-related genes in the same cluster (fig. 7*B*). These genes are citrate synthase 5 (cs5), aconitase 1 (aco1), isocitrate dehydrogenase 3 (idh3), 2-oxoglutare dehydrogenase (ogdh) E1 and E2 subunits, and succinate dehydrogenase 1-1 (sdh1-1). Interestingly, from all IDH used here only NAD^+-dependent idh-3, which is strictly associated with the TCA cycle (Nunes-Nesi et al. 2013), showed an augmentation in its expression (fig. 7*B*). In addition, the transcript of this IDH isoform clearly increased after exposure for at least 3 h to drought stress. Indeed, under abiotic stresses situation such as drought, oxidative, and salt stress augmentation in the transcript levels of ogdh and sdh1-1 as well as idh3 was observed in root tissues. Whether these genes have at least partial redundant functions, particularly under stress situations, or whether the similar pattern of expression due to any transcription factor is unknown.

In contrast to the situation observed in roots, the TCA cycle-related genes were highly upregulated under various stresses in shoots (fig. 7*A*). Notably, when comparing the data obtained within upregulated genes, we can observe that the mitochondrial fumarase gene is highly upregulated in response to both drought and salt stresses, which are stresses well known to impact photosynthesis due to, in several cases, stomatal closure (Deikman et al. 2012; Daszkowska-Golec and Szarejko 2013). In close agreement, and as discussed above, antisense inhibition of fumarase leads to decreased photosynthesis and biomass in tomato plants associated

Fig. 5.—Continued

online. Coresponse connection was performed using coefficient values calculated by a microarray data set of all experiments covering 1,388 microarray data (*B* and *C*); "hormone treatment" covering 326 data (*D* and *E*), "developmental stages" covering 237 data (*F* and *G*), and "stress experiments" covering 298 data (*H* and *I*). Analyses were performed with cut-off value of $r > 0.5$ for (*B*), (*D*), (*F*), and (*H*) and of $r > 0.7$ for (*C*), (*E*), (*G*), and (*I*). Connections between each genes were prepared by "intersection of sets "search. The output files which were formatted with ".net" file from PRIMe database were later used to drawn the networks using Pajek software (Batagelj and Mrvar et al. 1998). Abbreviations and locus names of genes used are available in supplementary table S1, Supplementary Material online.



Candidate cluster 01

CI01: At1g14150 Photosynthetic NDH subcomplex L
CI02: At5g58260 NADH dehydrogenase complex
CI03: At2g15535 Low-molecular-weight cysteine rich 10
CI04: At1g26480 14-3-3-like protein gF 14 iota
CI05: At3g23270 Regulator of chromossome condensation family
CI06: At2g13620 Cation/H(+) antiporter 15
CI07: At5g04140 Ferredoxin-dependent glutamate synthase 1
CI08: At5g09660 peroxissomal NAD-malate dehydrogenase 2
CI09: At1g15980 NAD(P)H dehydrogenase subunit 48
CI10: At1g68010 Hydroxypyruvate reductase (HPR)
CI11: At4g38970 Fructose-biphosphate aldolase 2
CI12: At1g42970 Putative glyceraldehyde-3-phosphate
CI13: At3g46750 Hypotheical protein
CI14: At4g17690 Peroxidase superfamily protein
CI15: At3g03080 Zinc-biding dehydrogenase family protein
CI16: At3g22650 F-box and association interaction domain
CI17: At3g63140 Chloroplast stem-too binding protein
CI18: At5g36700 2-phosphoglycolate phosphatase 1
CI19: At1g09340 Putative RNA-biding protein
CI20: At3g28770 uncharacterized protein

Candidate cluster 02

CII01: At4g35670 Pectin lyase-like superfamily protein
CII02: At3g17720 PLP-dependent transferases family protein
CII03: At4g25950 Putative V-ATPase G-subunit
CII04: At2g33690 Late embryogenesis abundant protein
CII05: At2g29790 Maternaly expressed family protein
CII06: At1g04670 Hypotheical protein
CII07: At2g04675 Unkown protein
CII08: At2g32890 Protein raif-like 17
CII09: At1g68750 Phosphoenolpyruvate carboxylase 4
CII10: At5g46940 Invertase/pectin methylesterase inhibitor
CII11: At3g09930 R20748 putative lipase acylthydrolase
CII12: At3g21970 Domain of unkown function (DUF26)
CII13: At3g17220 Pectin methyltranferase inhibitorr 2
CII14: At3g51070 Methyltransferase PMT27
CII15: At3g21930 Domain of unkown function
CII16: At1g20130 GDSL-like lipase/acylhydrolase superfamily
CII17: At3g22000 Domain of unkown function (DUF26)

Fig. 6.—Coexpression analysis as a tool to identify candidate genes involved on mitochondrial metabolism. Framework for coregulation network analysis ($r > 0.70$) of 84 preselected known genes encoding 40 mitochondrial TCA cycle (green circle), 24 mitochondrial carriers (blue circle), and 22 stress response (black circle) genes using coexpression PRIMe database and Pajek software. The candidate genes were listed by a combinatorial method of "intersection of sets" and "interconnection of sets" using the PRIMe website (http://prime.psc.riken.jp/, last accessed October 13, 2014). Candidate genes were found by an "intersection of sets" search with a threshold value with a coefficient of $r > 0.70$ queried by intraconnection between all query genes. A coexpression network, including candidate genes (37 genes) and queried genes (84 genes), was reconstructed by a "union of sets" search with $r > 0.70$ using the PRIMe database. The output files that were formatted with a ".net" file from the PRIMe database and networks were drawn using Pajek software (http://vlado.fmf.uni-lj.si/pub/networks/pajek/, last accessed October 13, 2014). For a complete description of the gene names, see supplementary table S2, Supplementary Material online.

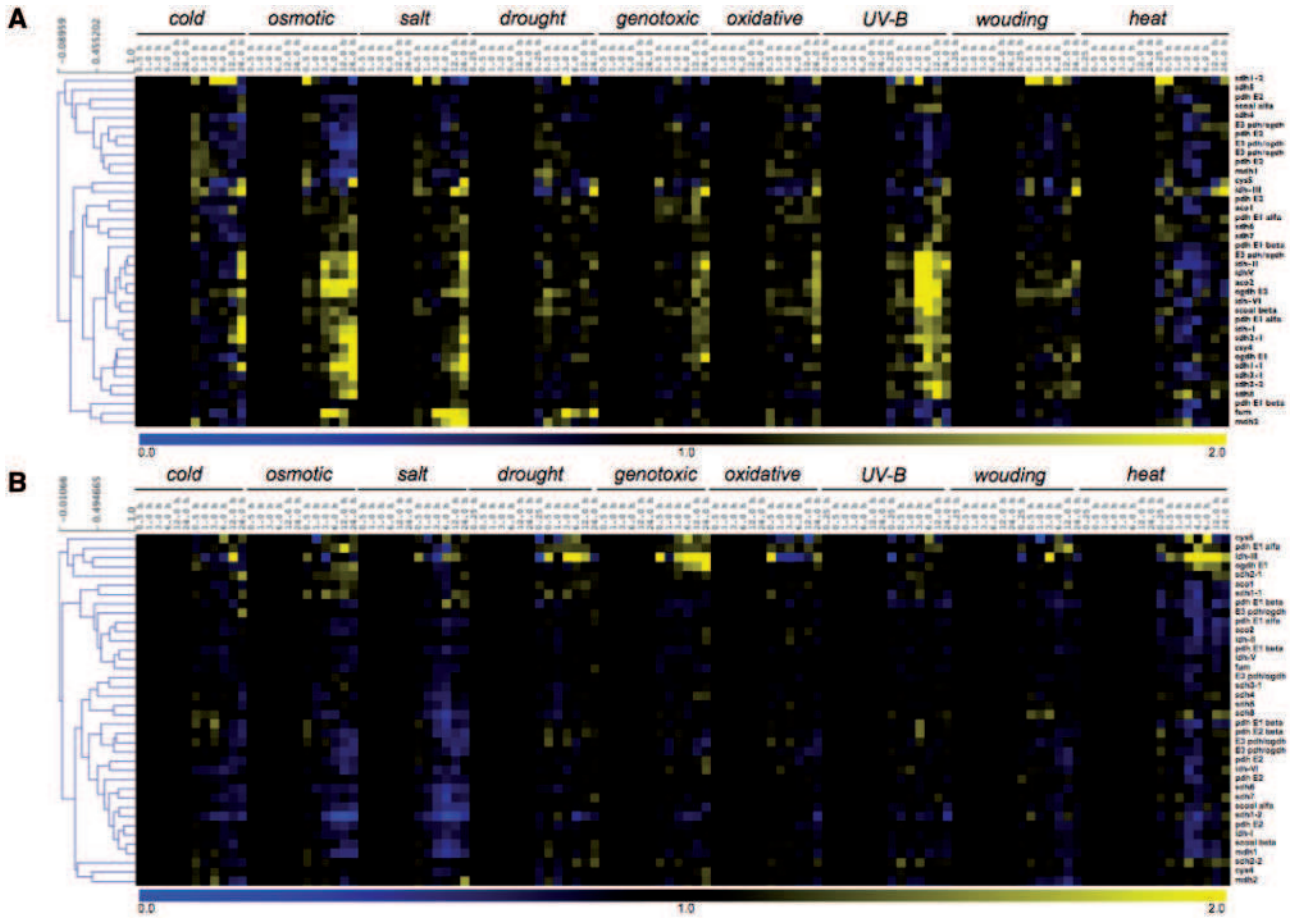


Fig. 7.—Coexpression analysis of genes encoding TCA cycle genes found in *A. thaliana* under a range of stress situations. Heatmap of gene expression data and clustering of the corresponding gene expression data set retrieved of The Bioarray Resource for Plant Biology (BAR, http://bar.utoronto.ca/welcome.htm, last accessed October 13, 2014) using e-Northerns w. Expression Browser platform was performed with MultiExperiment Viewer software (MeV software; Saaed et al. 2003) using "AtGenExpress—Stress Series" data set. The analysis was performed with 48 TCA cycle-related genes of *A. thaliana* using the expression in (*A*) shoot and (*B*) root tissues. For a complete description of the gene names, see supplementary table S1, Supplementary Material online.

with impairment in stomatal function (Nunes-Nesi et al. 2007), whereas antisense inhibition of the iron-sulfur subunit of SDH in tomato plants culminated in higher photosynthesis rate as well as increased whole plant biomass (Araújo, Nunes-Nesi, et al. 2011). These results provided strong evidence to support that modulation of malate and fumarate concentration through genetic manipulation of the mitochondrial metabolism can greatly influence stomatal function and photosynthesis itself in an abscissic acid independent manner. Similarly, during salt stress, fumarase seems to be strongly coexpressed with the mitochondrial MDH 2 (fig. 7*A*). In good agreement with our putative data showing an increase of fumarase and MDH 2 genes during various stresses, it has been recently demonstrated that after 6 h of salt treatment there was a reduction and increase on MDH 2 transcripts and protein amount, respectively, whereas fumarase transcripts and proteins are both reduced (Jacoby et al. 2011). This increment of mitochondrial MDH protein abundance following 6 h of salt treatment suggests that MDH 2 displays rapid responses to stress. Accordingly, salt stress acts decreasing water potential of soil leading to physiological effects that are similar to those seen during drought stresses (Munns 2002); however, in this case it is reasonable to assume that malate has a dual role being 1) required for stomatal closure as discussed above or 2) used as substrate by MDH to form NADH and subsequently ATP synthesis by mitochondrial electron chain transfer in order to relieve cellular damages caused by salt stress.

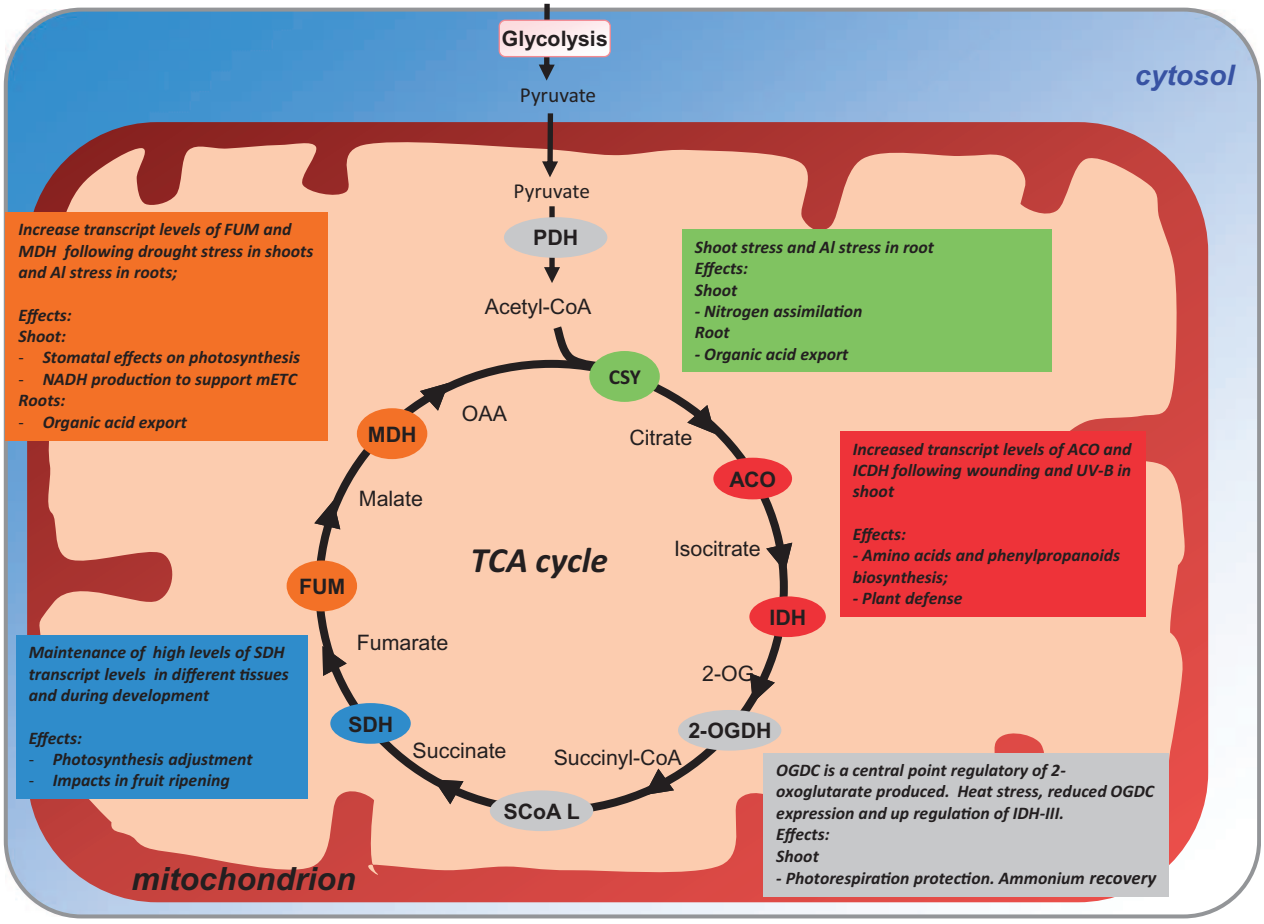


Fig. 8.—Schematic summary representation of tricarboxylic acid (TCA) cycle and its possible strategic for the metabolic engineering. By using bioinformatics approaches presented in figures 6 and 7 a number of possible research avenues for metabolic engineering with the TCA cycle enzymes are presented and discussed in the text. Abbreviations: citrate synthase (CS), aconitase (ACO), isocitrate dehydrogenase (ICDH), 2-oxoglutarate dehydrogenase complex (OGDC), succinyl-CoA ligase (SCoA L), succinate dehydrogenase (SDH), fumarase (FUM), and malate dehydrogenase (MDH).

Our data additionally demonstrated that UV-B stress leads to significant reductions in the expression of genes from the second half of the TCA cycle, such as fumarase and MDH simultaneously to increments on transcripts level of genes from the first half of the TCA cycle in shoot tissue (fig. 7*A*). This finding is in a good agreement with recent experimental evidence using UV-B stress under shoot tissue of *Arabidopsis* (Kusano et al. 2011). Accordingly, intermediates of the first half of the TCA cycle are deviated for the phenylpropanoid pathway reducing flux through the TCA cycle (Kusano et al. 2011) suggesting the operation of a modular TCA cycle in response to UV-B.

Concluding Remarks and Outlook

Although it is clear that coexpression analysis can be used to associate genes with certain biological functions, the remaining question is how reliable these predictions are which means, do the genes identified by coexpression analysis truly function together? There are currently several examples available in which such an approach has been successfully used to identify genes not previously associated with a given biological question (for reviews see Usadel et al. 2009; Bordych et al. 2013, and references therein). We are aware that coexpression analysis, performed individually, is not sufficient to yield lists of candidate genes that are short enough to be investigated in vivo. Nevertheless, as shown in our work, this analysis can be assumed as a reasonable first step to provide initial suggestion of putative candidates. We have no doubts that the coexpression approach has opened new venues for plant researchers and it seems to be true also for the TCA cycle in plants. The coming years will see many more gene expression data sets from specific cell types and other species, which should dramatically accelerate precise hypothesis generation in plant biology (Usadel et al. 2009; Bordych et al. 2013) and particularly within the TCA cycle.

Altogether our coexpression analyses coupled with phylogenetic trees provided an evolutionary explanation for

the modular operation of the TCA cycle according with physiological conditions. This is most likely due to the fact that prior endosymbiotic events some TCA enzymes were already present in eubacterial host and these enzymes seem to have being interconnected to other pathways during evolution, what explain, at least partially, the diversity of function for the isoforms of the TCA cycle enzymes. Notably, endosymbiotic process allowed that other enzyme closed the cycle at the same time that other pathways continued associated with TCA cycle and giving even more functions to this highly important and specialized pathway.

Accordingly, increasing our understanding on the evolution, organization, and function of the TCA cycle members seems to be an interesting way to address endosymbiotic gene transfer. Our results demonstrated that this was most likely the case for several of the mitochondrial enzymes of the TCA cycle, which clearly created a range of biochemical associations of this important pathway with others running in different cell compartments (fig. 8). It will thus be interesting in future studies to evaluate these processes temporally and simultaneously, to dissect both the cascades that control them and the consequence of energy-related processes on the life cycle of the plant. Notwithstanding this fact, the results here also illustrate that mitochondrial metabolism and particularly the TCA cycle in shoots are more dramatically altered with respect to a range of stress. Further work is still required to fully establish the mechanisms involved in this response; however, it is clear that the diverse biochemical phenotypes that have been observed (discussed above) cannot be explained in terms of the operation of a classical TCA cycle, as suppression of any of the enzymes would be expected to reduce the cyclic flux to a greater or lesser extent and thus lead to similar consequences (Sweetlove et al. 2010). Similar conclusions have previously been drawn in microbial and mammalian systems (Tian et al. 2005; Singh et al. 2009; Lemire et al. 2010), allowing us to postulate that the different steps in the TCA cycle have functions other than maintenance of cyclic flux, and that the fine metabolic balancing between these functions is likely to depend on the physiological context in which the pathway is operating. That being said, the far greater alterations observed in TCA cycle-related genes render both the cycle itself and the mitochondrial metabolism as whole as important targets for metabolic engineering and/or breeding strategies for the generation of plants capable of performing well in the future global climate changes scenario (fig. 8).

Supplementary Material

Supplementary data sets S1–S4 and tables S1 and S2 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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Running title: Lys biosynthesis deficiency alters growth and metabolism

Title:
Deficiency on Lysine biosynthesis culminates in both growth impairments and metabolic shifts in *Arabidopsis thaliana*

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Abstract

Although Lysine (Lys) donates electrons to the mitochondrial electron transport chain linking amino acid metabolism and the tricarboxylic acid (TCA) cycle, our understanding of Lys deficiency in plants and its connections with whole metabolism and growth is still limited. Here we used an Arabidopsis mutant with reduced activity of the Lys biosynthesis enzyme *L,L*-diaminopimelate aminotransferase (dapat) to elucidate the physiological and metabolic impact of Lys biosynthesis deficiency. Despite similar stomatal conductance and internal CO_2 concentration a decreases in photosynthetic rates and growth were observed in *dapat* plants. Whilst no differences in dark respiration between *dapat* and wild type (WT) plants were observed, lower storage and consumption of starch and soluble sugars during a diurnal cycle was observed in *dapat* plants. Even though *dapat* plants accumulated and degradated more protein in a diurnal cycle, no differences in total amino acids were observed. We observed a putative stress situation in this mutant associated with increase in amino acids related to stress conditions. Two-dimensional (IEF/SDS-PAGE) proteome analyses demonstrate alterations in several proteins associated with photosynthesis and photorespiration. In connection with the proteomic data, a high glycine/serine ratio was observed. Our findings demonstrated that stomatal limitations are not responsible for the decreased photosynthesis and growth of *dapat* mutants but rather biochemical alterations which mimics stress conditions and are associated to Lys deficiency.

Keywords: amino acid metabolism; alternative respiration; carbon partition; *L,L*-diaminopimelate aminotransferase;Lysine deficiency.

Introduction

Plant mitochondria play an essential and key role in the biosynthesis of cell ATP through oxidative phosphorylation. Accordingly, the tricarboxylic acid (TCA) cycle in the mitochondria is also extremely important in oxidizing acetyl-CoA into CO_2 to produce NADH, $FADH_2$ and ATP and carbon skeleton to be used in several other metabolic processes. Notwithstanding, compelling evidence have recently demonstrated that, although plant respiration is mainly dependent on carbohydrates oxidation (Plaxton and Podesta, 2006), under stress conditions (which affects carbohydrates supply), the metabolism is altered and other pathways are induced in order to provide alternative substrates to the respiratory processes (Ishizaki et al., 2005; Ishizaki et al., 2006; Araújo et al., 2010). Accordingly, it has been demonstrated that protein and amino acid degradation can sustain leaf respiration, particularly during senescence and/or stress situations (Møller and Kristensen, 2004; Araújo et al., 2010). In addition protein degradation can be important for respiratory metabolism under more common physiological circumstances (Bouma et al., 1994; Lehmeier et al., 2008). Notably, it has been demonstrated that in *Arabidopsis thaliana*Lysine (Lys) degradation occurs via a branched pathway (Araújo et al., 2010) partially similar to that described for the bacteria *Rhodospiril lumrubrum* (Ebisuno et al., 1975) and recently described in mammalian systems (Struys and Jakobs, 2010) with 2-hydroxyglutarate being produced via a pipecolate pathway and branched chain keto acids being produced via an, as yet undefined, aminotransferase. It is important to mention that Lys synthesized in the chloroplasts (Figure 1) and therefore the 2-hydroxyglutarate generated during Lys degradation must be imported into the mitochondria to be further oxidized to 2-oxoglutarate (Araújo et al., 2010).

The Electron-Transfer Flavoprotein / Electron-Transfer Flavoprotein: Ubiquinone Oxidoredutase (ETF/ETFQO) complex has been shown to be highly induced at a transcriptional level during dark-induced senescence (Buchanan-Wollaston et al., 2005) and oxidative stress (Lehmann et al., 2009), and under conditions in which free amino acids are present at high concentrations (Weigelt et al., 2008). By analyzing selected *Arabidopsis thaliana* mutants using enzymatic, metabolic, and isotope-labeling procedures we provide evidence that products derived from the ETF/ETFQO pathway (mainly aromatic and the branched-chain amino acids – BCAA, isoleucine, leucine, and valine) represent alternative electron donors at the mitochondrial level (Engqvist et al., 2009; Araújo et al., 2010). This donation of electrons occurs either directly by the transfer of electrons to the mitochondrial electron transport chain (mETC) via ETF complex or indirectly by the direct feeding of catabolic products into the TCA cycle (Araújo et al., 2010). To date, and in contrast to mammals only two alternative dehydrogenases (Isovaleryl-CoA dehydrogenase: IVDH and D-2 hydroxyglutarate dehydrogenase: D2HGDH) have been

demonstrated to be able to donate electrons to the ETF/ETFQO system (Araújo et al., 2010). Accordingly, Lys catabolism via either D2HGDH or IVDH suggests a potential connection between the TCA cycle and alternative respiration in maintaining energy metabolism supporting the growing evidence for strong network behaviour in the co-ordination of plant amino acid metabolism (Foyer and Noctor, 2003; Sweetlove and Fernie, 2005; Less and Galili, 2008; Araújo et al., 2010; Gu et al., 2010). Altogether this information reinforces the idea that Lys metabolism has a strong correlation not only with the TCA cycle but also with mitochondrial energy metabolism in general (Angelovici et al., 2011; Kirma et al., 2012; Galili and Amir, 2013).

Compelling evidence has demonstrated that composite branched pathways are generally responsible for the biosynthesis of amino acids and in particular branched aspartate metabolic network has been extensively studied in Arabidopsis plants in terms of the impact of enhancing biosynthesis fluxes and slowing down catabolic fluxes on the global transcriptome and primary metabolome (Karchi et al., 1994; Zhu and Galili, 2003; Angelovici et al., 2009; Less and Galili, 2009; Angelovici et al., 2011). In contrast, little is known concerning the biological impact of a deficiency in the biosynthesis of aspartate-family amino acids, as naturally occurs in response to stress. Interestingly, an Arabidopsis mutant selected for enhanced defence against a *Pseudomonas* pathogen, was demonstrated to be caused by a single amino acid substitution in the *LL*-diaminopimelate aminotransferase (*LL*-DAPAT) enzyme of Lys biosynthesis, significantly reducing its activity (Rate and Greenberg, 2001; Song et al., 2004). The mutation in *LL*-DAPAT gene (AT4G33680) also resulted in dwarfism, altered leaf morphology and enhanced accumulation of the stress hormone salicylic acid (SA), and was therefore originally named the "*Aberrant Growth and Death*" (agd2) mutant (Rate and Greenberg, 2001; Song et al., 2004).

Here, we have investigated the metabolic and physiologic impact of Lys deficiency by using an established Arabidopsis mutant with reduced activity of the Lys biosynthesis enzyme *LL*-DAPAT. Our results demonstrated that Lys deficiency is associated with growth impairment coupled with drastic decreases on photosynthetic rates. Furthermore, proteomic and metabolic analyses are suggestive of an imbalance in carbon and nitrogen metabolism during a diurnal cycle. The combined results are discussed in the context of current models of amino acids and protein mobilization in situations of limited energy supply being used as alternative source to mitochondrial respiration.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana wild type and the *dapat* plants used in this study were both of the Col ecotype (Col-0) (for further details, see Rate and Greenberg, 2001). Seeds were surface-sterilized and imbibed for 2 days at 4°C in the dark on 0.8% (*w*/*v*) agar plates containing half-strength Murashige and Skoog (MS) media (Sigma-Aldrich; pH 5.7). Seeds were subsequently germinated and grown at 22°C under short-day conditions (10 h light/14 h dark) with 65 μmol m^{-2} s^{-1}. For examination of phenotype, seedlings were transferred to soil 7 to 10 days after germination and placed in a growth chamber at similar growth conditions. The whole rosette leaves of 4-week-old plants were harvested for subsequent analysis.

Measurements of photosynthetic parameters

Leaf gas exchange measurements were performed with an open-flow gas exchange system (LI-6400 XT Li-Cor Inc., Lincoln, NE, USA). The net carbon assimilation rate (A), stomatal conductance to water vapour (g_s), and internal-to-ambient CO_2 concentration ratio (C_i/C_a) were performed after at least 2 hours illumination during the light period. The reference CO_2 concentration was set at 400 μmol CO_2 mol^{-1} air and gas exchange was determined under150 μmol photons m^{-2} s^{-1} at the leaf level (light saturation) of photosynthetically active photon flux density (PPFD). All measurements were performed at 25 °C and vapour pressure deficit was maintained at 2.0 ± 0.2 kPa, whilst the amount of blue light was set to 10% PFD to optimize stomatal aperture. For dark respiration measurements, plants were adapted for 30 min in the dark to avoid light-enhanced dark respiration.

Biochemical assays

Sampling was performed in the last hour of the day (end of the day - ED), or night (end of the night – EN), and completed within 1 h. For all these analyses, whole rosette leaves were collected after gas exchange measurements, flash frozen in liquid nitrogen and stored at −80 °C until analyzed. Each replicate represented the mean of three determinations on the same sample. Chlorophyll, total protein, total amino acid and nitrate contents were determined as previously described (Sienkiewicz-Porzucek et al., 2010). Malate and fumarate contents were determined as described before (Nunes-Nesi et al., 2007). The levels of starch, sucrose, glucose and fructose were determined exactly as described previously (Fernie et al., 2001).

Metabolite profiling

Metabolite extraction was performed essentially following a gas chromatography-mass spectrometry (GC-MS)-based metabolite profiling protocol of Lisec et al. (2006). In brief, Arabidopsis leaf tissues (~50 mg) were homogenized with liquid nitrogen and extracted in 1.400 µL of methanol, chloroform and water (2.5:1:1), and 60 µL of internal standard (0.2 mg ribitol mL^{-1} water) was subsequently added as a quantification standard. The samples were vortex for 30 min at 4 ºC. After this step, samples were centrifuged for 10 min at 13500*g* at 4 ºC. 1.0 mL of the supernatant was recovered to a new tube and it was mixed with 750 µL of ultrapure water and, vortex for 10 seconds. 200 µL of upper phase was transferred to a new tube and drier to vacuum. The derivatization, standard addition, and sample injection steps were carried out exactly as previously described (Lisec et al., 2006). Both chromatograms and mass spectra were evaluated using TAGFINDER software (Luedemann et al., 2008). Metabolites were identified in comparison to database entries of authentic standards (Kopka et al., 2005; Schauer et al., 2005). Identification and annotation of detected peaks followed the recommendations for reporting metabolite data described in Fernie et al. (2011) and are presented in Figure 6 and Supplemental Table 1.

2D gel electrophoresis

Total leaf proteins were homogenate in 900µL of ice-cold extraction buffer (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 100 mMKCl, 1% (w/v) DTT, 30% (w/v) sucrose; 2% PMFS) and vortex for 30 sec. The supernatant was recovered to new tube and added 900µL of ice-cold Tris buffered phenol (pH 8.0) and vortexed for 15 min at 4 ºC followed by centrifugation (3 min, 6000 *g*, 4 ºC). The phenolic phase was recovered to new tube and re-extracted with 900µL of ice-cold extraction buffer and vortexed for 30 sec followed by centrifugation (3 min, 6000 *g*, 4 ºC). The phenolic phase was further collected and precipitated overnight with 1 mL of 100 mM methanol/ammonium acetate at -20˚C. After precipitation, the pellet was centrifuged (30 min, 16000 *g*, 4 ˚C) and rinsed with ice-cold acetone/DTT (0.2%) at -20ºC for 1 h. The sample was air-dried and resuspended in lysisbuffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.8% IPG-buffer (Amersham Biosciences), 1% DTT). Protein was quantified by Bradford reagent (Bradford, 1976). 850µg of protein was diluted with a rehydration buffer (7 M urea, 2 M thiourea, 0.5% CHAPS, 10% glycerol, 0.002% bromophenol blue, 0.5% IPG-buffer) and loaded in strips from 18 cm pH 4-7 linear for 16 h. Isoelectric focalization(IEF) was carry out in IPGphor at 20 ºC with 50 µA/strip on the following conditions: 12 h at 200 V (step), 1 hour at 500 V (step), 600 Vh at 1000 V (gradient), 13500 Vh at 8000 V (gradient) and 18200 Vh at 8000 V (step). After IEF, strips were equilibrated for 15 min on equilibrium buffer (6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 50 mM Tris pH 8.8) containing 1% DTT. Immediately, strips were equilibrated for 15 min on equilibrium buffer

containing 4.5% iodoacetamide. The 2D electrophoresis was carried out at 15 ˚C in 12.5% of poliacrylamide gel using DaltSix System on the following conditions: 20 mA per strip for 30 min, 40 mA per strip for 6 h. The gel was fixed overnight and stained for 2 days in Coomassie Blue G-250 solution. Image acquisition was done using an ImageScanner III (GE Healthcare) and images were analyzed using ImageMaster 2D Platinum v. 7 software (GE Healthcare). Spot protein that differed in ANOVA (P<0.05) were excised to gel for tripsin digestion according Shevchenko et al., 1996.

MALDI-TOF/TOF Mass spectometry analysis and database searching

Tripysin digested proteins were further analyzed using a AB SCIEX TOF/TOF (MALDI-TOF/TOF MS) 4800 proteomics analyzer.The peak list obtained was analyzed using a local Mascot 2.2.07 against Uniprot_Arabidopsis_20140909 database considering a precursor tolerance of 0.1 Da for the product ions, allowing for deamination of asparagine and glutamine, methionine oxidation as a variable modification, carbamidomethylation as a fixed modification, two missed cleavages, and trypsin as the enzyme. The peptide and protein identification were statistically evaluated and validated at 90 % probability level using the Scaffold package (Proteome Software, Inc., Portland, OR, USA).

Experimental design and statistical analysis

The experimental design was completely randomized. Data were submitted to analysis of variance and tested for significant ($P < 0.05$) differences using Student's t tests. All the statistical analyses were performed using the algorithm embedded into Microsoft Excel®.

Results

Lys biosynthesis deficiency reduce Arabidopsis growth and switches physiological parameters

To investigate the relative contribution of Lys deficiency on growth and metabolism we have used an Arabidopsis mutant with reduced activity of the Lys biosynthesis enzyme *L,L*-Diaminopimelate aminotransferase (*dapat*) as well as its wild type control growing side by side. Analysis of the transgenic plants showed that they were clearly different from their wild types with respect to growth and development (Figure 2 and Table 1). Thus, a clear decrease of rosette area was observed for *dapat* plants in both short day and long day conditions (Figure 2A and 2B). This was followed by a lower total number of leaves, as well as a strong decrease in both fresh and dry weight accumulation in those plants (Table 1). Thus, Lys biosynthesis seems to be a limiting factor for normal growth in *Arabidopsis* plants. To study in more details the impact of Lys biosynthesis on plant growth we compared the development of the *dapat* plants with that of wild type. Germination kinetics were clearly delayed in *dapat* mutant plants (Figure 2C) as well as leaf emergence and bolting were slightly delayed, resulting in delayed growth of the inflorescence stems.

Given that the reduction on lysine biosynthesis resulted in plant growth reductions (Figure 2A), we asked whether this phenomenon is associated with an alteration in photosynthetic rates.First, gas exchange of fully expanded leaves was measured and we observed a significant reduction on both net photosynthesis (A) and stomatal conductance (g_s) levels (83% and 75%, respectively) in relation to wild type plants (Figure 3A and 3B). By contrast, no significant differences in internal CO_2 concentration (C_i) were found between Lys deficient and wild type plants (Figure 3C) indicating that decreased photosynthesis cannot be directly associated with stomatal limitations, but most likely associated with biochemical limitation. Interestingly, dark respiration rates (R_d) were not altered in *dapat* plants (Figure 3D). Altogether these results indicate that impairments on Lys metabolism seems to impact photosynthetic metabolism while respiration in maintained most likely by the supply of other alternative substrates in order to cope with this metabolic stressful situation, in good agreement with our initial hypothesis.

Lys biosynthesis is required for normal metabolism

To understand the reduced growth and photosynthesis phenotype observed in *dapat* plants we next investigated the levels of carbon metabolites including starch, soluble sugars, protein and amino acids at the end of the day (ED) and at the end of the night (EN) period (Figure 4). Briefly, the levels of carbohydrates oscillate consistently revealing one conspicuous feature that was clear when comparing the genotypes here analyzed (Figure 4). Thus, soluble sugars (sucrose, glucose and fructose, Figure 4A, 4B and 4C, respectively) showed a similar profile, with lower levels being

observed in *dapat* plants at both ED and EN when compared to WT plants. We additionally observed lower levels of starch in *dapat* plants at the ED (Figure 4D), in good agreement with the lower photosynthetic capacity (Figure 3A) observed in those plants. Interestingly, a reduced starch consumption during the night was observed in *dapat* plants and thus while about 7% of starch produced during the day was still present in WT plants, more than 20% of total starch synthesized was remained at the EN suggesting impairment in starch degradation which can be indirectly associated to the slow growth phenotype observed in Lys deficient plants. Unexpectedly low lysine plants contained higher levels of protein (Figure 4E) and amino acids (Figure 4F) at the ED with similar levels of both at the EN suggesting that during the night the consumption of protein seems to be higher in *dapat* plants (Figure 4E). Thus, protein levels decreased much more at EN in *dapat* mutant (62.4%) than wild type (72.7%) showing a higher consumption of protein during the night for *dapat* plants. Higher amino acids levels were observed both at ED and EN in *dapat* plants (Figure 4F). It should be noted, however, in relative terms, at EN the consumption of amino acids seemed to be lower in *dapat* plants (80% of total amino acids was left) than in WT plants (72% of total amino acids was left).

Given that both malate and fumarate can accumulate to very high level in Arabidopsis leaves (Araujo et al., 2011) we next determined the levels of these organic acids (Figure 05). In agreement with the changes observed for starch the levels of malate were increased in *dapat* mutants at the ED when comparing to WT plants (Figure 5A). By contrast, virtually no differences in the levels of fumarate were observed (Figure 5B). It should be kept in mind that higher levels of organic acids as observed here (Figure 5) coupled with similar respiration rates (Figure 3B) and differences in protein and amino acids suggest that these organic acids as well as amino acids and proteins support respiration at night in *dapat* plants. Thus, oscillation on organic acids levels, in special malate, along the diurnal cycle seem to suggest that they may be used for respiration as alternative respiratory substrates providing an important respiratory substrate specially under low carbohydrates situations (Gibon et al., 2009).

Metabolome analysis reveals a reprograming of *dapat* mutant metabolism

To explore the consequences of the deficiency of Lys biosynthesis on the major primary pathways of plant metabolism we next used an established gas chromatography-mass spectrometry (GC-MS) protocol (Lisec et al., 2006). The data obtained are displayed in false color in the heat map of Figure 6 in order to provide an easy overview (the full dataset is additionally provided as Supplemental Table 1). From this display, it is noticeable that there were considerable changes in the levels of metabolites both at the ED and EN in the lysine biosynthetic deficient *dapat* mutant. As would be anticipated the levels of Lys were increased in *dapat* plants at the ED and although

surprisingly, a strong reduction was observed at the EN for this amino acid. Furthermore, and in good agreement with previous metabolic characterization of this mutant (Rate and Greenberg, 2001) the levels of shikimic acid and salicylic acid were higher in *dapat* plants (Figure 6). It is important to mention that the increased levels of saliciylic acid were observed even when this mutant grew without pathogen attack or other stressfully conditions (Rate and Greenberg, 2001). The reduced expression of the *dapat* gene resulted in an accumulation of metabolites at the ED known to be related to stress response such as proline and ß-alanine (2.75- and 6.09-fold, respectively). In addition, putrescine, another metabolite relate to stress as (Wulff-Zottele et al., 2010), followed the same behavior with increased levels (3.45-fold) at the ED. Furthermore, ornithine, which increased 5.17-fold at the ED, is a central metabolite involved in polyamines biosynthesis as putrescine (Majumdar et al., 2013) or proline biosynthesis. Taken together these findings suggest that a mutation on *dapat* gene may results a putative stress situation even when the plants are growing under optimal conditions.

It is of interest that the aspartate-family amino acids aspartate, methionine and isoleucine increased significantly at the ED in *dapat* plants. In addition, isoleucine decreased at the EN, similarly to the situation observed for Lys. Perhaps one of the most interesting metabolic changes was the behavior of tryptophan which increased more than 8-fold at the ED whereas reduced to 0.65 (relative level) at the EN. Tryptophan as well as other aromatic amino acids has been suggested as potential alternative substrates for respiration (Araújo et al., 2011b). Collectively, these data suggest that both aspartate-derivate amino acids (e.g., lysine and isoleucine) and tryptophan may function as alternative substrate to fulfil respiratory demands in *dapat* plants. However, given that other aromatic amino acids only changed slightly along a diurnal cycle it seems that not all of them are able to work as alternative substrate under our experimental condition. Our metabolic profile also showed that mutation on *dapat* culminated in increased levels of glutamine and glutamate at the ED (5.13- and 2.66-fold, respectively). Interestingly, serine and glycine showed an opposite behavior and thus at the ED glycine showed increased levels while serine decreased. In turn, at the EN glycine had reduced levels whereas serine accumulated.

Other change of note in our metabolite profile related carbon metabolism was the increased (2.51-fold) levels of maltose at the ED which also presented higher levels than the wild type plants at the EN. Sorbitol was significantly reduced only at the EN (0.35-fold).

Lysine deficiency impacts the proteome in Arabidopsis leaves

Since the *dapat* mutant possesses a deficiency in Lys biosynthesis and we demonstrated that this mutant has substantial altered level of the total protein and amino acids, we next extracted total soluble proteins from shoots of the *dapat* mutant and its respective WT control and analyzed the

total protein content by SDS-PAGE followed by coomassie blue staining (Supplementary Figure 1). Although the genotypes possess comparable levels of total soluble protein (Figure 4E), these analyses revealed considerable differences in specific proteins between these genotypes at the ED and EN (Supplemental Figure 1). We thus observed significant differences in both patterns and intensities of the protein bands observed in SDS PAGE stained with Coomassie blue, indicating that flux through the Lys biosynthesis pathway may operate in a considerable excess to that required for protein synthesis. This finding has encouraged us to carry out a robust proteome analysis through 2D electrophoresis (IEF/SDS-PAGE) tandem by MALDI TOF-TOF (Figure 7). We identified 71 proteins that changed their expression level in *dapat* plants by analyzing the proteome profile at both ED and EN (Table 2 and Supplementary Table 2). For easy of shake of first described the results obtained at the ED and after the results at EN are presented and the results are always presented in terms of changes that took place in *dapat* plants in relation to WT ones.

By analyzing proteins at ED our proteome approach we were able to identify several proteins related to photosynthesis (involved in both light- and Calvin-Benson cycle-reactions) and 1 protein related to photorespiration. In this context, proteins involved on photosystem I and II showed a differential expression pattern. Thus, although proteins related to photosystem II such as LHCb-II and Oxygen Evolving Complex (subunit 33 kDa) decreased their abundance, proteins such as Ferredoxin-NADP oxidoreductase 2 (FNR2; At1g20020) located on PSI and ATP synthase gamma chain 1 (At4g04640) increased their amount at the ED in *dapat* plants. Interestingly, enzymes of the Calvin-Benson cycle were also affected on *dapat* mutant plants. In this vein, we observed that RubisCO large chain and seduheptulose 1,7 biphosphatase increased (7.15- and 5.8-fold, respectively) whereas RubisCO small chain 1a, phosphoribulokinase (PRK), GAPDHA as well as RubisCO activase reduced their abundance (Table 2. In addition, redox related proteins that regulate photosynthesis and others chloroplastidic reactions such as Thioredoxin m4, 2-Cys peroxidase A- and B-type, thioredoxin superfamily protein and plenty of gluthatione - S – transferaseisoforms (e.g. GSF2, GSF8, GFS9 and GSF18) were also identified and most of them were down regulated on this time point analyzed. Regarding to photorespiration, only HPR1 was differently expressed in *dapat* plants which showed a reduction on the level of this protein.

Our proteomic approach performed at the ED also provided some insights into amino acid metabolism. Thus, we found an increase in GOGAT protein which can explain, at least partially, increased levels of glutamate and glutamine in *dapat* plants (Figure 6). Moreover, we also found proteins related to hormone metabolism such as *S*-Adenosylmethionine synthase (SAM) 2 and 3 which decreased and increased, respectively. These enzymes are related with the synthesis of both polyamines and ethylene. Lipoxigenase 2, which is related to jasmonate metabolism, also increased at the ED. Collectively, these findings demonstrated that not only the metabolism of the

phytohormone SA is affected on *dapat* plants, but also that a range of different hormones seems to be altered in this mutant.

When analyzing the changes on proteome at the EN we observed the presence of 34 spots corresponding to 29 proteins differentially abundant in *dapat* plants (Table 2). Interestingly, most of those proteins, which were up regulated proteins in *dapat* plants, are related to energy metabolism and particularly located on mitochondria (e.g GDC, SHMT and CS4, see Table 2). Accordingly, up-regulation of peptidase and Chaperon CPN60-like 1, which are related to import and folding of novel mitochondrial proteins, demonstrate that mitochondrial metabolism is more active and shown a higher proteome plasticity than wild type plants. We also observed that citrate synthase 4 was the only up regulated protein belonging to the TCA cycle. In addition, FBPK2 and cytosolic malic enzyme 2 increased at the EN suggesting an augmentation of the energetic metabolism in *dapat* plants. In agreement with this, increased levels of proteins related to NADH production such as GDC subunit H, SHMT4 and FDH suggests that a metabolic reprogramming is occurring in *dapat* plants most likely to use alternative energy sources such as amino acids. Furthermore, the decrease on alcohol dehydrogenase 3 may suggests a reduced glycolysis on *dapat* at night giving further support for the contention that a disorder on carbon breakdown is most likely taking place in this mutant.

Discussion

By using an established Lys deficient mutant (*dapat*) we here provide further evidence of the pivotal importance of this amino acid as depicted by the growth impairments and photosynthesis reductions observed. Intriguingly our results also highlight an interesting and novel aspect of Lys metabolism showing how the deficiency on the biosynthesis of Lys culminated in physiological and metabolic changes in *Arabidopsis*. It is important to mention however that the importance of Lys metabolism is not without precedence given that both the role of this mutant specifically has already been demonstrated in response to biotic stress (Rate and Greenberg, 2001; Song et al., 2004) and that Lys has been demonstrated as an alternative substrate for respiration in plants under carbon limitation (Araújo et al., 2010). The different levels of the total protein observed in the *dapat* mutant and its WT control genotype (Figure 3F) indicate that the significant reduction in DAPAT activity, occurring in the *dapat* mutant, has a major impact not only on the overall rates of protein synthesis and the pattern of proteins visualized following Comaisse staining (Supplemental Figure 1) but also in the protein consumption during the night, as depicted in our by 2D electrophoresis approach (Figure 7). Altogether these results indicate that protein synthesis is also similar, or even higher, on a qualitative basis in the wild type and mutant. These results imply that plants can

synthesize Lys in considerable excess to the amount required for the synthesis of the total protein. Here we further demonstrated one previously unrecognized role of *dapat* and additionally Lys biosynthesis by showing its importance in controlling growth and primary metabolism, with impacts on protein synthesis and degradation.

Our data demonstrated that the net photosynthesis was negatively affected in plants with decreased levels of Lys (Figure 2).The reduction in photosynthesis rates may explain, at least partially, the lower accumulation of starch at the end of the light period in *dapat* plants (Figure 3). Reductions on photosynthesis level are regulated either by stomatal movements or by biochemical parameters associated with photosystem and Calvin-Benson cycle. Nonetheless, internal CO_2 concentration was similar between *dapat* and its WT genotype suggesting that reductions in CO_2 assimilation might be caused by biochemical impairment/adjustment. Here, we were able to further investigate this photosynthetic impairment in a molecular level by using one proteomic approach (Figure 7and Table 2). Our findings demonstrated a decrease on protein of PSII and an increase on protein of PSI as well as ATP synthase gamma chain 1 (Table 2). This opposite behavior may be an indicative of the occurrence of cyclic flux allowing the maintenance of chloroplastidic electron transfer chain in order to enhance ATP synthesis. This finding is further supported by a disorder on redox proteins that regulate photosynthesis and others chloroplastidic reactions. Bearing this on mind, a reduction of PSII associated with the specific reduction of TRX m4, as previously observed (Serrato et al., 2013) may explain the cyclic electron flow taking place in *dapat* mutant. Furthermore, the reduced levels of enzymes of the Calvin-Bensoncycle such as GAPDH and PRK might culminate with decreased flux through this cycle. Accordingly, PRK/GAPDH forms a complex mediated by CP12 protein which has been demonstrated to be a regulatory step of the Calvin-Benson cycle (Marri et al., 2005; Serrato et al., 2013). It is important to mention that when CP12 is in its oxidized form, this complex shows lower activity (Marri et al., 2009). Moreover, PRK/CP12/GAPDH complex has its activity regulated by thethioredoxin system (Howard et al., 2008; Marri et al., 2009). Thus, as mentioned above, our findings demonstrated a decrease on the levels of the thioredoxin system and this down regulation most likely is associated with impairments on PRK/CP12/GAPDH, which become inactive and, consequently, reduce the Calvin-Benson cycle flow. In this scenario, where there is a decrease of proteins of the Calvin-Benson cycle as well as RubisCO content and activity, the reduced power generated as NADPH, which is normally used for CO_2 assimilation, might be redirected to others reaction. Here, we hypothesize that the NADPH produced following the chloropastic electron chain is used by the NADP-dependent malate dehydrogenase to produce malate. In good agreement, our organic acids measurements showed an accumulation of malate at the ED (Figure 4B). Accordingly impairments in stomatal conductance have been associated with alteration on organics acids metabolism as

observed in both fumarase and succinate dehydrogenase antisense lines (Nunes-Nesi et al., 2007; Araújo et al., 2011a). Thus, high malate levels as observed in *dapat* plants can indirectly be linked with stomatal impairments in those plants. However, given that there is some uncertainty about the origin of these organic acids and therefore apoplastic measurement might be required to further clarify this issue. Notably alterations on photosynthesis and respiration have also been observed following chemical inhibition of branched chain metabolism by herbicides leading to clear imbalances on carbon and nitrogen metabolism (Zabalza et al., 2013), as observed here.

The starch turnover gave us insights into how Lys biosynthesis dysfunction in *dapat* mutant affects primary metabolism once we observed that higher levels of starch at EN and the decreased rate of starch breakdown during the night indicating that both accumulation and turnover of starch is altered in *dapat* plants (Figure 3D). The reduced capability of *dapat* plants to degraded starch can lead to a putative starchless condition, which may culminated in a reduced growth (Figure 2A and Table 1). In good agreement with that, the tightly regulation between starch turnover and growth has been extensively demonstrated (Gibon et al., 2009; Sulpice et al., 2009; Andriotis et al., 2012; Ragel et al., 2013) and that fast or incomplete exhaustion of starch culminated in reduced plant growth rate (Stitt and Zeeman, 2012). Additionally although starch turnover is positively correlated with protein content (Gibon et al., 2009; Sulpice et al., 2009) we have observed a negative association between protein and starch levels in *dapat* plants. It should be mention, however, that the augmentation on protein content during the light period may lead to an increment on energy cost to sustain amino acid and protein synthesis (Hachiya et al., 2007; Piques et al., 2009; Raven, 2012) which can represent a large source of ATP consumption, leading also to growth reduction and may explain a suggested cyclic electron flow on photosystem (Table 2). In this vein it can be assumed that *dapat* plants is most likely unable to efficiently degraded starch, and we thus hypothesize that under Lys deficiency *dapat* plants uses alternative substrate for sustain mitochondrial respiration and ATP synthesis since that our data showed that *dapat* mutant and its WT presented similar level of dark respiration (Figure 2D). Accordingly, high protein turnover and similar balance on amino acids levels along diurnal cycle as observed in *dapat* plants suggests that nitrogen metabolism as a whole and indeed protein degradation provide an alternative source of electrons to the mitochondrial electron transport chain. When taken together our results demonstrated that growth reduction observed in *dapat* mutant is most likely due to an imbalance on storage and breakdown of carbon and nitrogen sources uncoupling growth from both carbon and metabolism.

Although Lys deficiency led to growth inhibition, this cannot be associated with a lack of respiratory substrates since a lower consumption of carbohydrates during the night was observed coupled with higher levels of both protein and amino acids (Figure 3). It is reasonable to assume that growth arrest observed here following Lys deficiency can be associated to a general

impairment in metabolism, which does not allow the utilization of available respiratory substrates at the expected rate, despite similar dark respiration, but usage alternative substrates where amino acids seem become essential to support respiration. In fact there is a growing body of evidence suggesting that Lys pathway can be assumed as a respiratory bypass associated with 2-oxoglutarate production (Araújo et al., 2010; Engqvist et al., 2011; Boex-Fontvieille et al., 2013). However, it should be kept in mind that the pool of Lysis not that large and its biosynthetic rate is most likely low (Boex-Fontvieille et al., 2013).Following this assumption, our GC-MS data provided several insights about amino acids turnover along diurnal cycle (Figure 6). The daily fluctuation of both glutamate and glutamine in *dapat* plants indicates a strong variation on glutamate content. Intriguingly, the pattern observed in this mutant is clearly different from the observed in several reports where glutamate normally suffers a small oscillation in its content along a diurnal cycle (Stitt et al., 2002; Gibon et al., 2006). In fact, glutamate level was increased only when leaves were fed with 2-oxoglutarate (Hogdes, 2002; Schneidereit et al., 2006; Forde and Lea, 2007). Thus, one reasonable explanation for the changes in glutamate observed in *dapat* plants is the increase on Alanine:2-oxoglutarate aminotransferase (At1g23310) detected by 2D approach at the ED which is likely capable to produce glutamate. In this vein, it is reasonable to assume that to keep basal levels of glutamine into the cells, *dapat* plants make use of glutamate as precursor of glutamine, proline and ornithine synthesis which in turn increased at the ED (Figure 6). In fact, glutamate and their amino acids-derivated decreased at the EN. Thus, diminishing these amino acids during the dark period suggest that they can be turn converted into 2-oxoglutarate that goes into TCA cycle allowing the production of NADH and ATP. It has been demonstrated that in Arabidopsis cell suspension growing in presence of proline there is an increased activity of both proline dehydrogenase and glutamate dehydrogenase to maintain the production of 2-oxoglutarate as final product which may be completely oxidized in TCA cycle to support energy production (Schertl et al., 2014). Thus, glutamate and its derivative are likely an energetic source to supply organic acids in this mutant and, consequently, when plant cells are under energy deprivation. Interestingly, glycine and serine levels were altered during the day/night cycle. Thus, whereas the levels of glycine were reduced at the end of the day an accumulation of serine level was observed. In agreement with that, our 2D approach revealed increases in both GDC and SHMT4at the end of the night. Accordingly, GDC has been showed to be present in two distinct expression peaks along the diurnal cycle: four hours after onset light and at the end of night (Lee et al., 2010).It is important to mention however, that GDC subunits found during the light period shows a different pI suggesting that GDC in light might be found as degradation product due to photorespiration and ROS production by this path (Lee et al., 2010). Thus, it is reasonable to suggest that a glycine-dependent respiration becomes an important path in energy production in this mutant under optimal

conditions. In addition, increase on FDH at the EN suggests the involvement of formate in respiration. In both cases it seems clear that *dapat* mutant plants display metabolic impairments and therefore alternative substrates to maintain respiration are required. In addition, other respiratory pathways (e.g. ETF/ETFQO) might be up regulated in our conditions. In support to this statement, our metabolite profile demonstrated that both isoleucine and Lys decreased drastically at the EN (Figure 6) which may be catabolized by IVDH and D-2HGDH, respectively. Furthermore, the maintenance of similar respiratory rates in *dapat* plants at night time might also occur through malate oxidation that is accumulated during thelight period. In this case, the increased levels of NADH-dependent ME cytosolic coupled with simultaneous increased on citrate synthase, the first commissioned step of TCA cycle, and the metabolite profiles described above, allows us to postulate an independent pathway for the maintenance of respiration in situations where Lys is somehow limiting. The results presented here suggests that there is an alternative and elegant pathway in which the malate accumulated at end of day is exported to cytosol, during the night, being later converted to pyruvate by the action of the NADH-dependent ME cytosolic enabling it to enter into to the mitochondria and consequently be used by the TCA cycle, and therefore supporting normal rates of respiration.

Bearing this on mind, it is reasonable to assume that alternative pathways of plant respiration are also induced when there are impairments on Lys biosynthesis which seems to mimic a putative stress condition. Indeed, alternative pathways of respiration are induced under several environmental stress and developmental associated stress that lead to a restriction in the provision of carbon for mitochondrial respiration and are coordinated with a general increase in protein degradation and amino acid metabolism (Araújo et al., 2011b). Lysine as well as branch-chain amino acids can be used as alternative substrate to the mitochondrial respirations in a process mediated by the electron transfer flavoprotein/ electron transfer flavoprotein ubiquinone oxidoreductase ETF/ETFQO system (Araújo et al., 2010) as well as by other respiratory bypasses (Boex-Fontvieille et al., 2013). While somewhat preliminary the results presented here suggest that Lys deficiency leads to energy limitation and alterations not only in primary metabolism but also in the global proteome. Identification of metabolites as well as protein involved in these responses might be of crucial importance in defining the key role of Lys metabolism in controlling cellular homeostasis. The findings of this study clearly indicate that further analyses of Lys metabolism are likely to identify additional components of the electron transport chain and their role in the cross-compartment pathway of Lys degradation and its connection with respiratory bypasses.

Although Lys metabolism has been studied for almost 40 years (Azevedo and Arruda, 2010), it seems highly possible that enhancing our understanding of Lys catabolism can provide potential application for metabolic engineering application for increments of free Lys levels in

crops seeds (Azevedo and Arruda, 2010; Galili and Amir, 2013). In summary, the results of this study indicates that (*i*) Lys deficiency leads to growth impairment which is most likely associated to a reduced respiratory substrate usage and (*ii*) in order to avoid starvation under Lys deficiency an extensive metabolic reorganization takes place highlighting a potential role for Lys (and other amino acids) as signals controlling gene expression aspects of this reprogramming. Given the well-recognized connections between Lys metabolism and alternative pathways of respiration (Kirma et al., 2012) it is likely that much more research effort need be expended before the metabolic control underlying this complex process is understood beyond a cursory level.

Concluding Remarks

There is clearly a lack of information concerning the biological impact of Lys deficiency in regarding to its biosynthesis, as naturally occurs in response to stress. However, *dapat* (*LL*-diaminopimelate aminotransferase) mutant used in this study have been well characterized in defense response against a *Pseudomonas* pathogen infection (Rate and Greenberg, 2001; Song et al., 2004). In this study we report that Lys biosynthesis deficiency resulted in an exquisite molecular and, consequently, physiological reprogramming. This reprogramming resulted in a reduced photosynthesis that is in good agreement with the dwarf phenotype (Figure 2A) and is supported by previous results (Rate and Greenberg, 2001 and Song et al., 2004). When taken together with our work here, it seems reasonable to suggest that *dapat* plants growth in a constant stress condition. This point of view is supported by the contention that plants under stress conditions use alternative substrates for the maintenance of respiration and ATP synthesis (Araújo et al., 2010 and 2011b). In summary, our data provide compelling evidence that alternative substrates are used when there is an imbalance on Lys biosynthesis allowing the proper functioning of plant respirations. Further studies are still required to fully understand this metabolic phenotype which mimics stress conditions as revealed by the smaller shoot growth of *dapat* mutants.

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Tables

Table 1.Growth parameters observed in *dapat* plants. Plants deficient on Lys biosynthesis showed a reduced aerial biomass with respect to the WT during vegetative growth (4-week-old-plants)

	Rossette size (diameters mm)	Numbers of leaves	Fresh weight (mg)	Dry weight (mg)	% WRT
WT	32.31 ± 1.34	17.46 ± 0.44	0.1203 ± 0.08	0.0123 ± 0.01	0.1040 ± 0.01
dapat	21.39 ** ± 1.04	12.46 ** ± 0.30	0.0403 ** ± 0.01	0.0048 ** ± 0.01	0.1293 * ± 0.01

Values are presented as average ± SE of at least thirteen independent biological replicates per genotype; an asterisk (*) and (**) indicated values that were determined by the Student's *t*-test to be significantly different ($P< 0.05$) or ($P<$

Table 2. Identification of protein with altered abundance in *dapat*with respect to the WT along a diurnal cycle. Proteins were separated by IEF/SDS-PAGE and spot were analyzed by MALDI TOF-TOF (n=3) *p*-value < 0.05.
ND[a] . non detected in wild type (Col0); ND[b]non detected in dapat mutant

Spot	Locus	Name	Fold	p-value
End of Day				
60	At2g45790	Phosphomanomutase	ND[a]	0.0021
59	At3g48870	HSP93-III	ND[a]	0.0244
1	AtCg00490	Ribulose bisphosphate carboxylase large chain	7.1519	0.0228
2	At3g55800	Sedoheptulose-1.7-bisphosphatase	5.8696	0.0083
3	AtMg01190	ATP synthase subunit 1. ATP1	4.0432	0.069
4	At5g13850	Nascent polypeptide-associated complex subunit alpha-like protein 3	1.7853	0.0459
5	At2g36880	S-adenosylmethionine synthase 3 (MAT3)	1.7631	0.0107
6	At1g57720	Translation elongation factor EF1B	1.7075	0.0397
7	At4g02520	Glutathione S-Transferase phi 2	1.6004	0.0069
8	At1g42970	Glyceraldehyde 3- phosphatedehydrogenasesubunit b	1.5973	0.0083
9	At3g01500	beta carbonic anhydrase 1	1.5916	0.0193
10	At3g59970	Methylenetetrahydrofolate reductase 1	1.5300	0.0500
11	At4g16143	Importin alpha isoform 2	1.4309	0.0242
12	At1g09780	2.3-biphospoglycerate-independent phosphoglycerate mutase	1.3960	0.064
13	At5g25980	Glucoside glucohydrolase 2	1.3851	0.0445
14	At2g45140	Lipoxygenase 2	1.3793	0.060
15	At2g27720	60S acidic riosomal protein family	1.3676	0.0329
16	At4g04640	ATP synthase gamma chain 1. chloroplastic	1.3638	0.0288
17	At5g42020	Luminal binding protein (BiP2)	1.2800	0.0034
18	At1g23310	Alanine 2-oxoglutarate aminotransfarase 1	1.2626	0.0125
19	At2g30860	Glutathione S-Transferase phi 9	1.2419	0.0130
20	At3g11630	2-Cys peroxiredoxin (2-Cys PrxA)	1.2416	0.0357
21	At2g47730	Glutathione S-Transferase phi 8	1.1736	0.0016
22	At2g45140	Lipoxygenase 2	1.1517	0.0142
23	At1g20020	Ferredoxin--NADP reductase. leaf isozyme 2. chloroplastic	1.1364	0.0031
24	At5g24490	30S ribosomal protein	0.8957	0.0130
25	At3g55440	Cytosolic triose phosphate isomerase	0.8886	0.0690
26	At3g01500	beta carbonic anhydrase 1	0.8596	0.0344
27	At4g25100	Fe-superoxide dismutase	0.8483	0.0112
28	At1g32060	Phosphoribulokinase	0.8449	0.0075
29	At3g01500	Putative beta-carbonic anhydrase betaCA1	0.8194	0.0032
30	At3g11630	2-Cys peroxiredoxin (2-Cys PrxA)	0.7797	0.0574
31	At4g24280	Chloroplast heat shock protein 70-1	0.7579	0.0292
32	At3g09440	Heat shock 70 kDa protein 3	0.7367	0.0163
33	At1g01090	Pyruvate dehydrogenase E1 alpha subunit	0.7242	0.0592
34	At3g01500	beta carbonic anhydrase 1	0.7216	0.0293
35	At3g50820	Oxygen envolving complex subunit 33 Kda	0.7202	0.0355
36	At3g15360	Thioredoxin M-type 4. TRX-M4	0.7151	0.0401
37	At5g39570	Uncharacterized protein	0.6994	0.0310
38	At5g25980	Beta glucosidase 37	0.6862	0.0012
39	At4g01850	S-adenosylmethionine synthase 2	0.6586	0.0493

40	At2g39730	Rubisco activase	0.6551	0.0580
41	At1g68010	NADH-dependent hydroxypyruvate reductase 1	0.6392	0.0456
42	At2g38230	Pyridoxine biosynthesis 1	0.6208	0.0107
43	At5g06290	2-Cys peroxiredoxin (2-Cys Prx B)	0.5735	0.0547
44	At3g63540	Thylakoid lumenal 19 kDa protein	0.5616	0.0337
45	At5g53490	Thylakoid lumenal 17.4 kDa protein	0.5429	0.0195
46	At1g54270	Member of eIF4A - eukaryotic initiation factor 4A	0.5140	0.0441
47	At5g52310	Cold regulated 78 / responsive to dessication 29A	0.5124	0.0197
48	At5g39570	Uncharacterized protein	0.3539	0.0469
49	At5g17920	Methionine synthesis 1	0.3509	0.0051
50	At3g52960	Thioredoxin superfamily protein	0.0886	0.0030
51	At1g31180	Isopropylmalate dehydrogenase 3	ND[b]	< 0.001
52	At1g78330	Glutathione S-Transferase Tau 19	ND[b]	< 0.001
53	At3g50820	Oxygen envolving complex subunit 33 Kda	ND[b]	< 0.001
54	At1g53850	20S Proteasome alpha subinit E1	ND[b]	0.0069
55	At2g34430	Light-harvesting chlorophyyll-protein complex II subunit b1	ND[b]	0.0072
56	At3g26650	Glyceraldehyde 3- phosphate dehydrogenase subunit a	ND[b]	0.0252
57	At1g67090	Ribulosebiphosphate carboxylase samall chain 1a	ND[b]	0.0254
58	At2g34430	Light-harvesting chlorophyyll-protein complex II subunit b1	ND[b]	0.0396
End of Night				
61	At1g32470	Glycine cleavage system H protein 3. mitochondrial	ND[a]	< 0.001
62	At5g63400	Adenylate kinase 4	ND[a]	0.002757882
63	At4g38970	Probable fructose-bisphosphate aldolase 2. chloroplastic	ND[a]	< 0.001
64	At5g14200	3-isopropylmalate dehydrogenase 1. chloroplastic	ND[a]	< 0.001
65	At1g51980	Probable mitochondrial-processing peptidase subunit alpha-1	ND[a]	< 0.001
66	At5g11670	NADP-dependent malic enzyme 2	ND[a]	< 0.001
67	At3g45140	Lipoxygenase 2. Chloroplastic	ND[a]	< 0.001
68	At1g29930	Chlorophyll a-b binding protein 1. chloroplastic	ND[a]	< 0.001
69	AtCg00490	Ribulose bisphosphate carboxylase large chain	3.7305	< 0.001
70	At4g13930	Serine hydroxymethyltransferase 4	3.1073	0.002116139
71	At4g04640	ATP synthase gamma chain 1. chloroplastic	3.0485	0.000185067
72	AtCg00490	Ribulose bisphosphate carboxylase large chain	2.111	0.002139099
73	At5g14780	Formate dehydrogenase. Mitochondrial	1.796	0.000638415
74	At3g45140	Lipoxygenase 2. Chloroplastic	1.7279	0.001603881
75	At5g39570	Uncharacterized protein	1.6958	0.000211135
76	At5g37600	Glutamine synthetase cytosolic isozyme 1-1	1.6922	0.000164
77	At4g02520	Glutathione S-transferase F2	1.5208	0.000530939
78	At2g44350	Citrate synthase 4. Mitochondrial	1.36	9.91E-06
79	At3g48870	Chaperone protein ClpC2. chloroplastic	1.2211	0.000394181
80	At1g32470	Glycine cleavage system H protein 3. mitochondrial	1.1909	0.001854308
81	At2g33210	Chaperonin CPN60-like 1. mitochondrial	1.1839	3.25E-05
82	At1g21750	Protein disulfide isomerase-like 1-1	1.17	5.31E-06
83	At1g20020	Ferredoxin--NADP reductase. leaf isozyme 2. chloroplastic	1.1479	0.000504754
84	At2g37660	Uncharacterized protein At2g37660. chloroplastic	0.8234	0.001054948
85	At5g43940	Alcohol dehydrogenase class-3	0.7418	0.000351807
86	At5g27380	Glutathione synthetase. Chloroplastic	0.7312	1.58E-05
87	At5g24780	Vegetative storage protein 1	0.5834	0.000176055
89	At1g02930	Glutathione S-transferase F6	0.5808	2.06E-05
90	At5g38420	Ribulose bisphosphate carboxylase small chain 2B. chloroplastic	0.5295	0.002254416
91	At1g42970	Glyceraldehyde-3-phosphate dehydrogenase GAPB. Chloroplastic	0.4115	0.001024379
92	AtCg00490	Ribulose bisphosphate carboxylase large chain	0.3387	0.001005402
94	At5g66190	Ferredoxin--NADP reductase. leaf isozyme 1. chloroplastic	ND[b]	< 0.001
95	At5g27380	Glutathione synthetase. Chloroplastic	ND[b]	< 0.001

Figure legend

Figure 1.Schematic representation of lysine biosynthesis and degradation. Lysine is synthezised in chloroplast using aspartate and pyruvate as precursor. DHDPS is the first enzyme to lysine biosynthesis which it is required pyruvate export from cytosol to chloroplast. End point of lysine catabolism is on mitochondria. Lysine degrataion is carried out by two enzymes LSR/SDH or D-2HGDH generating either acetyl-CoA or 2-oxglutarate, respectively, in which feed the TCA cycle (dashed arrows). Lysine function is also as electron donor to support ATP sysnthesis through ETF / ETFQO. In summary, this schematic representation shows a close relationship between chloroplast and mitochondria on lysine metabolism. DAPAT enzyme is highlighted in red represent mutation point in Arabidopsis mutant used in this study. DHDPS, dihydrodipicolinate synthase; LKR/SDH, lysine-ketoglutarate reductase/ saccharopine dehydrogenase; D-2HGDH, 2-hydroxyglutarate dehydrogenase; ETF/ETFQO, electron transfer flavoprotein/electron transfer flavoprotein:-ubiquinone oxidoreductase. Enzymes related to biosynthesis of other amino acids. CGS, cystathionine gamm-synthase; TS, threonine synthase; TD, treonine deaminase.

Figure 2. Differences between wild type (WT) and *dapat* phenotypes. (A) *Arabidopsis thaliana* grown up on short day as described in materials and methods. (B) Arabidopsis thaliana grown up on 12 hours light and 12 hours dark photoperiod and 150 μmols $m^{-2}{\cdot}s^{-1}$ during 4 weeks. Indeed this growth condition results bigger plants, but *dapat* plants show a differential morphologic when compare with wild type plants. (C) WT and *dapat* seeds germination assay. Lysine deficiency on dapat mutant resulting in a delay germination of mutant seeds as reported by Angelovici et al (2010) using other loss-of-function lysine mutant seeds. (D) *Arabidopsis thaliana* seedlings of WT and *dapat* of 5 days-old after germination start. While WT resulted in all seed germinated, a few number of *dapat* seed was unable to germinate.

Figure 3. Changes on gas-exchange performance measurements in wild type (WT) and mutant (dapat). (A) Photosynthesis. (B) Stomatal conductance to water vapor. (C) Internal CO_2 concentration. (D) Dark respiration. Bars are means ± SE from five biological replicates, asterisk (**) designate that significantly differ ($p < 0.01$) by the t-test.

Figure 4. Metabolites variation along diurnal cycle. Metabolites were mesuared using all rossete of Arabidopsis of 4 weeks-old. Plants were harvest in two points: End of night (EN) and End of day (ED) time. (A) Starch. (B) Sucrose. (C) Glucose. (D) Fructose. (E) Protein. (F) Amino acids. Values are average of five independent biological replicates. Asterisk (*) and (**) designate that significantly differ ($P < 0.05$) or ($P < 0.01$) by the t-test, respectively.

Figure 5. Effect of deficiency on lysine biosynthesis along diurnal cycle on malate (A) and fumarate (B) level. Values are average of five independent biological replicates. Asterisk (**) designate that significantly differ ($P < 0.01$) by the t-test.

Figure 6. Metabolic profiling of *dapat* mutant along diurnal cycle: (ED) End of day and (EN) end of night. (A) Amino acids. (B) other metabolites. Values are average of four independent biological replicates. Color squares designate that significantly differ ($p< 0.05$) by the t-test. Metabolites in red were up-regulated and blue were down-regulated.

Figure 7. 2D gel maps of rossete of Arabidopsis in two points: (A) End of day and (B) End of night (ED) time. Proteins were extracted by phenol follow methanol/ammonium acetate precipitation and separated by IEF/SDS-PAGE then stained with CBB-250G (see material and methods). An equal amounte (850μg) of total protein was load on each gel strip and resolved of 18 cm length in 4-7 pH linear gradient range. Spots highlighted designate that significantly differ ($p< 0.05$) by the t-test. Red and blue numbers shows up-regulated and down-regulated spots, repectively.

Figure 1.

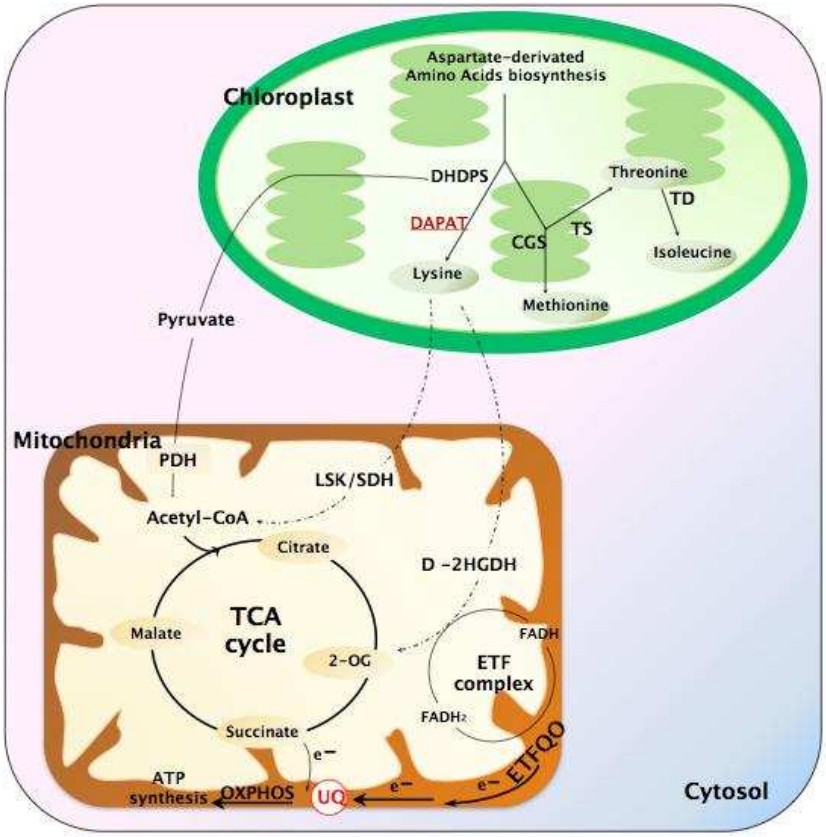


Figure 2.

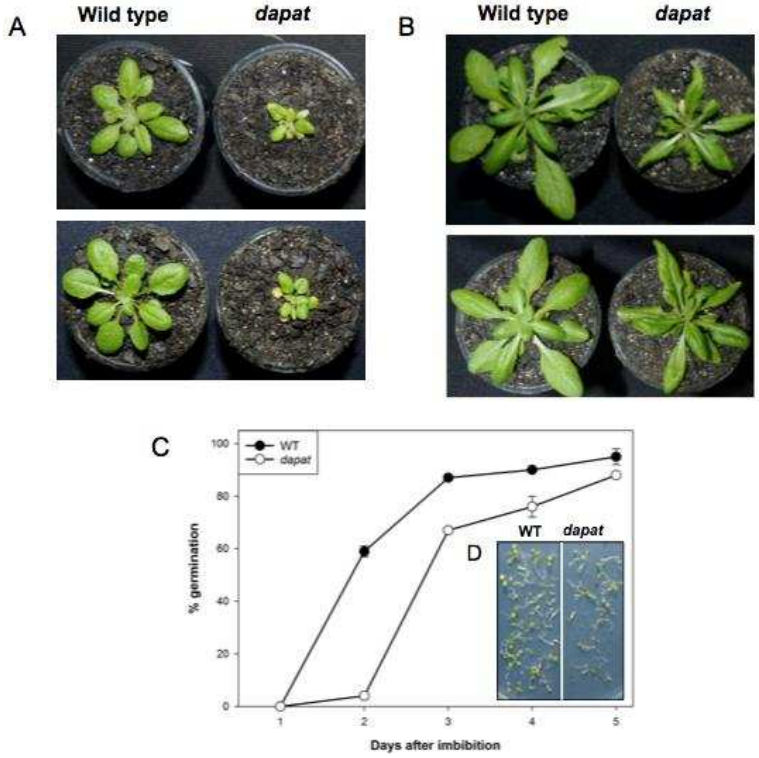


Figure 3.

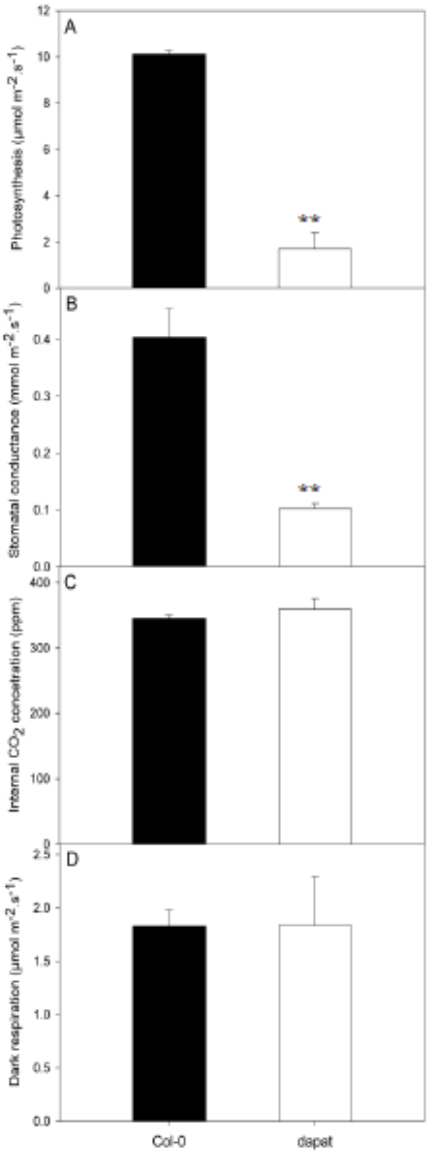

A
B
C
D
Photosynthesis (µmol m-2 s-1)
Stomatal conductance (mmol m-2 s-1)
Internal CO2 concetration (ppm)
Dark respiration (µmol m-2 s-1)
**
**
Col-0
dapat

Figure 4.

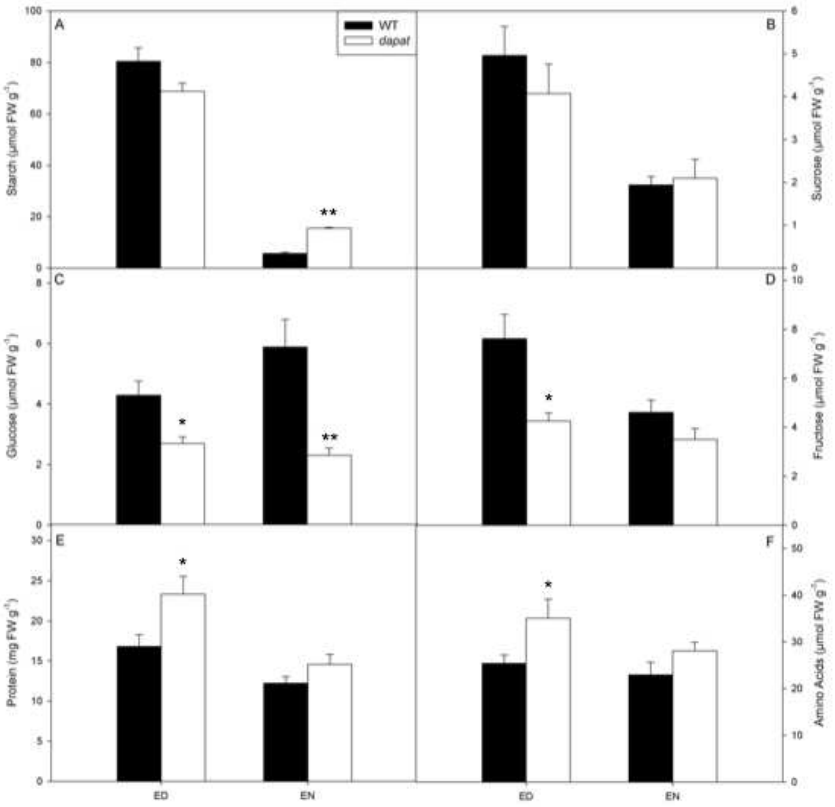


Figure 5.

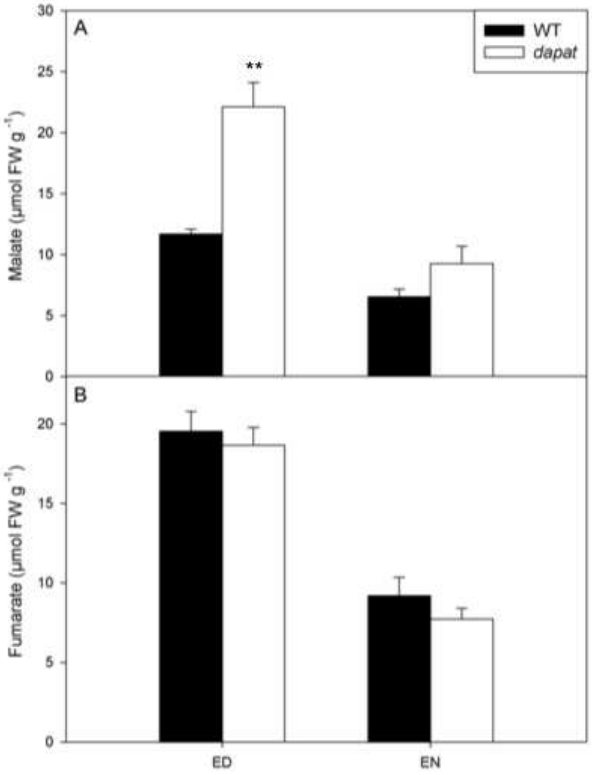
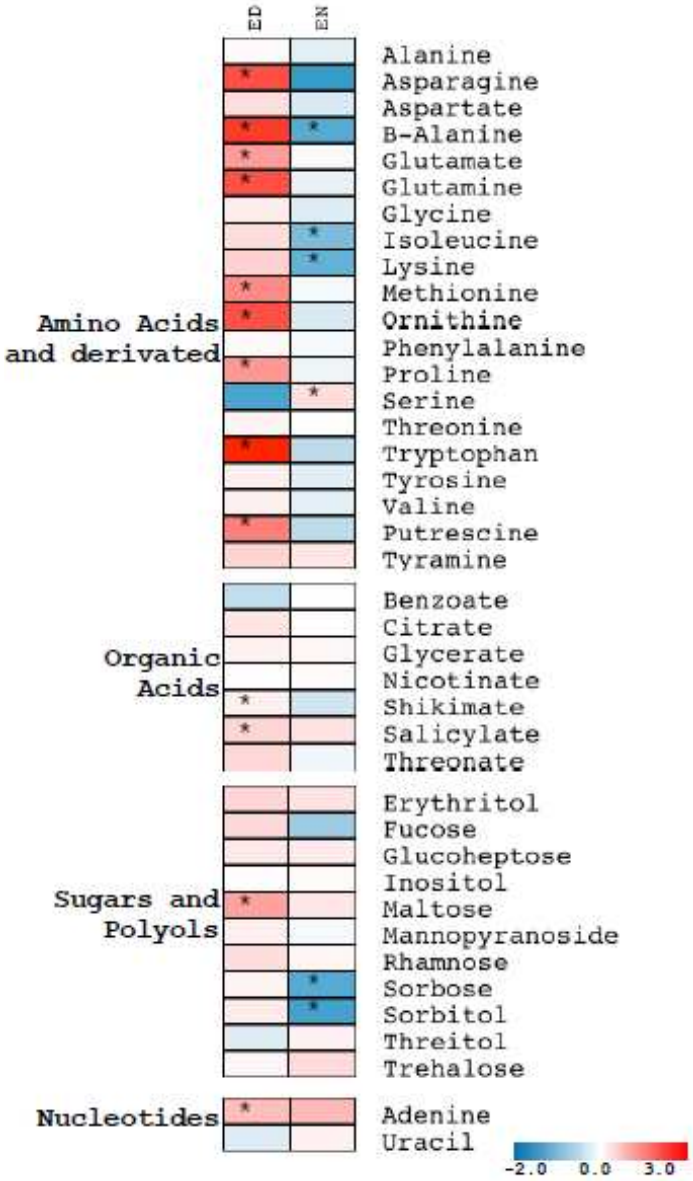


Figure 6.


ED
EN
Alanine
Asparagine
Aspartate
B-Alanine
Glutamate
Glutamine
Glycine
Isoleucine
Lysine
Methionine
Ornithine
Phenylalanine
Proline
Serine
Threonine
Tryptophan
Tyrosine
Valine
Putrescine
Tyramine
Amino Acids and derivated
Benzoate
Citrate
Glycerate
Nicotinate
Shikimate
Salicylate
Threonate
Organic Acids
Erythritol
Fucose
Glucoheptose
Inositol
Maltose
Mannopyranoside
Rhamnose
Sorbose
Sorbitol
Threitol
Trehalose
Sugars and Polyols
Adenine
Uracil
Nucleotides
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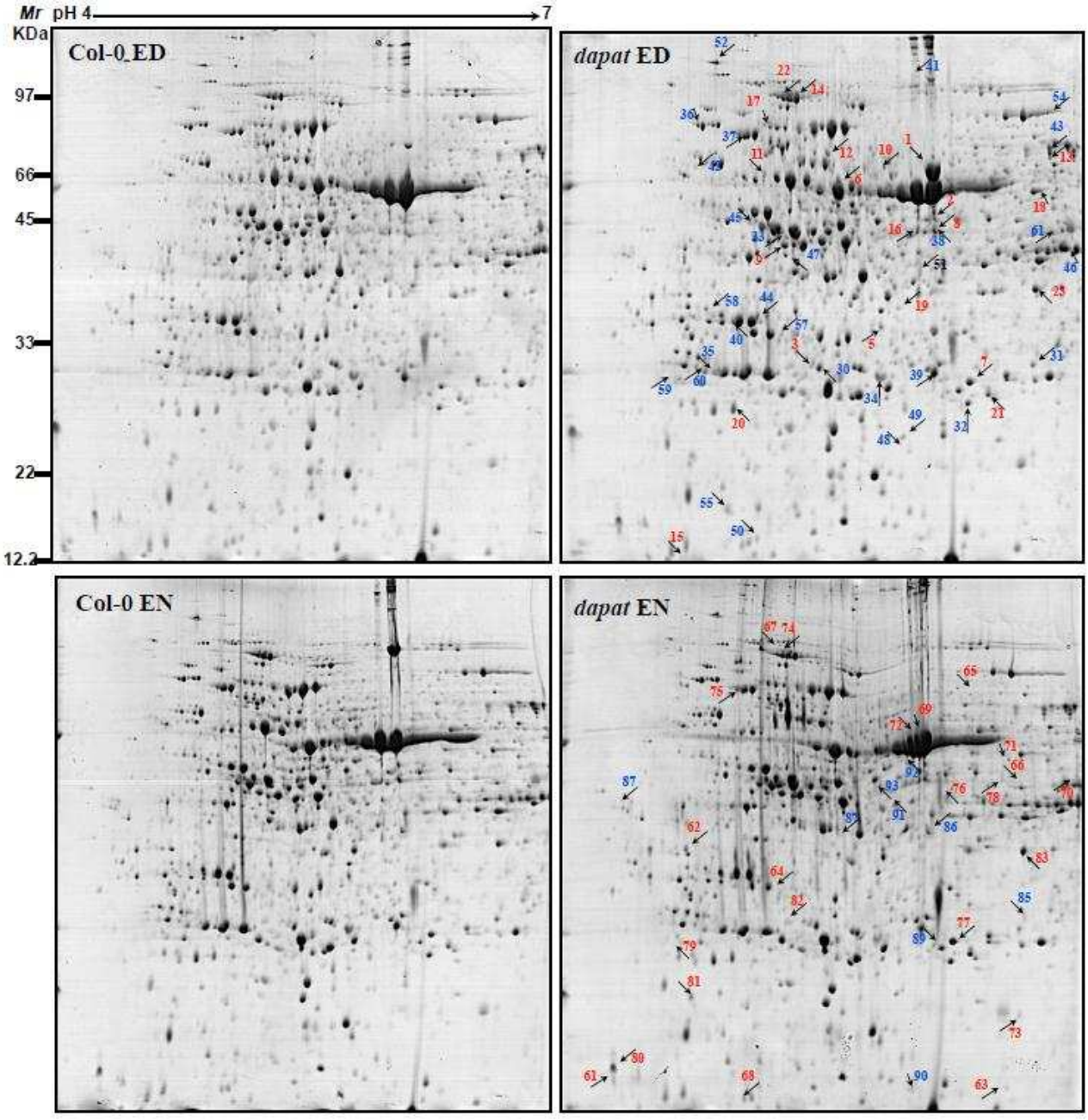
0.0
3.0

Figure 7



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Running title: Arabidopsis cell culture under carbon starvation

Title:

Amino acids show different effects on OXPHOS system activity under carbon starvation in Arabidopsis cell suspensions

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Abstract

Plant respiration is mostly dependent on carbohydrate to synthesize ATP. However, during stress situation plant cells also use amino acids as alternative substrates to donate electrons via the ETF/ETFQO complex to the mitochondrial electron transport chain (mETC). Given to this, here we investigated changes on oxidative phosphorylation (OXPHOS) system in Arabidopsis thaliana cell culture under carbon (C) starvation supplied with a range of amino acids. Induction of isovaleryl-CoA dehydrogenase (IVDH) activity was observed under carbon starvation which was associated with increased amounts of IVDH protein detected by immunoblotting. Furthermore, activities of the protein complexes of the mETC were reduced under carbon starvation. We also observed that OXPHOS system activity behavior is differently affected by different amino acids. Collectively, our results support the contention that ETF/ETFQO is an essential pathway to donate electrons to the mETC and that amino acids are alternative substrates to maintain respiration under C starvation.

Keywords: Alternative respiration; amino acids catabolism; energy deprivation; mitochondria; stress response

Introduction

The primary role of the mitochondrion in the plant cell, similarly in all eukaryotic cells, is to synthesize ATP through cellular respiration (Millar et al., 2005), reducing equivalents and metabolic intermediates for use in biosynthetic pathways elsewhere within the cell, which is well established (Araújo et al, 2012; Huang et al., 2013). It should be mentioned however that the importance of mitochondrial function in the physiology and development of higher plants is also demonstrated by the fact that mutations in several genes of the mitochondrial genome frequently lead to cytoplasmic male sterility (Chase, 2007) and by the association of plant mitochondria in programed cell death (Yao et al., 2004; Reape et al., 2008). In fact, plants exhibit several unique features within respiratory metabolism, including multiple entry points from sucrose to starch, the duplication of pyrophosphate and ATP-dependent phosphorylation of fructose-6-phosphate, complementation between cytosol, plastid and mitochondria, the loss of regulation of glycolysis by kinetic effects of ATP on phosphofructokinase and pyruvate kinase reactions, and the presence of non-phosphorylating transport systems and alternative pathways of respiration (Fernie et al., 2004; Millar et al., 2011). It is important to note that the main components of plant respiration, including enzymes of the tricarboxylic acid (TCA) cycle and the proteins constituents of the mitochondrial electron transport chain (mETC), have been identified and characterized in several plant species (Millar et al., 2011; Araújo et al., 2012). However, our understanding of the regulation of the responses of both classical and alternative pathways of plant respiration in response to changing environmental conditions and developmental programs is still limited.

Although it has been recently demonstrated that the oxidation of alternative substrates is of high importance during several stress situations which affects carbohydrate supply (Araújo et al., 2010, Araújo et al., 2011, Engqvist et al., 2011, Grüssel et al., 2014), plant respiration is mostly dependent on carbohydrate oxidation (Paxton and Podesta, 2006). Carbohydrate oxidation inside the mitochondria is carried out by two distinct but coupled pathways which are: (*i*) the TCA cycle which generatesreductant power such as NADH and $FADH_2$ and (*ii*) the mitochondrial oxidative phosphorylation (OXPHOS) system which covers the mETC, consisting of four large protein complexes, commonly referred to as complex I to IV. The electron transfer through these complexes is tightly coupled to the ATP synthase (Complex V) and use the electron donates by NADH and $FADH_2$ to phosphorylate ADP to ATP (Millar et al., 2005; Dudkina et al., 2006; Millar et al., 2011). It is important to mention, however, that under some circumstances (e.g., natural senescence or environment stresses) carbohydrate levels are reduced forcing the plant cells to use alternative substrates to support ATP synthesis (Araújo et al., 2011). In this scenario, it has been demonstrated that both isovaleryl-CoA dehydrogenase and (*D*)-2-hydroxyglutarate dehydrogenase

provide electrons to the plant ubiquinol pool via the electron transfer flavoprotein (ETF)-ETF:ubiquinone oxidoreductase (ETF/ETFQO) complex (Ishizaki et al., 2005; Araújo et al., 2010). As such, protein degradation can be an important source of alternative substrates for respiratory metabolism being directly connected with energy supply for plant survival especially under sub-optimal conditionsmaintaining mitochondrial energy metabolism active (Ishizaki et al., 2005; Araújo et al., 2012).

It seems clear that when respiration uses alternative substrates some features commonly observed are distinct from those of the classical respiration, which use carbohydrate as substrate. For instance, the type II NAD(P)H dehydrogenases and the alternative oxidase, which are energy bypass proteins, circumvent the proton pumping protein complexes I (the rotenone-inhibited NADH dehydrogenase) and III and IV (mediating the cytochrome pathway of oxygen consumption), thereby uncoupling electron transport from ATP synthesis. Moreover, pathways linking branched chain amino acids (BCAA) and lysine degradation to electron donation to the mETC via the action of alternative dehydrogenases have been identified in land plants. During the action of the alternative dehydrogenases electronsare donated directly to the ETF/ETFQO and after transferred to the ubiquinone pool without passing by the Complexes I and II (Araújo et al., 2010; Kleessen et al., 2012). Additionally, at least in mammals the presence of 11 dehydrogenases able to donate electrons to this alternative pathway has been demonstrated (Beckmann and Frerman, 1985). In plants however, only two major dehydrogenases - isovaleryl-CoA dehydrogenase and (*D*)-2-hydroxyglutarate dehydrogenase - that oxidize BCAA (isoleucine, leucine and valine) and lysine, respectively, have been characterized to date (Enqvist et al., 2009; Araújo et al., 2010). Nevertheless, by using a combination of genetic, biochemical, and molecular approaches Ehylmalonic encephalopathy protein 1 (ETHE1) has been further identified as another enzyme able oxidize cysteine and methionine connecting it to the ETF/ETFQO system (Grußel et al., 2014). Moreover, proline dehydrogenase, which is located in the inner mitochondrial membrane, has been suggested to possibly donate electrons to mETC (Schertl et al., 2014). Collectively, these findings suggest that the ETF/ETFQO complex, similarly to the situation observed in mammalian, can be regarded as a branch of the mETC. Notwithstanding, there is a current lack of information concerning the identification of alternative dehydrogenases able to link alternative substrates to the ETF/ETFQO system.

By contrast to the situation in mammals, in which fatty acid and proteins are important respiratory substrates, with the exception of certain specialized cells, plant respiration is primarily dependent on carbohydrate oxidation (Paxton and Podesta, 2006). This fact notwithstanding, under certains situations, when carbohydrate supply is limited, the plant cell metabolism is modified and required enzymes are active to degraded alternative respiratory substrates (Araújo et al., 2011;

Obata and Fernie, 2012). In fact, it has been demonstrated that a range of environmental stress are able to induce the oxidation of alternative substrates for plant respiration (Zhou et al., 2009; Karvchik and Bernstein, 2013). In plants, degradation of amino acids for maintenance of respiration has been shown to be important during carbon deprivation (Araújo et al., 2010) and metabolite analysis of the mitochondrial composition following oxidative stress suggested that by increasing amino acid catabolism plants might compensate for a reduced electron supply from the TCA cycle (Obata et al., 2011).The exact role of these alternative pathways during stress situations has not been fully confirmed yet, but it is generally associated to its ability to decrease the production of reactive oxygen species in mitochondria, mainly through the alternative oxidase (Maxwell et al., 1999; Fiorani et al., 2005: Xu et al., 2011). Notably not only ETF/ETFQO enzymes (see above) but also other alternative enzymes, mainly IVDH, have been demonstrated to be up-regulated under a range of environmental stresses (Zhou et al., 2009; Karvchik and Bernstein, 2013). We are aware that plant metabolism is highly reorganized under a range of different stress conditions including salt, cold, drought and oxidative stress (Kaplan et al., 2004; Gong et al., 2005; Kaplan et al., 2007; Sanchez et al., 2012; Siahpoosh et al., 2012; Schulz et al., 2015), allowing plants to continue to produce indispensable metabolites whilst preventing the accumulation of reactive oxygen species. Thus, we here postulate that the functionally translated portion of the genome plays an essential role in plant stress, and therefore extended proteomic studies can provide a finer picture of protein networks involved in metabolic pathways important for cellular detoxification and tolerance mechanisms. Bearing this in mind, studies aiming to better understand the role of alternative substrates for electron supply to the mETC under carbon limitations were performed. For this reason and in an attempt to elucidate the physiological role of the enzymes of the alternative respiration and how exactly the OXPHOS system is reorganized following carbon limitation a proteomic, metabolic and physiological approach was undertaken. To this end, we carried out a range of experiments using Arabidopsis cell culture following short carbon starvation and supplied with different amino acids as alternative substrates. Our preliminary results demonstrated an opposite behavior among members of OXPHOS system and provide evidence that changes in amino acids sources are critical for a metabolic adaptation of Arabidopsis cells, especially for the mETC. The results obtained are discussed in the context of our current understanding of the transient reconfiguration of metabolism following stress situations.

Material and Methods

Arabidopsis thaliana cell suspension and carbon starvation stress

All experiments were carried out using *Arabidopsis thaliana* from the Columbia ecotype (Col-0). Establishment and maintaining of the cell culture was carried out as described in Sunderhaus et al. (2006). Briefly, *A. thaliana* seeds were surface sterilized and imbibed for 2 days at 4 °C in the dark on 0.8% (*w*/*v*) agar plates containing half-strength Murashige and Skoog (MS) media (pH 5.7). Seeds were subsequently germinated under short-day conditions (8 h light/16 h dark) with 100 µmol m^{-2} s^{-1}. After 10 days, seedlings were cutted in small pieces (3 cm^2) and laid out on plates with B5 media (pH 5.7) containing 3% sucrose (*w*/*v*), 0.0001% (*w*/*v*) 2,4-dichloropenoxyacetic acid and 0.00001% (*w*/*v*) kinetin. The B5 plates were stored in darkness for 3 weeks and then cells were transferred to B5 liquid media as described above. To maintain the cells in optimal conditions, the medium was renovated once a week.

Carbon starvation was induced by removing sucrose from the media and supplying it with a range of amino acids for 24 h. To this end, 6 different treatment were carried out: control (as already described above), Proline (exactly as in Schertl el al. (2014), which was used as apositive control containing 3% sucrose plus 50 mMProline), no sucrose, BCAA (without sucrose plus 10 mM BCAA; 10 mM each: isoleucine, leucine and valine), Lysine (without sucrose plus 30 mM lysine) and Arginine (without sucrose plus 30 mM arginine).

Mitochondria isolation by ultracentrifugation in Percoll gradient

Mitochondria isolation was performed according Schertl et al. (2014). Arabidopsis cells were disrupted in a Waring blender in buffer containing 450 mM sucrose, 15 mM3-(*N*-morpholino) propanesulfonic acid (MOPS), 1.5 mM EGTA, 0.6 (*w*/*v*) polyvinylpyrrolidone (PVP-40), 0.2% bovine serum albumin (BSA), 0.2% phenylmethylsulfonyl fluoride (PMSF), pH 7.4 (KOH).To remove cell debris the suspension was centrifuged twice for 5 min at 2700 g and once for 10 min at 83000 g. Afterwards, the supernatant containing the mitochondrial fraction was transferred to a new tube for a high speed centrifugation for 10 min at 17000 g. The mitochondria pellet was resuspended in 3 mL of washing buffer containing 300 mM sucrose, 10 mM MOPS, 1 mM EGTA, 0.2 mM PMSF, pH 7.2(KOH). The mitochondria suspension was carefully placed on Percoll gradients and centrifuged for 90 min at 70000 *g* using an ultracentrifuge. After centrifugation mitochondria band was collected into a new tube using a Pasteur pipette. The mitochondria fraction was washed three times in resuspension buffer containing 400 mM mannitol, 10 mMTricine, 1 mM EGTA, 0.2 mM PMSF, pH 7.2 (KOH) at 14300 *g* for 10 min. All the steps were carried out at 4 °C. Isolated mitochondria was aliquotedand stored at -80 °C.

Measurement of mitochondrial enzyme activity

All the assays were carried out in final volume of 300 µLat 25 °Cusing an Epoch Microplate Spectrophotometer (Biotech, Winooski, VT, USA). The mitochondrial suspension stored at -80 °C was used to quantify mitochondrial enzyme activity. First, mitochondrial suspension was thawed and then centrifuged for 10 min at 13500 rpm. The supernatant (soluble fraction) was recovered in a new microtube and protein quantification was carried in both fractionsby Bradford method. IVDH activitywas monitored by following the reduction of dichlorophenolindophenol DCIP (E = 19.1 mM^{-1} cm^{-1}) at 600 nm. The reaction mixture contained 250 mM Tris-HCl pH 7.5, 1 mM KCN, 166 µM FAD, 0.06 mM DCIP, 1 mM PMS and20 µg of soluble mitochondrial proteins. The reaction was started by adding 67 µM Acyl-CoA.

To determineOXPHOS system activities we used membrane protein. Complex I activity was measured according Zhou et al. (2003) and Singer (1974) in an assay mixture containing3 µg of membrane protein, 50 mM Tris-HCl pH 7.4, 0.5 mM $K_3Fe(CN)_6$ and 0.2 mM NADH). The reaction was started by adding the protein bonding membrane and the reduction of $K_3Fe(CN)_6$ (E = 1 mM^{-1} cm^{-1}) was monitored at 420 nm.

Complex II was measured according Birch-Machin et al. (1994). For this assay, 15 µg of membrane protein was added to (50 mMTris-HCl pH 7.4, 5 mM $MgCl_2$, 20 mM succinate, 0.3 mM ATP, 0.5 mM SHAM, 100 µM decylubiquinone (oxidased), 2 mM KCN. The reaction was started with 50 µM DCIP and the activity of Complex II was monitored by the reduction of DCIP (E = 19.1 mM^{-1} cm^{-1}) at 600 nm.

Complex IV activity was carried out according Birch-Machin et al. (1994). The assay mixture contained50 mM KH_2PO_4 pH 7.4, 15 µM cytochrome c (reduced with sodium dithionite) and 0.3 mMdodecylmatoside. Protein (2 µg of membrane proteins) was used as a start and oxidation of cytochrome c (E = 19 mM^{-1} cm^{-1}) was monitored at 550 nm.

SDS-PAGE

One-dimensional SDS page was carried out exactly as in Laemmli (1970). Mitochondria aliquots kept at -80 °C were used to carry out SDS-PAGE to separed protein by molecular mass. Before gel loading, mitochondria aliquot was centrifuged for 10 min at 17000 g at 4 °C. Afterwards, soluble and membranefractions were transferred to new tubes. Mitochondrial proteins were quantified as in Bradford (1976). Samples (10 µg) were mixed with loading buffer containing 8% (*w*/*l*) SDS, 250 mM Tris-HCl, pH 6.8, 40% (*v*/*l*) glycerol, 0.002% (*w*/*l*) bromophenol blue, mercaptoethanol and 0.05% (*w*/*l*) DTT and then incubaded by 5 min at 70 °C before application in pre-cast (8 x 8 cm) Tris-glycine SDS-PAGE (Life technology, USA).The running conditions were

as following: 125 V, 30 mA by about 2 h in glycine buffer (0.248 M Tris; 1.92 M Glycine; 1% (*w*/*l*) SDS; pH not adjusted).

Immunoblotting

Proteins separated in polyacrylamide gels were, immediately, transferred to nitrocellulose membrane using a Semi-Dry Transfer system (Bio-Rad, USA). The transfer of proteins was carried out according Schertl et al. (2014) at 12 V (max), 400 mA for about 90 min using transfer buffer (25mM Tris, 192 mM glycine, 1.3 mM SDS, 20% Methanol). The blots were rinsed several timers in TTBS buffer (0.1 M Tris-HCl pH 7.5, 0.155 M NaCl and 0.01% (*v*/*v*) Tween 20) to remove all methanol and then incubated with 1: 1000 primary antibody dilution in TTBS buffer for about 16 h at room temperature. Antibodies for OXPHOS subunits are described in Peters et al. (2012) which are the 51 KDa subunit (Complex I), SDH1-1 (Complex II), alpha-MPP (Complex III), COX2 (Complex IV) and ß-subunit (Complex V).Proline dehydrogenase (ProDH) and IVDH antibodies were described in Schertl et al. (2014) and Gu et al. (2010), respectively.ETFQO antibody was used following technical instruction of Proteintech, USA. Subsequently, membranes were incubated for 2 h with a 1: 5000 diluted secondary antibody directly coupled to the horseradish peroxidase. All blottes were detected using the ECL prime chemiluminescence detection kit from GE Healthcare (Munich, Germany).

Statistical analysis

Data were submitted to analysis of variance and tested for significant ($P < 0.05$) differences using Student's *t* tests. All the statistical analyses were performed using the algorithm embedded into Microsoft Excel®.

Results

Isovaleryl-CoA dehydrogenase is induced following carbon starvation

To investigate the behavior of alternative enzymes of respiration following carbon starvation we first analyzed the activity of IVDH, which was differently affected by our treatments(Figure 1A). As expected, the strongest induction of IVDH activity was observed in absence of sucrose (-Suc) and in presence of BCAA (-Suc+ BCAA) which culminated with increments of 2.2 and 1.8-fold, respectively, in IVDH activity (Figure 1A). Both treatments in presence of sucrose (+Suc and +Suc+Proline) were characterized by a basal IVDH activity, suggesting that proline is not directly linked with the electron donation to the mETC through the IVDH enzyme. IVDH activity at –Suc+Lys shower similar level with treatments in presence of Sucrose since lysine is catabolised by D2HGDH.

To investigate whether the changes in IVDH activity were caused by enzyme activation and/or *de novo* synthesis of IVDH we carried out an immunoblotting experiment. Mitochondrial fractions were separated by SDS/PAGE, transferred to nitrocellulose membranes by electroblotting and probed using an antibody directed against IVDH which showed immunopositive bands at about 42 kDa. In good agreement with the enzymatic activity assay, we further demonstrated that IVDH was detected in mitochondrial fractions from all treatments and more importantly that enhanced levels of IVDH protein were observed only in -Suc and -Suc+ BCAA treatments (Figure 1B).

OXPHOS system shows a different behavior under carbon starvation when suppied with a range of amino acids

To investigate whether the cultivation of Arabidopsis cells following carbon starvation could culminate in changes on the activity of complexes of the mETC as well as in dehydrogenases located on the inner mitochondrial membrane we analyzed the activity of Complex I, II, and IV. Interestingly, carbon starvation resulted in an opposite effect on OXPHOS system activity in which we could observe that while the activities of Complex I and II were reduced the activity of Complex IV was enhanced following carbon limitation (Figure 2). We observed thatin absence of Sucrose there was a tendency to diminish Complex I activity, although this statistically, significant only for –Suc, -Suc+BCAA, and -Suc+Arginine in comparison to our control (+Suc; Figure 2A). We also observed a similar behavior for the Complex II activity although only the -Suc+BCAA treatment differed significantly to the control treatment (Figure 2B). It should be mentioned that in both cases the -Suc+BCAA treatment presented the lowest activity. By contrast to the situation observed for Complex I and II, the activity of Complex IV was higher than the control in all treatments(significantly only in -Suc+BCAA and -Suc+Lysine; Figure 2C). Given the changes

observed in their activity we next investigated whether it was correlated with protein amount. To this end, immunoblotting were carried out to explain the possible regulation of OXPHOS complexes by using specific antibodies against some subunits of the respiratory complexes (Peters et al., 2012). Surprisingly, no changes on the amounts of the subunits tested were observed (Figure 3A-3D), indicating that the changes observed in enzyme activity (Figure 2) were most likely associated with post translational mechanisms.

We next evaluated the expression pattern of other two mitochondrial membrane e enzymes by immunoblotting (Figure 4). The inner mitochondrial membrane enzyme ETFQO, which accepts electrons from alternative dehydrogenase such as IVDH and donate them to the ubiquinone pool, did not showed significant changes in protein level following our treatments (Figure 4A). Additionally, immunoblotting using the antibody directed against the Arabidopsis proline dehydrogenase (ProDH) was carried out and immunopositive bands were observed alltreatments less in +Suc (Figure 4B). Intriguingly, although proline treatment (+Suc+Proline) induces ProDH, in accordance with previous results (Schertl et al., 2014), this activation on protein level observed was higher in the –Suc+ BCAA treatment.

DISCUSSION

During the last decade we have witnessed an increase on our knowledge concerning the key function on amino acid sensing and the importance it has on virtually every aspect of plant metabolism (Galili et al., 2011; Kirma et al., 2012; Galili et al., 2014). This fact notwithstanding, our current understanding of the connections between protein degradation and amino acid catabolism during carbon starvation in feeding electrons into the mETC remains fragmented.In this vein, a suitable approach to increase our knowledge concerning carbon starvation and its effect onmitochondrial metabolism in general is the use of *Arabidopsis* cell suspension. Here, we took advantage of this method by removing sucrose from the media, inducing a carbon starvation, and simultaneously supplying the media with different amino acids. This approach revealed novel insights into the metabolic acclimatatory mechanisms through which the supply of alternative substrates might promote changes to the mETC.Our results demonstrated that changes on mETC take place following carbon starvation and that those alterations are modified in function of the amino acid supplied in the medium. Collectively our results also indicate these changes are apparently associated with the alternative donation of electrons to the ubiquinone pool which is mainly mediated by dehydrogenases such as IVDH and D-2HGDH. Firstly, by measuring the activity of IVDH we could confirm that our experimental conditions were able to induce changes on mitochondrial energy metabolism (Figure 1A). As it could be expected, the two treatments

containing sucrose (+Suc; used as our control) and Proline (+Suc+Proline), which was used here as positive control (Schertl et al., 2014) displayed the lowest (basal) IVDH activity. In good agreement with previous report (Araújo et al., 2010), BCAA were able to promote the highest induction on IVDH activity followed by -Suc treatment. The highest activation of IVDH in presence ofBCAA is in accordance with a cumulative effect of both carbon starvation and the addition of the substrate for this enzyme. It should be mentioned, however, that in absence of sucrose we also observed a strong induction on IVDH activity. It is reasonable to assume that this result are most likely related to the processes of protein degradation and recycling of amino acids (Araújo et al., 2011) and thusfree BCAA released following protein degradation are used for alternative respiration. Although protein degradation might release only a relatively small amount of free amino acids it is possible that this could be enough to induce IVDH activity given that it presents a low K_m value of about 1.0 μM (Weigelt et al., 2008; Mohsen and Vockley, 2015). IVDH activity was not altered in presence of lysine even though it has been described as an electron donor to the ETF/ETFQO system (Araújo et al., 2010). Indeed, lysine catabolism has been extensively associated to mitochondrial metabolism and energy production by the action of both reductase/sacharopine dehydrogenase(LKR/SDH) or D2HGDH which provide acetyl-CoA and 2-oxoglutarate, respectively (Zhu and Galili, 2004: Enquivist et al., 2009, 2011, Galili, 2011; Kirma et al., 2012). The action of theses enzymes, whichare thought to operate in functionally similar albeit independentfrom the IVDH pathway,will also culminated with the donation of electrons to the ETF/ETFQO system (Kleessen et al., 2012). Given that lysine treatment did not culminate indifferences in Complex II activity (Figure 2B) it seems reasonable to assume that both pathways of lysine catabolism act together to support the classical mETC. Remarkably, our findings provide additional evidence corroborating the recent suggestion that alternative pathways of respiration are induced under carbon starvation (Araújo et al., 2011) and offers also strong evidence for the validation of the mathematical model for alternative substrates and dehydrogenases associated with the ETF/ETFQO system(Kleesen et al. 2012). One conspicuous feature concerning the increased IVDH activity is its correlation with the enhancement of IVDH protein amount as detected by immunoblotting assays (Figure 1B). Thus, it seems that IVDH activity must be tightly regulated by *de novo* synthesis and that carbon starvation is able to trigger IVDH transcription. In fact, extended darkness in Arabidopsis, which resulted in decrease of carbohydrate level,was already described to stimulate transcription of IVDH (Buchanan-Wollaston et al., 2005; Ishizaki et al., 2005). To the best of our knowledge this is the first demonstration of alterations in the activity of the mitochondrial complexes in plants following carbon starvation and perhaps more importantly that during carbon limitations the presence of amino acids, such as Lysine and the BCAA, can induce changes in the mitochondrial activity. Although it is well known that carbon starvation trigger

alternative respiration (Ishizaki et al., 2005, 2006; Araújo et al., 2010; Grüßel et al., 2014) our results add novel insights into the regulation of mitochondrial metabolism by showing that amino acids act differently on the mETC.

We further investigated the behavior of the mitochondrial respiratory complexes under carbon starvation and how different amino acids affect the mETC (Figure 2). First, we have observed a reduction of Complex I activity in absence of sucrose and notably that in presence of BCAA the lowest activity of this complex (Figure 2A). Carbon starvation is already shown decrease $NADH/NAD^+$ ratio which mightexplain, at least partially, a reduction of Complex I activity. In addition, we evaluated whether this reduction in the activity of the Complex I was associated with the reduction on the amount of protein. To this end, we focused on the 51 KDa subunit of the Complex I by immunoblotting assay and observed no changes in the amount of protein. The reasons for this may be twofold: (*i*) the 51 k Da subunit might not be the best target for evaluating structural changes on Complex I and thus it is likely that we need to investigate othersubunits of this complex and/or (*ii*) the 51 KDa subunit is associated with posttranslational modification (PTM) required in response to stress situations such as carbon starvation. In fact, the 51 KDa subunit was shown be a PTM target by phosphorylation (Haezlewwod et al., 2004; Braun et al., 2014). Moreover, reductions in the $NADH/NAD^+$ ratio and carbon starvation in mice (*Mus muscullus*) were described to induce PTM mainly by acetylation mediated by sirtuins (Ahn et al., 2008). It has been also recently demonstrated that in ArabidopsisPTM by acetylation are mediated by sirtuins (Finkemeier et al., 2011; König et al., 2014). In addition, components of the OXPHOS system have been suggested as potential targets of sirtuins and importantly among these targets it was shown that Complex I is strongly affected (Finkemeier et al., 2011). Our results also demonstrated that Complex II activity is clearly reduced in the presence of BCAA as the only carbon source (Figure 2B). Collectively, this results together with the activity of both IVDH and Complex I indicate that BCAA is able to promote strong alterations on the plant OXPHOS system which is clearly associated with the fact that the electrons donate by those amino acids go into the mETC by the ETF/ETFQO system directly to ubiquinone skipping Complex I and II, given that the catabolic products of the BCAAs degradations feed electrons directly into the TCA cycle.

By contrast to the situation observed for the first complexes of the mETC, Complex IV demonstratedan opposite pattern in whichenhancement of its activity in response to the imposed treatments was observed (Figure 2C). This fact notwithstanding, it should be mentioned that only BCAA and lysine had differed significantly from the control. It is reasonable to assume that under carbon starvation all the electron flow is channeled into the mECT to ATP synthesis since other alternative uncoupling dehydrogenases (e.g., alternative oxidase) might present a lower activity following carbon starvation.

In summary, we have presented compelling evidence that following carbon starvation changes in the OXPHOS system in Arabidopsis mitochondria are evident and that the responses are variable according the amino acid that is used as a carbon source during such stress condition. Interestingly, BCAA and Lysine treatment are able to differentially affect Complex I and II, although a similar response for the Complex IV was observed. Our results also demonstrated that there is a positive correlation between IVDH activity and protein amount detected by immunoblotting. Collectivelly, these data give evidences concerning OXPHOS system regulation where ETF/ETFQO is of pivotal importance to stress response.

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Figure legend

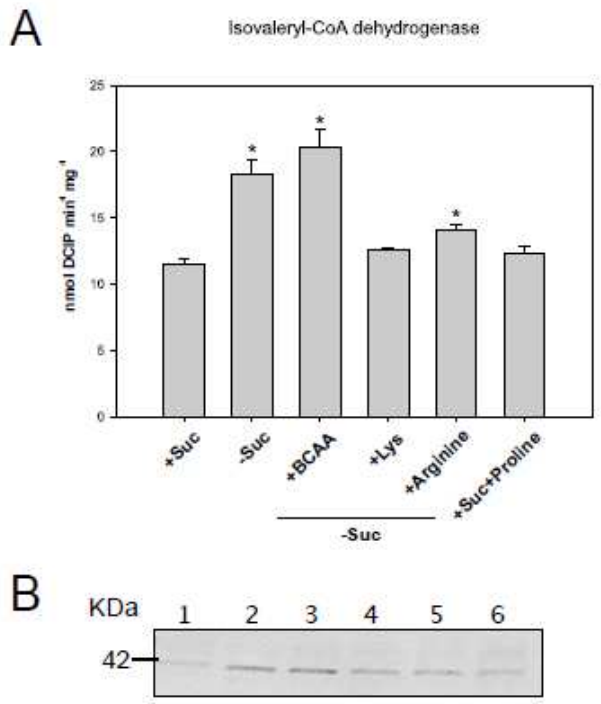
Figure 1. Induction of Isovaleryl-CoA dehydrogenase in Arabidopsis cell suspension following carbon starvation. (A) Activity of Isovalery-CoA dehydrogenase (IVDH) was measured in mitochondria isolated fromArabidopsis cells. Values are means of three biological repetitions and asterisks indicates values that were determined by the Student's *t*-test to be significantly different ($P< 0.05$) from the three biological repetitions. (B) Identification of IVDH by immunoblotting. Mitochondrial protein isolated from Arabidopsis cells were immunoblotted with IVDH antibody and the position of molecular weight markers are indicated on the left.Samples in the column are: 1) plus sucrose; 2) less sucrose; 3) less sucrose plus 10 mM BCAA; 4) less sucrose plus 30 mM Lysine; 5) less sucrose plus 30 mM Arginine; 6) sucrose plus 50 mMProline according Schertl et al., 2014.

Figure 2. Changes on OXPHOS system in Arabidopsis cell suspension under carbon starvation for 24 hours. (A) Activity of Complex I and NADH alternative dehydrogenase of Arabidopsis suspension cell grown for 24 hours under carbon starvation measured by photometric assays. (B) Activity of Complex II of Arabidopsis suspension cell grown for 24 hours under carbon starvation measured by photometric assays. (C) Activity of Complex IV of Arabidopsis suspension cell grown for 24 hours under carbon starvation measured by photometric assays Columns represents average of three biological repetitions and asterisks designate that significantly differ ($P < 0.05$) by t-test.

Figure 3. Immunoblotting of OXPHOS system subunits of Arabidopsis cell suspension under carbon starvation. Samples in the column are: 1) plus sucrose; 2) less sucrose; 3) less sucrose plus with 10 mM BCAA; 4) less sucrose plus with 30 mM Lysine; 5) less sucrose plus with 30 mM Arginine; 6) plus sucrose plus with 50 mMProline according Schertl et al., 2014.

Figure 4. Immunoblotting of ETFQO and ProDH in Arabidopsis cell suspension under carbon starvation. Samples in the column are: 1) plus sucrose; 2) less sucrose; 3) less sucrose plus with 10 mM BCAA; 4) less sucrose plus with 30 mM Lysine; 5) less sucrose plus with 30 mM Arginine; 6) plus sucrose plus with 50 mMProline according Schertl et al., 2014.

Figure 1.



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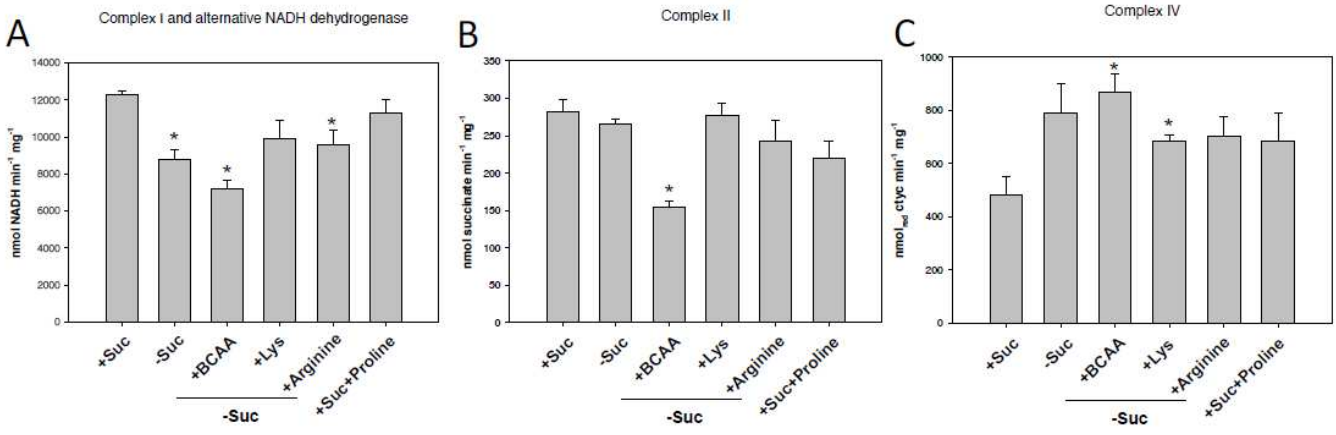
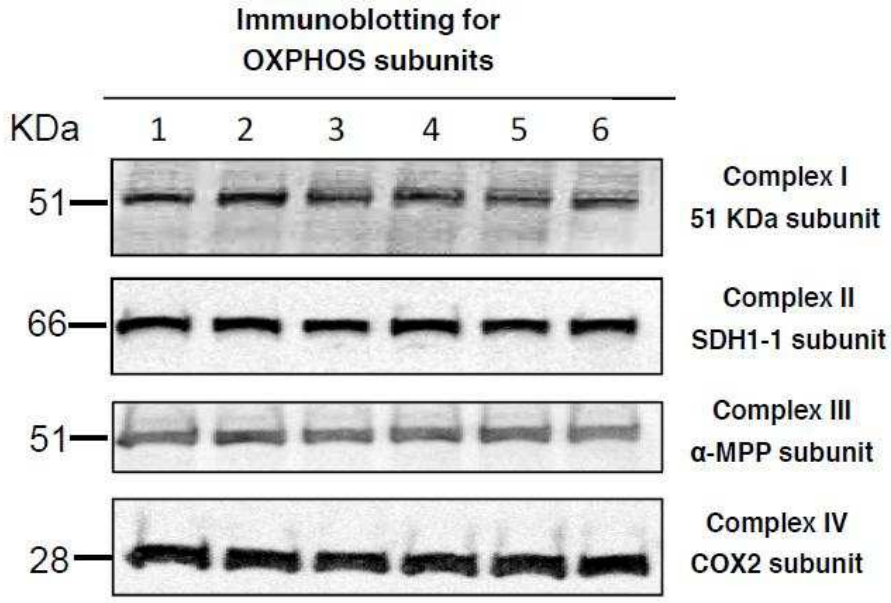


Figure 3.



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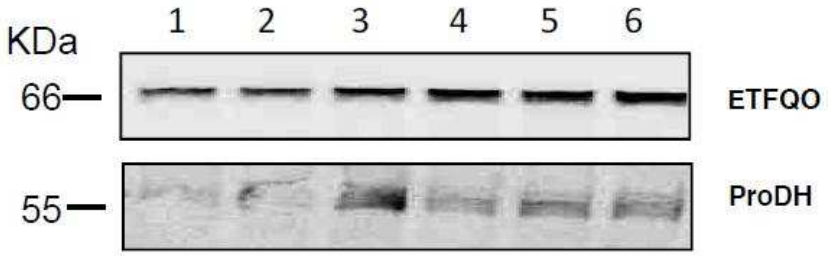


3. CONCLUDING REMARKS AND OUTLOOK

The main goal of the work presented here was to obtain a compreenhesive picture of how the TCA cycle evolved and to which extend its alternative pathways interact to adjust to different cellular and metabolic requirements. To this end different experimental approaches were used and

although each chapter has an independent discussion focusing on the results described within it, this final section discusses this thesis in its integrity and provides details of its findings in a broader context. Notably, compelling evidence is provided within this thesis that amino acids and energy metabolism are strictly associated. It should be mentioned that, although each chapter present their own conclusion, potential regulatory modes governing respiratory function are presented with the main concepts that were developed during the course of this study.

When considered together the results presentedhere clearly demonstrated that the alternative respiration is more complex than previously thought.Byinvestigating the evolutionary history of all TCA cycle genes was possible to validate a model for the origin of the TCA cycle genes in plants. In addition, novel insights into the usage of alternative substrates and the evolution of TCA cycle are provided, highlighting the complexity and specificity of plant respiration. Within the results presented in the Chapter 1, by using bioinformatics approaches we could infer that the TCA cycle function, whichallowsplant cell to synthesize ATP, is at the same time tightly connected with other pathways within the cell. By using a range of bioinformatics approach it was possible to find candidate genes that are co-expressed with the TCA cycle genes as well as with othermitochondrial stress related and mitochondrial carriersgenes (Chapter 1, Figure 6). Although some of theserepresent logical candidates for involvement in stress tolerance and have already been showed to be connected with energy metabolism, several other candidate genes have either unknown function (At24g04670, At3g28770) or perhaps more interesting, never been associated with energy metabolism such as At2g33690 (Late embryogenesis abundant protein). These findings related to the usage of bioinformatics provide further evidence thatthis approach can be assumed as a good target to retrieve, from public databases available,is likely to enable us to pursue new research avenues in order to increase our understanding of the complex networks governing the role of mitochondrial function within energy metabolism in general.

Lysine shows a multifaceted function within plant cell and even thought compelling evidences demostranting its involvement in a range of different functions, our understanding concerning its importance on overall metabolism remains limited. By using an Arabidopsis mutant for Lys biosynthesis, *L*,*L*-diaminopimelate aminotrasnferase (dapat), it was observed that Lys is required for normal plant growth and thus, although Lys seems to be synthetized in excess (Figure 4 and 7), its biosynthesis deficiency resulted in a dwarf phenotype (Figure 2) and also impairments during germination (Figure 2C and D). This aberrant phenotype resulted in drastic changes on molecular level which are relatively similar to stressed plants. Indeed, the *dapat* phenotype resembles to defective-photorespiratory mutants in a good agreement with our omics data. In addition,the low rate of starch turnover in this mutant suggests that the usage of alternative substrates is occouring to maintain respiration. The results obtained highlight a tight connection of

Lys metabolism with many other pathways within the cell and indicates that further studies are clearly required to fully understand how exactly lysine is connected with whole plant metabolism.

As stated before, the mechanism by which amino acids are used as alternative substrate to support respiration had been elucidated over the last years. Both IVDH and D2HGDH are able to donate electron following amino acids degradation to the ETF/ETFQO system, which are further used to generatethe electrochemical gradient in the mitochondrial intermembrane space which is converted in ATP by ATP synthase. In this context, ETFQO is an electron acceptor from amino acids catabolism located on mitochondrial inner membrane carrying then directly to ubiquinone pool.Although mETC function is very well estabilished, our knowlegment concerning its function and behavior under stress situations (e.g. carbon starvation) remains limited. It was further proposed to investigate the involvement of differents amino acids on fuel filling the mETC in a process mediated by alternative pathways of respiration. The results obtained demonstrated that the OXPHOS system is differently affected by carbon starvation and that the response is amino acids dependent. That being said,the results presented here suggests that further analysis of mitochondrial metabolism following carbon starvation are likely to identify additional components of the electron transport chain, and to define their role in the cross-compartiment pathway of amino acids degradation and its usage by alternative pathways in land plants.

In summary, evidence was provided regarding energy metabolism in *Arabidopsis thaliana* in different growth and physiological situations.To this end, several experimental approaches were performed allowing us to obtain a comprehensive view concerning classical and alternative respiration and how they may be interconnected. Whilst the precise nature of the interaction between energy metabolism, TCA cycle evolution, and amino acids mediating growth could not be observed in the present study, it remains an exciting topic for future research.

4. SUPPLEMENTARY DATA

Supplementary data CHAPTER 1 - Evolution and function of the TCA cycle.

Protein sequences used to build phylogenetic tree correspondent to Figures 1 - 4 can be download at http://gbe.oxfordjournals.org/content/early/2014/10/01/gbe.evu221/suppl/DC1

Supplemental DataSet 1. Protein sequences of pyruvate dehydrogenase (PDH), 2- oxoglutarate dehydrogenase (OGDH), 2-oxoglutarate decarboxylase (OGDC) from cyanobacteria, bacteria, yeast, green algaes, animals and plants.

Supplemental DataSet 2. Protein sequences of citrate synthase, aconitase and isocitrate dehydrogenase from cyanobacteria, bacteria, yeast, green algae, animals and plants.

Supplemental DataSet 3. Protein sequences of succinyl-CoA ligase and succinate dehydrogenase from cyanobacteria, bacteria, yeast, green algaes, animals and plants.

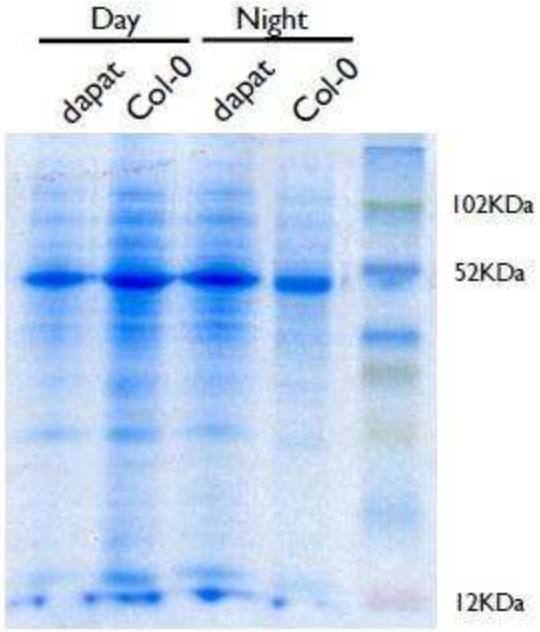
Supplemental DataSet 4. Protein sequences of fumarase and malate dehydrogenase from cyanobacteria, bacteria, yeast, green algaes, animals and plants.

Supplemental Table S1.Number and locus of nuclear genes encoding TCA cycle related genes in Arabidopsis thaliana and their subcellular localization. Genes retrieved from TAIR, NCBI and KEGG database.

Supplemental Table S2.List of candidate genes co-expressed involved in mitochondrial response associated with mitochondrial TCA cycle, carriers and stress response genes.

Supplementary data CHAPTER 2 - Lys biosynthesis deficiency alters growth and metabolism.

Supplemental Figure S1. Differential protein profiling in dapat mutants compared with the wild type at end of day and at end of night. Separation of proteins on SDS-PAGE gel.



Supplemental Table S1. Relative metabolite content of shoot of Arabidopsis mutants dapat and its wild tyoe (WT) along diurnal cycle as measured by GC-MS. Values are means ± SE of four independent samplings.

Name Analyte	Metabolite Class	Mol formula	Tag Mass	Col0 ED	Standart desviation	dapat ED	Standart desviation	t-test	Col0 EN	Standart desviation	dapat EN	Standart desviation	t-test
Guanidine	Amino acids	CH5N3	146	1	2,006470419	2,332767237	2,156644453	0,341296744	1	0,963670924	1,869024743	1,731308913	0,355463301
Valine	Amino acids	C5H11NO2	144	1	0,866187463	1,173564978	0,377328799	0,729347151	1	0,150970021	0,839469609	0,126501312	0,18507397
Glycerol	Polyols (triols)	C3H8O3	205	1	1,627206903	2,062583564	1,891119676	0,368790406	1	0,606490118	1,038675242	0,610342139	0,922414322
Isoleucine	Amino acids	C6H13NO2	158	1	1,092632276	2,366542432	1,130701439	0,167275291	1	0,359689185	0,648242191	0,092841934	0,011435155
Glycine	Amino acids	C2H5NO2	174	1	0,54689821	1,212753965	0,344247647	0,551654007	1	0,251368006	0,806398148	0,264401091	0,329370619
Serine	Amino acids	C3H7NO3	204	1	0,848519467	0,352581954	0,149390404	0,262975891	1	0,179418789	1,410746067	0,17585301	0,047257496
Proline	Amino acids	C5H9NO2	142	1	0,746873191	2,748921649	0,456334197	0,007148247	1	0,355087239	0,891513227	0,118247636	0,583186077
Glycerate	Alcohols (polyols)	C3H6O4	133	1	0,1897652	1,150993498	0,066238215	0,190294201	1	0,1270733	1,093479198	0,041825548	0,211751255
Glycerate	Alcohols (polyols)	C3H6O4	133	1	0,82241001	1,623376946	0,390676779	0,16431369	1	0,204940843	0,9559725	0,108568721	0,682394415
Benzoate	Organic Acid	C7H5O2	179	1	0,898237338	0,761988482	0,414939451	0,605291819	1	0,386725958	0,860878639	0,190611965	0,491133681
Alanine	Amino acids	C3H7NO2	248	1	0,464406822	1,067962464	0,509257697	0,850168931	1	0,371238957	0,83467294	0,310623119	0,520035488
Threonine	Amino acids	C4H9NO3	218	1	0,323618901	1,080574682	0,15185134	0,716137102	1	0,124860852	1,048761497	0,144965093	0,652109052
Nicotinate	Organic Acid	C6H4NO2	180	1	0,177001438	1,031861484	0,200648749	0,819680881	1	0,284577711	1,056880079	0,259686338	0,766134432
Uracil	Amina	C4H4N2O2	241	1	0,211025327	0,806783078	0,565663026	0,604512377	1	0,414223964	1,16445027	0,584623125	0,662364738
Alanine, beta	Amino acids	C3H7NO2	248	1	0,369121326	6,09291791	2,339673862	0,01475984	1	0,433781627	0,457516385	0,146638502	0,0495543
Erythritol	Alcohols (polyols)	C4H10O4	217	1	0,767684078	1,780832153	0,306555503	0,067642813	1	0,398628322	1,128451683	0,36888959	0,611269256

Threitol	Polyols (triols)	C4H10O4	217	1	0,767684078	1,780832153	0,306555503	0,067642813	1	0,398628322	1,128451683	0,36888959	0,611269256
Aspartate	Amino acids	C4H7NO4	232	1	0,567834565	1,428393583	0,265108642	0,220566974	1	0,291813253	0,907488054	0,407834761	0,724831374
Threonate	Organic Acid	C4H8O5	292	1	0,647444711	1,071310007	0,259221543	0,824886915	1	0,234135874	0,90771464	0,343633137	0,63305183
Methionine	Amino acids	C5H11NO2S	142	1	0,474520719	3,145062711	0,498690371	0,002246242	1	0,330935853	0,931486098	0,37191241	0,811130072
Salicilate	Organic Acid	C8H8O3	267	1	0,258139916	1,53451976	0,231806266	0,021627034	1	0,182904525	1,265948395	0,356315224	0,17590256
Ornithine	Amino acids	C5H12N2O2	174	1	0,638988078	5,177580103	2,845415796	0,048125862	1	0,32387517	0,786480043	0,607961996	0,619823043
Pyroglutamate	Organic Acid	C5H7NO3	156	1	0,536936668	1,134105119	0,492352723	0,691422136	1	0,3720855	1,214918758	0,537589036	0,483297499
Glutamate	Amino acids	C5H9NO4	246	1	0,65037768	2,665862127	0,661130511	0,035829969	1	0,093212648	0,963765108	0,259178045	0,829602268
Rhamnose	Sugars	C6H12O5	117	1	0,60896093	1,4708835	0,568300999	0,241780183	1	0,409548614	1,13911581	0,36927965	0,588147988
Putrescine	Polyamines	C4H12N2	174	1	0,268335187	3,4574194	1,315862316	0,033876066	1	0,494820627	0,652004837	0,194256601	0,266431969
Fucose	Sugars	C6H12O5	117	1	0,575429236	1,301340288	0,523310534	0,41154638	1	0,423631436	1,065459795	0,362701533	0,799596638
Phenylalanine	Amino acids	C9H11NO2	218	1	0,540430757	1,079850884	0,343260119	0,81880914	1	0,184636345	0,955943573	0,117630651	0,712980497
Asparagine	Amino acids	C4H8N2O3	188	1	0,409749504	5,1883288	1,623153207	0,007925044	1	0,482945128	0,322735208	0,050936542	0,064615634
Mannopyranose	Sugars	C6H12O6	204	1	0,450375296	1,375101683	0,204316545	0,128331137	1	0,094828443	0,933432058	0,147218518	0,420029774
Sorbose	Polyols (triols)	C6H12O6	217	1	0,527235596	1,356942693	0,487712342	0,298724903	1	0,384866969	0,601331794	0,391447732	0,143053323
Shikmate	Organic Acid	C7H10O5	204	1	0,099594957	1,17388886	0,098761363	0,047836716	1	0,029640238	0,930630581	0,159364736	0,499582901
Sorbose	Polyols (triols)	C6H12O6	217	1	0,529730031	1,309673573	0,527721395	0,381498207	1	0,422269825	0,604543182	0,421645333	0,17666402
Sorbitol	Sugars	C6H14O6	217	1	0,529730031	1,309673573	0,527721395	0,381498207	1	0,122269825	0,604543182	0,021645333	0,017666402
Citrate	Organic Acid	C6H5O7	273	1	0,625807519	1,673511313	0,350406603	0,109444681	1	0,227018186	1,092901792	0,48355559	0,039572447
Glutamine	Organic acids	C5H10N2O3	156	1	1,683712098	1,874652771	1,238948832	0,434751542	1	0,364297686	0,653764929	0,389245654	0,241664241
Lysine	Amino acids	C6H14N2O2	156	1	0,98384201	1,584862551	0,305094871	0,381066467	1	0,13872228	0,407829036	0,128410794	0,005595065
Galactonate	Polyols (triols)	C6H11O7	217	1	0,507022538	1,269711262	0,438061864	0,394370052	1	0,344330693	1,062281455	0,348630773	0,783466058
Dehydroascorbate	Organic acids	C6H6O6	316	1	0,203882254	1,113427464	0,081412566	0,341286201	1	0,244111643	1,068137085	0,155201774	0,644390164
Galactonate	Polyols (triols)	C6H11O7	292	1	0,479395726	1,663791556	0,223387939	0,02296933	1	0,143261308	1,600943965	1,04826982	0,239752892

Gluconate	Organic acids	C6H12O7	305	1	0,479395726	1,663791556	0,223387939	0,02296933	1	0,143261308	1,600943965	1,04826982	0,239752892
Tyramine	Amina	C8H11NO	174	1	0,617148637	1,630725822	0,424856579	0,096556834	1	0,269037418	1,073933651	0,317981419	0,701816095
Benzoate	Organic Acid	C7H52O2	266	1	0,534383094	1,297344917	0,500340704	0,390290293	1	0,366533889	0,932504252	0,278643411	0,751479441
Isositol, myo	Sugars	C6H12O6	305	1	0,067902556	1,021964984	0,079147831	0,650233338	1	0,070681582	1,004914151	0,154924138	0,950132417
Tyrosine	Amino acids	C9H11NO3	218	1	0,964143552	1,300273172	0,730463202	0,63721766	1	0,334113621	0,670364956	0,300811546	0,163326971
Glucoheptose	Sugars	C5H5N5	217	1	0,495384009	1,298498167	0,434038732	0,340530751	1	0,278765345	1,140843456	0,235833303	0,413516164
Adenine	Amina	C5H5N5	264	1	0,788279622	1,667184245	0,936242504	0,2575849	1	0,675670181	1,176587341	0,63818428	0,682142029
Cinnamate	Polyols (triols)	C9H8O2	293	1	0,630173394	1,209602518	0,684403958	0,627993146	1	0,492508994	1,134503584	0,508897755	0,682258632
Tryptophan	Amino acids	C11H12N2O2	202	1	0,826016848	8,819062007	2,746992204	0,001584887	1	0,297609674	0,651052903	0,375908577	0,275980383
Maltose	Sugars (disaccharides)	C12H22O11	204	1	0,717845531	1,681232909	0,086818109	0,023751914	1	0,745133804	1,320065039	1,018420579	0,586171037
Threalose	Sugars (disaccharides)	C12H22O11	361	1	0,682154094	0,940217625	0,308071871	0,862695372	1	0,338033099	1,258273979	0,233704012	0,197553205
Turonose	Sugars (disaccharides)	C7H14O7	361	1	0,721391343	1,639876382	0,735657089	0,202369461	1	0,465681131	1,367716366	0,656721781	0,336990659
Galactinol	Alcohols (polyols)	C12H22O11	204	1	0,540876817	1,415882686	0,812571163	0,368641939	1	0,282569642	0,962456267	0,377909044	0,863215998
Galactinol	Alcohols (polyols)	C12H22O11	204	1	0,509836629	1,757031258	0,991463602	0,167404107	1	0,521104067	1,108365314	0,458847696	0,736105465
Raffinose	Sugars (trisaccharides)	C18H32O16	361	1	0,658991769	1,347318667	0,666604421	0,431391495	1	0,52363649	0,781976209	0,243933359	0,423218938

Supplemental Table S2. Identification of proteins with altered abundance in shoot Arabidopsis mutant dapat compared to wild type along diurnal cycle

Spot	Locus	Name	UniProtKD ID	ratio	p-value	spectra total	spectra total (%)
End of Day							
60	At2g45790	Phosphomanomutase	O80840	ND[a]	0.0021	3	5.08
59	At3g48870	HSP93-III	F4JF64	ND[a]	0.0244	6	7.41
1	AtCg00490	Ribulose bisphosphate carboxylase large chain	O03042	71.519	0.0228	9	14.8
2	At3g55800	Sedoheptulose-1.7-bisphosphatase	P46283	58.696	0.0083	10	28.6
3	AtMg01190	ATP synthase subunit 1. ATP1	P92549	40.432	0.069	3	17.6
4	At5g13850	Nascent polypeptide-associated complex subunit alpha-like protein 3	Q6ICZ8	17.853	0.0459	2	5.71
5	At2g36880	S-adenosylmethionine synthase 3 (MAT3)	Q9SJL8	17.631	0.0107	2	5.56
6	At1g57720	Translation elongation factor EF1B	C0Z2J4	17.075	0.0397	4	16.7
7	At4g02520	Glutathione S-Transferase phi 2	P46422	16.004	0.0069	3	16.7
8	At1g42970	Glyceraldehyde 3- phosphatedehydrogenasesubunit b	P25857	15.973	0.0083	4	9.76
9	At3g01500	beta carbonic anhydrase 1	P27140	15.916	0.0193	5	12.2
10	At3g59970	Methylenetetrahydrofolate reductase 1	Q9SE60	15.300	0.0500	4	7.27
11	At4g16143	Importin alpha isoform 2	F4JL11	14.309	0.0242	10	18.9
12	At1g09780	2.3-biphospoglycerate-independent phosphoglycerate mutase	O04499	13.960	0.064	8	19
13	At5g25980	Glucoside glucohydrolase 2	Q9C5C2	13.851	0.0445	8	20
14	At2g45140	Lipoxygenase 2	Q9SHC8	13.793	0.060	11	16.7
15	At2g27720	60S acidic riosomal protein family	P51407	13.676	0.0329	2	25
16	At4g04640	ATP synthase gamma chain 1. chloroplastic	Q01908	13.638	0.0288	3	9.68
17	At5g42020	Luminal binding protein (BiP2)	Q39043	12.800	0.0034	13	30.2
18	At1g23310	Alanine 2-oxoglutarate aminotransfarase 1	D7KNE6	12.626	0.0125	3	7.89
19	At2g30860	Glutathione S-Transferase phi 9	O80852	12.419	0.0130	3	10.7
20	At3g11630	2-Cys peroxiredoxin (2-Cys PrxA)	Q96291	12.416	0.0357	3	30
21	At2g47730	Glutathione S-Transferase phi 8	Q96266	11.736	0.0016	2	11.1
22	At2g45140	Lipoxygenase 2	Q9SHC8	11.517	0.0142	11	16.7
23	At1g20020	Ferredoxin--NADP reductase. leaf isozyme 2. chloroplastic	Q8W493	11.364	0.0031	6	11.8
24	At5g24490	30S ribosomal protein	Q94K97	0.8957	0.0130	2	10
25	At3g55440	Cytosolic triose phosphate isomerase	P48491	0.8886	0.0690	5	13.5
26	At3g01500	beta carbonic anhydrase 1	P27140	0.8596	0.0344	5	12.2
27	At4g25100	Fe-superoxide dismutase	P21276	0.8483	0.0112	4	11.4

28	At1g32060	Phosphoribulokinase	P25697	0.8449	0.0075	2	13.3
29	At3g01500	Putative beta-carbonic anhydrase betaCA1	P27140	0.8194	0.0032	5	12.2
30	At3g11630	2-Cys peroxiredoxin (2-Cys PrxA)	Q96291	0.7797	0.0574	4	12.1
31	At4g24280	Chloroplast heat shock protein 70-1	Q9STW6	0.7579	0.0292	9	27.3
32	At3g09440	Heat shock 70 kDa protein 3	O65719	0.7367	0.0163	3	10.3
33	At1g01090	Pyruvate dehydrogenase E1 alpha subunit	O24457	0.7242	0.0592	2	7.41
34	At3g01500	beta carbonic anhydrase 1	P27140	0.7216	0.0293	6	13
35	At3g50820	Oxygen envolving complex subunit 33 Kda	Q9S841	0.7202	0.0355	6	14.6
36	At3g15360	Thioredoxin M-type 4. TRX-M4	Q9SEU6	0.7151	0.0401	2	14.3
37	At5g39570	Uncharacterized protein	Q9FKA5	0.6994	0.0310	3	16.7
38	At5g25980	Beta glucosidase 37	Q9C5C2	0.6862	0.0012	10	19.6
39	At4g01850	S-adenosylmethionine synthase 2	P17562	0.6586	0.0493	2	10
40	At2g39730	Rubisco activase	P10896	0.6551	0.0580	5	23.8
41	At1g68010	NADH-dependent hydroxypyruvate reductase 1	Q9C9W5	0.6392	0.0456	6	20
42	At2g38230	Pyridoxine biosynthesis 1	O80448	0.6208	0.0107	2	5.71
43	At5g06290	2-Cys peroxiredoxin (2-Cys Prx B)	Q9C5R8	0.5735	0.0547	10	19.6
44	At3g63540	Thylakoid lumenal 19 kDa protein	P82658	0.5616	0.0337	2	5.41
45	At5g53490	Thylakoid lumenal 17.4 kDa protein	P81760	0.5429	0.0195	6	20.7
46	At1g54270	Member of eIF4A - eukaryotic initiation factor 4A	P41377	0.5140	0.0441	5	14.3
47	At5g52310	Cold regulated 78 / responsive to dessication 29A	Q06738	0.5124	0.0197	4	16.7
48	At5g39570	Uncharacterized protein	Q9FKA5	0.3539	0.0469	1	8.33
49	At5g17920	Methionine synthesis 1	O50008	0.3509	0.0051	6	15.8
50	At3g52960	Thioredoxin superfamily protein	Q949U7	0.0886	0.0030	3	15.8
51	At1g31180	Isopropylmalate dehydrogenase 3	Q9SA14	ND[b]	< 0.001	2	5.26
52	At1g78330	Glutathione S-Transferase Tau 19	Q9ZRW8	ND[b]	< 0.001	2	2.21
53	At3g50820	Oxygen envolving complex subunit 33 Kda	Q9S841	ND[b]	< 0.001	4	9.52
54	At1g53850	20S Proteasome alpha subinit E1	O81149	ND[b]	0.0069	5	14.3
55	At2g34430	Light-harvesting chlorophyyll-protein complex II subunit b1	Q39142	ND[b]	0.0072	2	12.5
56	At3g26650	Glyceraldehyde 3- phosphate dehydrogenase subunit a	P25856	ND[b]	0.0252	5	10.2
57	At1g67090	Ribulosebiphosphate carboxylase samall chain 1a	P10795	ND[b]	0.0254	2	2.56
58	At2g34430	Light-harvesting chlorophyyll-protein complex II subunit b1	Q39142	ND[b]	0.0396	1	3.12
End of night							
61	At1g32470	Glycine cleavage system H protein 3. mitochondrial	Q9LQL0	ND[a]	< 0.001	3	11.5
62	At5g63400	Adenylate kinase 4	O82514	ND[a]	0.002757882	3	13.6

63	At4g38970	Probable fructose-bisphosphate aldolase 2. chloroplastic	Q944G9	ND[a]	< 0.001	7	11.5
64	At5g14200	3-isopropylmalate dehydrogenase 1. chloroplastic	Q9FMT1	ND[a]	< 0.001	4	23.5
65	At1g51980	Probable mitochondrial-processing peptidase subunit alpha-1	Q9ZU25	ND[a]	< 0.001	2	6.67
66	At5g11670	NADP-dependent malic enzyme 2	Q9LYG3	ND[a]	< 0.001	2	7.41
67	At3g45140	Lipoxygenase 2. Chloroplastic	P38418	ND[a]	< 0.001	10	20.4
68	At1g29930	Chlorophyll a-b binding protein 1. chloroplastic	P04778	ND[a]	< 0.001	2	7.69
69	AtCg00490	Ribulose bisphosphate carboxylase large chain	O03042	37.305	< 0.001	6	17.1
70	At4g13930	Serine hydroxymethyltransferase 4	O23254	31.073	0.002116139	2	4
71	At4g04640	ATP synthase gamma chain 1. chloroplastic	Q01908	30.485	0.000185067	5	10.4
72	AtCg00490	Ribulose bisphosphate carboxylase large chain	O03042	2.111	0.002139099	9	14.8
73	At5g14780	Formate dehydrogenase. Mitochondrial	Q9S7E4	1.796	0.000638415	3	3.82
74	At3g45140	Lipoxygenase 2. Chloroplastic	P38418	17.279	0.001603881	10	20.4
75	At5g39570	Uncharacterized protein	Q9FKA5	16.958	0.000211135	7	20.6
76	At5g37600	Glutamine synthetase cytosolic isozyme 1-1	Q56WN1	16.922	0.000164	3	10.7
77	At4g02520	Glutathione S-transferase F2	P46422	15.208	0.000530939	7	13.5
78	At2g44350	Citrate synthase 4. Mitochondrial	P20115	1.36	9.91E-06	4	21.3
79	At3g48870	Chaperone protein ClpC2. chloroplastic	Q9SXJ7	12.211	0.000394181	6	7.41
80	At1g32470	Glycine cleavage system H protein 3. mitochondrial	Q9LQL0	11.909	0.001854308	3	11.5
81	At2g33210	Chaperonin CPN60-like 1. mitochondrial	Q8L7B5	11.839	3.25E-05	3	17.6
82	At1g21750	Protein disulfide isomerase-like 1-1	Q9XI01	1.17	5.31E-06	3	17.6
83	At1g20020	Ferredoxin--NADP reductase. leaf isozyme 2. chloroplastic	Q8W493	11.479	0.000504754	5	20.8
84	At2g37660	Uncharacterized protein At2g37660. chloroplastic	O80934	0.8234	0.001054948	5	10.2
85	At5g43940	Alcohol dehydrogenase class-3	Q96533	0.7418	0.000351807	3	15.8
86	At5g27380	Glutathione synthetase. Chloroplastic	P46416	0.7312	1.58E-05	10	20
87	At5g24780	Vegetative storage protein 1	O49195	0.5834	0.000176055	3	8.57
89	At1g02930	Glutathione S-transferase F6	P42760	0.5808	2.06E-05	4	6.9
90	At5g38420	Ribulose bisphosphate carboxylase small chain 2B. chloroplastic	P10797	0.5295	0.002254416	3	5.15
91	At1g42970	Glyceraldehyde-3-phosphate dehydrogenase GAPB. Chloroplastic	P25857	0.4115	0.001024379	3	8.57
92	AtCg00490	Ribulose bisphosphate carboxylase large chain	O03042	0.3387	0.001005402	2	5.71
94	At5g66190	Ferredoxin--NADP reductase. leaf isozyme 1. chloroplastic	Q9FKW6	ND[b]	< 0.001	8	20
95	At5g27380	Glutathione synthetase. Chloroplastic	P46416	ND[b]	< 0.001	3	16.7