An L,L-diaminopimelate aminotransferase mutation leads to metabolic shifts and growth inhibition in Arabidopsis

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Abstract

Lysine (Lys) connects the mitochondrial electron transport chain to amino acid catabolism and the tricarboxylic acid cycle. However, our understanding of how a deficiency in Lys biosynthesis impacts plant metabolism and growth remains limited. Here, we used a previously characterized Arabidopsis mutant (dapat) with reduced activity of the Lys biosynthesis enzyme L,L-diaminopimelate aminotransferase to investigate the physiological and metabolic impacts of impaired Lys biosynthesis. Despite displaying similar stomatal conductance and internal CO2 concentration, we observed reduced photosynthesis and growth in the dapat mutant. Surprisingly, whilst we did not find differences in dark respiration between genotypes, a lower storage and consumption of starch and sugars was observed in dapat plants. We found higher protein turnover but no differences in total amino acids during a diurnal cycle in dapat plants. Transcriptional and two-dimensional (isoelectric focalization/SDS-PAGE) proteome analyses revealed alterations in the abundance of several transcripts and proteins associated with photosynthesis and photorespiration coupled with a high glycine/serine ratio and increased levels of stress-responsive amino acids. Taken together, our findings demonstrate that biochemical alterations rather than stomatal limitations are responsible for the decreased photosynthesis and growth of the dapat mutant, which we hypothesize mimics stress conditions associated with impairments in the Lys biosynthesis pathway.

Keywords: Alternative respiration, amino acid, carbon partition, L,L-diaminopimelate aminotransferase, lysine biosynthesis, primary metabolism.

Introduction

Plant mitochondria play a pivotal role in the biosynthesis of cellular ATP through oxidative phosphorylation. The tricarboxylic acid (TCA) cycle in the mitochondria is crucial in oxidizing acetyl-CoA to produce NADH, FADH2, ATP, and carbon skeletons to be used in other metabolic processes (Fernie et al., 2004; Millar et al., 2011; Araújo et al., 2012).
Compelling evidence has demonstrated that plant respiration is mainly dependent on carbohydrate oxidation (Plaxton and Podestá, 2006), but under stress conditions (which affect carbohydrate supply), metabolism is altered and alternative pathways are induced to provide substrates to the respiratory processes (Ishizaki et al., 2006; Araújo et al., 2010; Galili et al., 2014; Hildebrandt et al., 2015). It has been demonstrated that protein and amino acid degradation are highly effective at sustaining leaf respiration, particularly during senescence and/or stress situations (Moller and Kristensen, 2004; Araújo et al., 2011b; Hildebrandt et al., 2015; Galili et al., 2016). Additionally, 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, lysine (Lys) degradation occurs via a branched pathway (Araújo et al., 2010), partially similar to that described for the bacterium Rhodospirillum rubrum (Ebisuno et al., 1975) and for mammalian systems (Struys and Jakobs, 2010). In this pathway, 2-hydroxyglutarate is produced via the pipoculate pathway, and branched chain keto acids are produced via an as yet undefined, aminotransferase. It is important to note that Lys is synthesized in the chloroplasts (Fig. 1) and therefore must be transported to the mitochondria and then degraded to 2-hydroxyglutarate and further oxidized to 2-oxoglutarate (Engqvist et al., 2009; Araújo et al., 2010).

The electron-transfer flavoprotein (ETF)–electron-transfer flavoprotein:ubiquinone oxidoreductase (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), as well as under conditions in which free amino acids are present at high concentrations (Weigelt et al., 2008). By analysing select Arabidopsis mutants using enzymatic, metabolic, and isotope-labelling procedures it was demonstrated that the substrates used by the ETF–ETFQO pathway [mainly the branched–chain amino acids (BCAAs) isoleucine, leucine, and valine, as well as Lys] represent alternative electron donors at the mitochondrial level (Engqvist et al., 2009; Araújo et al., 2010). This donation occurs either directly, by the transfer of electrons to the mitochondrial electron transport chain (mETC) via the ETF complex, or indirectly, by the supply of substrates to fuel the TCA cycle (Araújo et al., 2010). To date, only two alternative dehydrogenases [savorseryl-CoA dehydrogenase (IVDH) and D-2-hydroxyglutarate dehydrogenase (D2HGDH)] have been demonstrated to be able to donate electrons to the ETF–ETFQO system in plants (Araújo et al., 2010). Accordingly, Lys catabolism via either D2HGDH or IVDH suggests a potential connection between the TCA cycle and alternative respiration in the maintenance of energy metabolism. This fact supports the growing evidence of strong network behaviour in the co-ordination of amino acid metabolism (Sweetlove and Fernie, 2005; Less and Galili, 2008; Araújo et al., 2010; Gu et al., 2010). Taken together, 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 the branched aspartate metabolic network has been extensively studied (Karchi et al., 1994; Zhu and Galili, 2003; Angelovici et al., 2009; Less and Galili, 2009; Angelovici et al., 2011; Clark and Lu, 2015). By contrast, little is currently known concerning the biological impact of a deficiency in the biosynthesis of aspartate–family amino acids, as naturally occurs in response to stress (Less and Galili, 2008; Baena-González and Sheen, 2008). Interestingly, an Arabidopsis mutant selected for enhanced defence against Pseudomonas syringae (Rate and Greenberg, 2001; Song et al., 2004) was further demonstrated to be unequivocally caused by a single amino acid substitution in the L,L-diaminopimelate aminotransferase (LL-DAPAT) enzyme of Lys biosynthesis, significantly reducing its activity (Hudson et al., 2006). Coupling bioinformatics with elegant biochemical results, it has been demonstrated that plants use a variant of the bacterial pathway for Lys production mediated uniquely by LL-DAPAT (Hudson et al., 2005). Remarkably, this discovery in Arabidopsis demonstrated the presence of an unnoticed mechanism for Lys synthesis in nature (McCoy et al., 2006). The mutation in the LL-DAPAT gene (AT4G33680) also resulted in dwarfish, 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 investigated the metabolic and physiological impact of impaired Lys biosynthesis by using this established Arabidopsis mutant (hereafter referred to as dapat) with reduced activity of the Lys biosynthesis enzyme LL-DAPAT. Our results demonstrate that the mutation in the LL-DAPAT gene culminated in growth impairments coupled with decreases in photosynthesis and maintenance of respiration. Furthermore, transcriptomic, proteomic, and metabolic analyses are suggestive of an imbalance in diel carbon and nitrogen metabolism. We additionally investigated the metabolic response of other mutants involved in amino acid metabolism, transport, or signalling to demonstrate that metabolite changes are triggered specifically by the DAPAT mutation.

Materials and methods

Plant material and growth conditions

Arabidopsis wild-type (WT) and the dapat [previously referred to as aberrant growth death 2 (agd-2)] characterized by Rate and Greenberg (2001) 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 d at 4 °C in the dark on 0.8% (w/v) agar plates containing half-strength Murashige and Skoog medium (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 150 µmol photons m⁻² s⁻¹. For phenotype examination, agar-initiated seedlings were subsequently transferred to soil 7–10 d after germination and placed in a growth chamber under similar growth conditions as above. The whole rosette leaves of 4-week-old plants were harvested for subsequent analysis.

We additionally used the following mutant lines: (i) dhdps-2 (At2g45440), a T-DNA insertional line in the gene encoding dihydrodipicolinate synthase in the Ws allele background, which displays relatively lower Lys synthesis but consequently a strongly enhanced threonine synthesis (Cracium et al., 2000); (ii) kin10 (At3g01090), a line carrying a mutation in the evolutionarily conserved protein kinase that targets a remarkably broad array of genes that orchestrate transcription networks, promoting catabolism and suppressing anabolism (Baena-González et al., 2007), and (iii) lmt1-1 (At5g40780), a Lys- and His-specific amino acid transporter (Hirner et al., 2006). All genotypes used here were cultivated...
under the same conditions described above and harvested at the same time of day in order to allow proper comparison.

**LL-Diaminopimelate aminotransferase enzyme activity assay**

DAPAT activity was analysed with the O-aminobenzaldehyde (OAB) assay described in Hudson et al. (2006). Frozen leaf material was ground into fine powder using a ball mill. Proteins were extracted in 100 mM HEPES–KOH (pH 7.6) from 20 mg of leaf material by vortexing. Following centrifugation at 22 000 g for 15 min at 4 °C, the supernatant was applied to an Amicon Ultra 30 000 MWCO filter unit (Millipore) for buffer exchange and concentration. The crude extract was concentrated by centrifugation at 14 000 g for 30 min at 4 °C and diluted with 450 µl of fresh 100 mM HEPES–KOH (pH 7.6) twice. Protein concentration in the concentrated crude extract was determined by Bradford protein assay kit (Bio-Rad). DAPAT activity was measured as the increase of absorbance at 440 nm in 1 ml of 100 mM HEPES–KOH (pH 7.6), 0.5 mM L,L-diaminopimelate (Sigma-Aldrich, 89469), 2 mM 2-oxoglutarate and 1.25 mg ml⁻¹ OAB (Sigma-Aldrich, A9628) following addition of concentrated crude extract.

**Measurement 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), and internal-to-ambient CO₂ concentration ratio (Cᵢ/Cₐ) were determined after at least 2 h illumination. The reference CO₂ concentration was set at 400 µmol CO₂ mol⁻¹ air and gas exchange was determined under 150 µmol photons m⁻² s⁻¹ at the leaf level 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% PPFD to optimize stomatal aperture. For dark respiration measurements, plants were adapted for at least 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). For all analyses, whole rosette leaves were collected, flash-frozen in liquid nitrogen and stored at −80 °C until analysed. Each replicate represented the mean of three determinations on the same sample. Chlorophyll, total protein, total free amino acid, and nitrate contents were determined as previously described by Sienkiewicz-Porzucek et al. (2010). Malate and fumarate contents were determined as described by Nunes-Nesi et al. (2007) and the levels of starch, sucrose, glucose, and fructose were determined as described by Fernie et al. (2001).

**RNA extraction and microarray analysis**

100 mg of harvested rosette leaves were used for total RNA extraction as described previously (Ruuska and Ohlrogge, 2001). Total RNA was treated with DNAses RQ-1 (Promega) and then RNA was amplified using two-cycle Affymetrix labelling, using the standard Affymetrix protocol. Hybridization, labelling, scanning, and data extraction were performed following standard Affymetrix protocols. Transcriptome analysis and annotation was performed using Partek® software (www.partek.com). Pre-processing was carried out using the Robust Microarray Averaging algorithm (Irizarry et al., 2003). Two-way ANOVA was performed and step-up correction was applied to correct from multiple comparisons (Hochberg and Benjamini 1990).
Differentially expressed genes were chosen according to step-up correction value <0.05 and a fold change >1.5 between genotypes. Over-representation analysis (enrichment) of the differently expressed genes was performed using the MapMan (Rasmussen et al. 2009) software tool. Changes in gene expression in *dapat* plants are further provided in Supplementary Tables S1, S2 at JXB online. Microarrays data were deposited at ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) as accession number E-MTAB-5315.

**Gene expression analysis**

Quantitative real-time PCR (qPCR) analysis was performed with three biological replicates, using gene-specific qPCR oligonucleotide pairs, designed with the Primer Express software (Applied Biosystems). *Ubiquitin C* (At5g25760) was used as the internal standard. The sequences of the specific oligonucleotides are provided in Supplementary Table S3. Reactions of the specific oligonucleotides are provided in Supplementary Table S3. Differentially expressed genes were chosen according to step-up correction value <0.05 and a fold change >1.5 between genotypes. Over-representation analysis (enrichment) of the differently expressed genes was performed using the MapMan (Rasmussen et al. 2009) software tool. Changes in gene expression in *dapat* plants are further provided in Supplementary Tables S1, S2 at JXB online. Microarrays data were deposited at ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) as accession number E-MTAB-5315.

**Metabolite profiling**

Metabolite profiling was performed based on the established gas chromatography–mass spectrometry (GC-MS) protocol of Lise et al. (2006). Both chromatograms and mass spectra were evaluated using TAGFINDER software (Luedemann et al., 2008). Metabolites were identified in comparison with 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 the full dataset is shown in Supplementary Table S4.

**2D gel electrophoresis**

Total leaf proteins were homogenized in 900 µl of ice-cold extraction buffer (50 mM Tris–HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 1% (w/v) 1,4-dithiothreitol (DTT), 30% (w/v) sucrose, 2% phenylmethysulfonyl fluoride) and vortexed for 30 s. The supernatant was recovered into a new tube and added to 900 µl of ice-cold Tris-buffered phenol (pH 8.0) and vortexed for 15 min at 4°C followed by centrifugation (3 min, 6000 g, 4°C). The phenolic phase was recovered into a new tube and re-extracted with 900 µl of ice-cold extraction buffer and vortexed for 30 s followed by centrifugation (3 min, 6000 g, 4°C). 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, 16,000 g, 4°C) and resuspended in ice-cold acetone–DTT (0.2%) at −20°C for 1 h. The sample was air-dried and suspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.8% IPG-buffer (Amersham Biosciences), 1% DTT). Protein concentration was determined by the Bradford reagent (Bradford, 1976). An 850 µg aliquot 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 of 18 cm, pH 4–7 linear for 16 h.

**Statistical analysis**

The experimental design was completely randomized. Data were submitted to two-way analysis of variance (ANOVA) and tested for significant (P<0.05) differences using Student's t test. All the statistical analyses were performed using an algorithm embedded in Microsoft Excel. In order to reduce the dimensionality of the metabolic dataset and identify the variables that explained a higher proportion of the total variance between the genotypes used here, a multivariate partial least-squares discriminant analysis (PLS-DA) (principal component analysis; PCA) with all metabolite data were used with the Excel add-in Multibase package (Proteome Software, Inc., Portland, OR, USA).

**Results**

**The DAPAT mutation reduces plant growth**

To investigate the contribution of impaired Lys biosynthesis on growth and metabolism, we have used an Arabidopsis mutant with reduced activity of the Lys biosynthesis enzyme DAPAT. Since DAPAT knockout plants are embryo lethal and this phenotype cannot be rescued by chemical application of Lys (Dobson et al., 2011), we concentrated our analysis on a single mutation in the LL-DAPAT gene resulting in an impairment of enzyme activity. Lower DAPAT activity was determined showing that the *dapat* mutant has only 10% of total DAPAT activity observed in WT plants (Fig. 2A). In good agreement with previous results (Song et al., 2004), *dapat* plants showed a clear decrease of rosette diameter (Fig. 2B; Table 1). This was coupled to a lower total number of leaves, as well as a strong decrease in both fresh and dry weight accumulation (Table 1).

To study in detail the impact of the DAPAT mutation on plant growth we next compared the development of WT and *dapat* plants. Germination kinetics displayed a clear delay in *dapat* mutant plants (Fig. 2C) whilst a small number of *dapat* seeds were incapable of germination (Fig. 2D).
Given that the DAPAT mutation resulted in impaired growth (Fig. 2B), we asked whether this phenomenon is associated with alterations in photosynthesis. We observed a significant reduction of both net photosynthesis ($A$) and stomatal conductance ($g_s$) levels (83% and 75%, respectively) in relation to WT plants (Fig. 3A, B). By contrast, no significant difference in internal CO$_2$ concentration ($C_i$) was found between dapat and WT plants (Fig. 3C) indicating that decreased photosynthesis cannot be directly associated solely with stomatal limitations, but is most likely associated with biochemical limitations. Dark respiration ($R_d$) was unaltered in dapat plants (Fig. 3D).

**Impact of DAPAT mutation on plant metabolism**

To further understand the phenotype observed in dapat plants, we next measured the levels of starch, soluble sugars, proteins, amino acids, and organic acids at the end of the day (ED) and at the end of the night (EN). Briefly, the levels of carbohydrates oscillated consistently, revealing one conspicuous feature that was clear when comparing the genotypes analysed here (Fig. 4). Sucrose was invariant between genotypes (Fig. 4B) whereas glucose and fructose (Fig. 4C, D, respectively) showed a similar pattern, with lower levels in dapat plants at both ED and EN when compared with WT plants. ANOVA was used to investigate statistical differences between genotypes and diurnal point (ED and EN). The mean separations were further compared by Student’s $t$-test and we observed similar statistical changes. There was a tendency for lower levels of starch in dapat plants at ED (Fig. 4A), in good agreement with the lower photosynthetic capacity (Fig. 3A). Interestingly, 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 remained at EN, suggesting impairment in starch degradation. Increased levels of malate were observed in dapat at ED compared with WT plants, reaching similar levels at EN (Fig. 4E). By contrast, no differences in the levels of fumarate were observed (Fig. 4F).

*dapat* plants contained higher levels of proteins (Fig. 5A) at ED with similar levels at EN suggesting that the consumption of protein during the night is higher in dapat plants (Fig. 5A). Indeed, when examining the decrease in protein levels between

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**Fig. 2.** Effects of lysine biosynthesis deficiency observed in dapat mutant plants. (A) L,L-Diaminopimelate aminotransferase (DAPAT) enzyme activity showing drastic reduction in mutant plants. (B) Arabidopsis plants grown in short day conditions as described in ‘Materials and methods’. (C) WT and dapat seed germination assay showing a delay in germination of dapat seeds. (D) Arabidopsis seedling establishment of WT and dapat 5 d after start of germination. While WT showed germination of all seeds, a few dapat seeds were unable to germinate. (This figure is available in colour at JXB online.)
ED and EN, protein levels decreased more at EN in the *dapat* mutant (62.4% of levels observed at the ED) than WT (72.7%), revealing higher protein consumption during the night for *dapat* plants. Higher levels of free amino acids were observed at ED in *dapat* plants (Fig. 5B). However, determination of free amino acid levels at EN revealed that total free amino acids was similar between the *dapat* plants (with 80% residual amino acid content) and WT plants (with 72% residual content). These findings are supported by ANOVA showing significant difference (*P*<0.05) for major classes of metabolites (both protein and amino acids; Fig. 5); results of ANOVA and Student’s *t*-test were similar. Both statistical treatments reinforce our idea that nitrogen compounds (protein and total free amino acids, described in Fig. 5) are accumulated at ED and broken down during the night to further support energy metabolism as discussed below. Thus, changes observed suggested that an extensive reprogramming may be occurring in the *dapat* mutants, and as a consequence we extended our analysis to a broad transcriptional, proteomic, and metabolic analysis.

**Transcriptome changes induced by the DAPAT mutation**

To elucidate the effects of the DAPAT mutation on the global transcriptome, whole rosettes of 4-week-old-plants grown under short day conditions were harvested and their mRNAs subjected to an Affymetrix ATH1 microarray analysis. As our initial results indicated that the DAPAT mutation impacts both photosynthesis and carbohydrate turnover (Figs. 3A, 4C) and are suggestive of higher protein degradation (Fig. 5A) most likely to support energy generation by alternative pathways of mitochondrial respiration, microarray analysis was carried out with samples harvested at EN. We focused on genes whose expression was significantly up- or down-regulated in the *dapat* mutant, compared with WT. It was found that the expression of 1982 and 1156 genes was significantly up- and down-regulated, respectively, in the *dapat* mutant compared with WT (Supplementary Tables S1, S2). These consistently up- and down-regulated genes were subjected to over-representation analysis using the tools embedded in the PageMan and MapMan software (Usadel *et al.*, 2006) and used to create an overview-of-metabolism diagram (Fig. 6; Supplementary Fig. S1). As would perhaps be expected, given that the *dapat* phenotype is due to a single amino acid substitution leading

| Parameter                  | WT               | *dapat*          |
|----------------------------|------------------|------------------|
| Rosette diameter (mm)      | 41.9 ± 1.2       | 18.8 ± 1.3       |
| Number of leaves           | 17.5 ± 0.5       | 12.5 ± 0.3       |
| Rosette fresh weight (mg)  | 0.1203 ± 0.08    | 0.0403 ± 0.01    |
| Rosette dry weight (mg)    | 0.0123 ± 0.01    | 0.0048 ± 0.01    |

Plants deficient in lysine biosynthesis showed a reduced aerial biomass with respect to the wild-type (WT) during vegetative growth (4 weeks old). Values are presented as mean ±SE of at least 13 independent biological replicates per genotype; bold indicates values that were determined by Student’s *t*-test to be significantly different (*P*<0.05) from the WT.

![Fig. 3](#) Changes in gas-exchange measurements in wild-type (WT) and mutant (*dapat*). (A) Photosynthesis. (B) Stomatal conductance to water vapor. (C) Internal CO2 concentration. (D) Dark respiration. Bars are means ±SE from five biological replicates; *significantly different (*P*<0.01) from WT by Student’s *t*-test.
LL-DAPAT mutation alters growth and metabolism

A range of stress-induced genes (BIN 20; 153 genes) were up-regulated, indicating that impaired Lys biosynthesis mimics a stressed plant phenotype. To further investigate the stress phenotype and accumulation of changes at the protein level in dapat plants, we performed a survey of genes related to the unfolded protein response (UPR) pathway, which could, at least partially, explain the dramatic visual phenotype. Notably, transcripts of UPR-related genes such as IRE1, BiP and transcription factors of the bZIP system were increased whereas the transcript levels of many genes of the UPR system were unaltered (see Supplementary Tables S1, S2). Therefore, caution must be shown in drawing any conclusions concerning the participation of the UPR system in determining the dapat phenotype. Furthermore, the transcriptome data suggested an early senescence phenotype in dapat plants. Accordingly, a group of transcription factors known as positive regulators of senescence were increased in dapat plants, such as ORE1 (4.42-fold change), ANAC29 (2.68-fold change), and ANAC016 (2.62-fold change). Also, the WRKY transcription factor family was up-regulated, as shown by the overenrichment analysis by PageMan in which WRKY45 increased 3.49-fold. Transcriptome data also revealed an increase of genes able to

Fig. 4. Effect of deficiency of lysine biosynthesis along diurnal cycle on starch (A), sucrose (B), glucose (C), fructose (D), malate (E), and fumarate (F) level. ED, end of day; EN, end of night. Values are means ±SE of five independent biological replicates. Asterisks designate values that were significantly different from WT (*P<0.05, **P<0.01) by Student’s t-test.

Fig. 5. Effect of deficiency on lysine biosynthesis along a diurnal cycle on the total protein (A) and amino acid level (B). Values are means ±SE of five independent biological replicates. Asterisks designate values that were significantly different from WT (*P<0.05, **P<0.01) by Student’s t-test.
destabilize chloroplast integrity, such as NYE1 and a recently identified chloroplast vesiculation (CV), which is involved in chloroplast degradation by an autophagy-independent pathway and senescence-associated vacuoles (SAVs) (Wang and Blumwald, 2014), presented here as unknown gene (Affymetrix ID 265913_at; Supplementary Table S1). Moreover, genes involved in protein degradation such as autophagy related genes (ATG) and SAVs showed a strong induction (Fig. 7A).

During the past years, it has been demonstrated that stress-induced senescence promotes amino acid catabolism supplying electrons to the mETC via the alternative pathway mediated by ETF–ETFQO (Araújo et al., 2011b; Hildebrandt et al., 2015). Hence, we next focused our analysis on amino acid metabolism and its association to the TCA cycle and ATP synthesis. Our transcriptome data showed a huge increase of genes encoding dehydrogenases associated to alternative respiration and amino acid catabolism, such as Branched chain aminotransferase 2 (BCAT2), IVDH, and Enoyl-CoA dehydrogenase (ECoAH), all involved at BCAA degradation; aldehyde dehydrogenase 7B4 (ALDH7B4), Lys-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH), and D2HGDH (Lys degradation); Persulfide dioxygenase (ETHE1) (sulphur amino acid degradation); and Glutamate decarboxylase (GAD1), Glutamate dehydrogenase (GDH1), and Fd-glutamate synthase (GLU2) (glutamate degradation) (Fig. 7A). Simultaneously, isoforms of TCA cycle genes were clearly up-regulated (Fig. 7A). Nevertheless, PDH E1 beta subunit showed reduced expression. These data indicated that amino acid degradation may feed organic acids into the TCA cycle most likely by anaplerotic reactions (Araújo et al., 2012). When considered together, our results suggest that Lys biosynthesis impairment caused by the DAPAT mutation leads to a molecular reprogramming that likely works by anticipating senescence in a putative stress situation leading to protein and amino acid degradation that ultimately support the activity of the oxidative phosphorylation system.

To validate our microarray data, we performed a qPCR analysis by selecting six genes related to amino acid catabolism: BCAT2, LKR/SDH, Threonine aldolase (THA1), Methionine gamma-lyase (MGL), IVDH, and D2HGDH. In addition, gene expression of these genes was performed at both ED and EN to evaluate their diurnal turnover. As shown in Fig. 7B, and in agreement with our transcriptomic analysis, the expression levels of all six genes were significantly higher in the dapat mutant, compared with WT, in both time points analysed and were also consistently higher at EN.
The leaf proteome is affected by the DAPAT mutation

Since we have demonstrated that the DAPAT mutation has substantially altered levels of total protein and free amino acids (Fig. 5), we decided to carry out a robust proteome analysis via 2D electrophoresis (IEF/SDS-PAGE) in tandem with MALDI TOF/TOF (see Supplementary Fig. S2). We identified 95 proteins that changed their abundance in dapat plants at both ED and EN (Tables 2, 3; Supplementary Table S5). For simplicity, we first describe here the results obtained at ED and after, the results at EN. Importantly, the results are always presented in terms of changes that took place in the dapat plants in relation to WT.

By analysing proteins at ED we were able to identify several proteins related to photosynthesis (involved in both light and Calvin–Benson cycle reactions) and one protein related to photorespiration that significantly changed their abundance. 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 (At1g20020), located on PSI, and ATP synthase γ chain 1 (At4g04640) increased their abundance at ED in dapat plants. Interestingly, enzymes of the Calvin–Benson cycle were also affected in dapat mutant plants. In this vein, we observed that Rubisco large chain and sedulheptulose 1,7-biphosphatase increased (7.15- and 5.8-fold, respectively) whereas Rubisco small chain 1a, phosphoribulokinase (PRK), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Rubisco activase reduced their abundance (Table 2). In addition, redox-related proteins involved in the regulation of photosynthesis and others chloroplastic reactions such as thioredoxin m4, 2-Cys peroxidase A- and B-type, thioredoxin superfamily protein and plenty of glutathione S-transferase isoforms (e.g. GSF2, GSF8, GSF9, and GSF18) were also identified and most of them were down-regulated. Regarding enzymes involved in photorespiration, only hydroxypyruvate reductase 1 was significantly reduced in dapat plants.

Our proteomic approach also provided some insights into amino acid metabolism at ED. We found an increase in glutamine:2-oxoglutarate aminotransferase (GOGAT) and also in proteins related to hormone metabolism such as S-adenosylmethionine synthase 2 and 3, which decreased and increased, respectively (Table 2). Lipoxigenase 2, which is related to jasmonate metabolism, also increased at EN.

When analysing proteome changes at EN, we observed the presence of 34 spots corresponding to 29 proteins differentially abundant in dapat plants (Table 3). Interestingly, most of those proteins, which accumulated in dapat plants, are related to energy metabolism and particularly located in the mitochondria [e.g. glycine decarboxylase (GDC), serine hydroxymethyl transferase (SHMT) and citrate synthase (CS4)] and also in proteins related to energy metabolism such as S-adenosylmethionine synthase 2 and 3, which decreased and increased, respectively (Table 2). Lipoxigenase 2, which is related to jasmonate metabolism, also increased at EN.

In addition, fructose-bisphosphate aldolase 2 (FBPK2) and cytosolic malic enzyme 2 (cME2) increased at EN, suggesting an augmentation of the energetic metabolism in dapat plants. In agreement, the increased levels of proteins related to NADH production such as GDC subunit H, SHMT4, and formate
Table 2. Identification of proteins with altered abundance in dapat with respect to the WT at the end of the day

| Spot | Locus       | Name                                             | Fold    | P-value |
|------|-------------|--------------------------------------------------|---------|---------|
| 1    | At2g45790   | Phosphomannomutase                               | ND\(^a\) | 0.0021  |
| 2    | At3g48870   | HSP90-III                                        | ND\(^a\) | 0.0244  |
| 3    | AtCg00490   | Rubisco large chain                              | 7.1519  | 0.0228  |
| 4    | At3g55800   | Sedoheptulose-1,7-bisphosphatase                 | 5.8696  | 0.0083  |
| 5    | AtMg01190   | ATP synthase subunit 1                           | 4.0432  | 0.069   |
| 6    | At5g13850   | NAC3                                             | 1.7853  | 0.0459  |
| 7    | At2g36880   | S-Adenosylmethionine synthase 3                  | ND\(^b\) | <0.001  |
| 8    | At1g57720   | Elongation factor EF1B                           | 1.7075  | 0.0397  |
| 9    | At3g48870   | HSP93-III                                        | 1.6004  | 0.0069  |
| 10   | AtCg00490   | Rubisco large chain                              | 1.5973  | 0.0083  |
| 11   | At3g55800   | Sedoheptulose-1,7-bisphosphatase                 | 1.5916  | 0.0193  |
| 12   | At3g59970   | Methyleneetrahydrololate reductase 1             | 1.5830  | 0.0500  |
| 13   | At4g16143   | Importin α isoform 2                             | 1.4309  | 0.0242  |
| 14   | At1g57720   | Phosphoglycerate mutase                          | 1.3960  | 0.064   |
| 15   | At4g04640   | ATPase γ chloroplastic                            | 1.3638  | 0.0288  |
| 16   | At5g24490   | 3OS ribosomal protein                            | 0.8957  | 0.0130  |
| 17   | At3g55440   | Triose phosphate isomerase                        | 0.8886  | 0.0690  |
| 18   | At3g01500   | Beta carbonic anhydrase 1                         | 0.8596  | 0.0344  |
| 19   | At4g25100   | Fe-superoxide dismutase                          | 0.8438  | 0.0112  |
| 20   | At1g32060   | Phosphoribulokinase                              | 0.8449  | 0.0075  |
| 21   | At3g01500   | Beta carbonic anhydrase 1                         | 0.8194  | 0.0032  |
| 22   | At3g11630   | 2-Cys peroxiredoxin                              | 0.7797  | 0.0574  |
| 23   | At4g24280   | HSP70                                            | 0.7579  | 0.0292  |
| 24   | At3g09440   | HSP70 protein 3                                  | 0.7367  | 0.0163  |
| 25   | At1g01090   | Pynuvate dehydrogenase E1α                       | 0.7242  | 0.0592  |
| 26   | At3g01500   | Beta carbonic anhydrase 1                         | 0.7216  | 0.0293  |
| 27   | At3g05820   | Oxygen evolving complex                           | 0.7202  | 0.0355  |
| 28   | At3g15380   | Thioredoxin M-type 4                             | 0.7151  | 0.0401  |
| 29   | At5g39570   | Uncharacterized protein                          | 0.6994  | 0.0310  |
| 30   | At5g25980   | Beta glucosidase 37                               | 0.6882  | 0.0012  |
| 31   | At4g01850   | S-Adenosylmethionine synthase 2                  | 0.6586  | 0.0498  |
| 32   | At2g39730   | Rubisco activase                                 | 0.6551  | 0.0560  |
| 33   | At1g66010   | Hydroxyprolyl reductase 1                         | 0.6392  | 0.0456  |
| 34   | At2g38230   | Pyridoxine biosynthesis 1                         | 0.6208  | 0.0107  |
| 35   | At5g06590   | 2-Cys peroxiredoxin                              | 0.5735  | 0.0547  |
| 36   | At3g63540   | Thylakoid lumen 19 kDa                           | 0.5616  | 0.0337  |
| 37   | At5g53490   | Thylakoid lumen 17.4 kDa                         | 0.5429  | 0.0196  |
| 38   | At1g54270   | eIF4A                                            | 0.5140  | 0.0441  |
| 39   | At5g2310    | Responsive to dessication 29A                    | 0.5124  | 0.0197  |
| 40   | At5g39570   | Uncharacterized protein                          | 0.3539  | 0.0469  |
| 41   | At5g17920   | Methionine synthesis 1                           | 0.3599  | 0.0051  |
| 42   | At3g52960   | Thioredoxin superfamily protein                  | 0.0886  | 0.0030  |
| 43   | At1g31180   | Isopropylmalate dehydrogenase 3                  | ND\(^b\) | <0.001  |
| 44   | At1g78330   | Glutathione S-transferase 19                     | ND\(^b\) | <0.001  |
| 45   | At3g50820   | Oxygen evolving complex                          | ND\(^b\) | <0.001  |
| 46   | At1g53850   | 2OS Proteasome α subunit E1                      | ND\(^b\) | 0.0069  |
| 47   | At2g34430   | LCB-II                                           | ND\(^b\) | 0.0272  |
| 48   | At3g26650   | Glyceraldehyde-3-phosphate dehydrogenase subunit a | ND\(^b\) | 0.0252  |
| 49   | At1g67090   | Rubisco small chain                              | ND\(^b\) | 0.0254  |
| 50   | At2g34430   | LCB-II                                           | ND\(^b\) | 0.0396  |

Proteins were separated by IEF/SDS-PAGE and spots were analysed by MALDI TOF/TOF (n=3). \(^a\) P<0.05.
\(^b\) ND: not detected in wild-type (Col-0).
\(^b\) ND: not detected in dapat mutant.
LL-DAPAT mutation alters growth and metabolism

Dehydrogenase suggest that metabolic reprogramming is occurring in dapat plants most likely to use alternative energy sources such as amino acids. Furthermore, the decreased levels of alcohol dehydrogenase 3 may suggest a reduced glycolysis in dapat at night, giving further support to the contention that a disorder of carbon breakdown is most likely taking place in this mutant.

Metabolome analysis reveals a metabolic reprogramming in the dapat mutant

To explore the consequences of the DAPAT mutation on the major primary pathways of plant metabolism, an established GC-MS protocol (Lisec et al., 2006) was used. The data obtained are displayed in a heat map (Fig. 8) in order to provide an easy overview (the full dataset is provided in Supplementary Table S4). From this display, it is noticeable that there were considerable changes in the levels of metabolites at both ED and EN in the dapat mutant. The levels of Lys in dapat plants at ED did not change, although surprisingly, a strong reduction was observed at EN for this amino acid. Furthermore, the levels of shikimic acid and salicylic acid were higher in dapat plants (Fig. 8). It is important to mention that the increased levels of salicylic acid were observed even when this mutant grew without pathogen attack or other stress conditions (Rate and Greenberg, 2001). The reduced DAPAT activity resulted in an accumulation of metabolites at ED known to be related to stress response such as proline and β-alanine (2.75- and 6.09-fold, respectively). In addition, putrescine, another stress-related metabolite (Wulf-Zottele et al., 2010), followed the same behaviour with increased levels (3.45-fold) at ED. Furthermore, ornithine, which increased 5.17-fold at ED, is a central metabolite involved in the biosynthesis of polyamines (Majumdar et al., 2013) and proline. Taken together, these findings suggest that the DAPAT mutation may result in a putative stress situation even when the plants are growing under optimal conditions.

Table 3. Identification of proteins with altered abundance in dapat with respect to the WT at the end of the night

| Spot | Locus | Name | Fold | P-value |
|------|-------|------|------|---------|
| 61   | At1g32470 | GDC subunit H | ND* | <0.001 |
| 62   | At5g63400 | Adenylate kinase 4 | ND* | 0.0027 |
| 63   | At4g38970 | Fructose-bisphosphate aldolase 2 | ND* | <0.001 |
| 64   | At5g14200 | 3-Isopropylmalate dehydrogenase 1 | ND* | <0.001 |
| 65   | At1g61980 | Mitochondrial-processing peptidase subunit α-1 | ND* | <0.001 |
| 66   | At5g11670 | NADP-dependent malic enzyme 2 | ND* | <0.001 |
| 67   | At3g45140 | Lipoxynase 2, chloroplastic | ND* | <0.001 |
| 68   | At1g29930 | LCBI-b | ND* | <0.001 |
| 69   | At1g00490 | Rubisco large chain | 3.7305 | <0.001 |
| 70   | At1g13930 | SHMT 4 | 3.1073 | 0.0021 |
| 71   | At4g04660 | ATP synthase γ chain 1. | 3.0485 | 0.0001 |
| 72   | At1g00490 | Rubisco large chain | 2.111 | 0.0021 |
| 73   | At5g14780 | Formate dehydrogenase. | 1.796 | 0.0006 |
| 74   | At3g45140 | Lipoxynase 2 | 1.7279 | 0.0016 |
| 75   | At5g39570 | Uncharacterized protein | 1.6958 | 0.0002 |
| 76   | At5g37660 | Glutamine synthetase isozyme 1 | 1.6922 | 0.0001 |
| 77   | At4g02520 | Glutathione S-transferase F2 | 1.5208 | 0.0005 |
| 78   | At3g14350 | Citrate synthase 4 | 1.36 | 9.91 × 10⁻⁶ |
| 79   | At3g08570 | Chaperone protein ClpC2 | 1.2211 | 0.0003 |
| 80   | At1g32470 | GDC subunit H | 1.1909 | 0.0018 |
| 81   | At2g33210 | Chaperonin CPN60-like 1 | 1.1839 | 3.25 × 10⁻⁶ |
| 82   | At1g17550 | Protein disulfide isomerase-like 1-1 | 1.17 | 5.31 × 10⁻⁶ |
| 83   | At1g20020 | Ferredoxin–NADP reductase, leaf isozyme 2 | 1.1479 | 0.0005 |
| 84   | At2g37660 | Uncharacterized protein | 0.8234 | 0.0010 |
| 85   | At5g43940 | Alcohol dehydrogenase 3 | 0.7418 | 0.0003 |
| 86   | At5g27380 | Glutathione synthetase | 0.7312 | 1.58 × 10⁻⁵ |
| 87   | At5g24780 | Vegetative storage protein 1 | 0.5834 | 0.0001 |
| 88   | At1g02930 | Glutathione S-transferase F6 | 0.5808 | 2.06 × 10⁻⁵ |
| 89   | At5g38420 | Rubisco small 2B, chloroplastic | 0.5295 | 0.0022 |
| 90   | At1g42970 | Glycerolaldehyde-3-phosphate dehydrogenase, chloroplastic | 0.4115 | 0.0010 |
| 91   | At1g00490 | Rubisco large chain | 0.3387 | 0.0010 |
| 92   | At4g02520 | Glutathione S-transferase F2 | ND* | <0.001 |
| 93   | At5g66190 | Ferredoxin–NADP reductase isozyme 1 | ND* | <0.001 |
| 94   | At5g27380 | Glutathione synthetase | ND* | <0.001 |
| 95   | At1g32470 | GDC subunit H | ND* | <0.001 |

Proteins were separated by IEF/SDS-PAGE and spots were analysed by MALDI TOF/TOF (n=3). P<0.05.

* ND: not detected in wild-type (Col-0).

ND: not detected in dapat mutant.
The aspartate-family amino acids aspartate, methionine, and isoleucine accumulated significantly at ED in *dapat* plants. In addition, isoleucine decreased at EN, similarly to the situation observed for Lys. Perhaps one of the most interesting metabolic changes was that of tryptophan, which increased more than 8-fold at ED, but was reduced to 0.65 (relative level) at EN. Our metabolic profile also showed that the DAPAT mutation culminated in increased levels of glutamine and glutamate at ED (5.13- and 2.66-fold, respectively). Interestingly, serine and glycine showed an opposite behaviour and thus at ED glycine showed increased levels while serine decreased. In turn, at EN glycine had reduced levels whereas serine accumulated. Given the major changes in amino acid content of *dapat* plants, the influence of each amino acid in the total amino acid pool of *dapat* plants compared with its respective WT is provided as Supplementary Fig. S3. The amino acid pool of *dapat* is over-represented by several amino acids including tryptophan, β-alanine, asparagine, and ornithine at ED. By contrast, at EN the amino acid pool is relatively more balanced in *dapat* plants. Thus, the major changes caused by the DAPAT mutation occur at the end of the light period.

**Related metabolic responses among *dapat*, *dh dps*2, *kin10*, and *lht* mutants**

To address whether the metabolite changes are triggered specifically by changes in Lys metabolism caused by the DAPAT mutation, we analysed metabolic responses of other mutants involved in (i) amino acid metabolism (*dh dps*2), (ii) amino acid transport (lht1-1), and (iii) stress signalling (*kin10*) (see Supplementary Tables S6, S7). *dh dps*2 mutants, with impairments in Lys biosynthesis via the disruption of another step of the pathway, have also been characterized by growth rate reductions, affecting both leaves and roots (Sarrobert et al., 2000). Growth reductions coupled with changes in plant architecture and lower yield have also been reported in double mutants of *dhps*-1 and *dhps*-2 (Jones-Held et al., 2012). In addition, lines carrying insertions in *LHT1* showed reduced growth compared with the WT (Hirner et al., 2006), whilst *KIN10* overexpression mutants displayed reduction of shoots and roots under normal growth conditions (Baena-González et al., 2007). We therefore selected mutant plants that have a similar growth phenotype to *dapat* mutants. In order to obtain further insight into the metabolic changes that were unequivocally caused by the DAPAT mutation, we first focused on Lys biosynthesis by comparing *dapat* and plants lacking dihydrodipicolinate synthase (DHDPS). DHDPS is the first enzyme of the Lys biosynthesis pathway, and the DHDPS2 gene (At2g45440) encodes one of two DHDPS isozymes that contributes to the majority of the total DHDPS activity in Arabidopsis. T-DNA insertion lines of DHDPS2 display relatively lower Lys biosynthesis, and, as a result of this, a strongly enhanced synthesis of threonine (Craciun et al., 2000). Analysis of the metabolic profile displayed significant differences between *dapat* and *dh dps*2 mutants (Supplementary Fig. S4; Supplementary Table S6). The significantly increased levels of amino acids in *dapat* were, however, not observed in *dhps*2 mutants. In fact, changes in amino acid composition have been previously observed mainly in the root tissue of *dhps*2 mutants (Sarrobert et al., 2000). In addition, higher levels of tryptophan were observed in *dapat* mutants, whereas in *dhps*2 mutants the levels of this amino acid were invariant. During adverse conditions the accumulation of aromatic amino acids (e.g. tryptophan and phenylalanine) suggest their potential roles either as metabolic precursors or under conditions of stress (Galili et al., 2016). Indeed increased levels of stress-related metabolites such as putrescine and proline were only observed in *dapat* mutants. In addition, the BCAAs and aromatic amino acids followed the same behaviour with higher levels only in *dapat* mutants.

Previous studies showed that modulation of biosynthesis and catabolic fluxes in the aspartate family pathway have a substantial influence on the TCA cycle (Angelovici et al., 2007).
depicted by our proteomic approach (Table 3; Supplementary Table S8). Although a detailed analysis of the entire data revealed no significant differences in sugars and organic acids between these genotypes, noticeable changes were found for amino acids (Supplementary Fig. S5). The levels of isoleucine, valine, Lys, and tyrosine are significantly higher only in kin10 and lht-1 mutants. The higher levels of these amino acids have been associated to energy stress situations and they play key role in energy generation via the ETF–ETFQO system or by feeding TCA cycle intermediates (Araújo et al., 2010; Barros et al., 2017; Hirota et al., 2018).

Despite the metabolic pattern of dapat, kin10, and lht-1 having a certain similarity, most amino acids increased only in dapat (Supplementary Fig. S5). Among them, glutamate, glutamine, and asparagine, precursors of the aspartate amino acid family, increased significantly more in dapat mutant plants suggesting that LL-DAPAT disruption leads to a specific readjustment of amino acid metabolism. Altogether, these results highlight that more pronounced changes in amino acid biosynthetic and catabolic pathways occur in dapat mutants, and they are in good agreement with our suggestion that mutation in the LL-DAPAT gene results in a unique molecular reprogramming.

Discussion

By using a mutant in the Lys biosynthesis pathway we provided evidence of the pivotal importance of Lys as depicted by the growth impairment and photosynthesis reduction observed. Our results also highlight a novel aspect of Lys metabolism showing that the DAPAT mutation culminated in physiological and metabolic changes. The differential turnover of total protein and free amino acids observed between the dapat mutant and WT along the diurnal cycle (Fig. 5) indicate that the significant reduction in DAPAT activity, occurring in the dapat mutant (Fig. 2), has a major impact on protein consumption during the night, as depicted by our proteomic approach (Table 3; Supplementary Fig. S2). Collectively, these results demonstrate a previously unrecognized role of DAPAT and additionally Lys biosynthesis by showing their importance in affecting growth and primary metabolism with direct impact on protein consumption.

Our data demonstrated that photosynthesis was negatively affected in dapat plants (Fig. 3A). The reduction in photosynthetic rates may explain, at least partially, the lower accumulation of starch at ED in dapat plants (Fig. 4A). Reduction in photosynthesis is regulated by either stomatal movements or biochemical parameters associated with photosystem components and Calvin–Benson cycle enzymes. Nonetheless, internal CO₂ concentration was similar between dapat and WT plants, suggesting that reductions in CO₂ assimilation are caused by biochemical impairments or adjustments. Our findings demonstrated a decrease in PSI proteins and an increase in both PSI protein and ATP synthase γ chain 1 (Table 2). This opposite behaviour may be indicative of the occurrence of cyclic electron flow allowing the maintenance of chloroplastic electron transfer to sustain ATP synthesis. This finding is further supported by a disorder of redox proteins that regulate photosynthesis and others chloroplastic reactions (see Supplementary Table S1, S2).

Notably, a reduction of PSII proteins associated with the specific reduction of thioredoxin m₄₅, as previously observed (Serrato et al., 2013), may explain the cyclic electron flow taking place in the dapat mutant. Furthermore, the reduced levels of enzymes of the Calvin–Benson cycle such as GAPDH and PRK (Table 2) might culminate with a decreased flux through this cycle. Accordingly, PRK–GAPDH forms a complex mediated by the protein CP12, which is a regulatory step of the Calvin–Benson cycle (Marri et al., 2005; Serrato et al., 2013). Remarkably, when CP12 is in its oxidized form, this complex shows lower activity (Marri et al., 2009). Moreover, the activity of the PRK–CP12–GAPDH complex is regulated by thioredoxin (Howard et al., 2008; Marri et al., 2009). Our findings demonstrated a decrease in the levels of proteins of the thioredoxin system and this regulation is most likely associated with impairments of PRK–CP12–GAPDH, which becomes inactive, consequently reducing 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 Rubisco activase (Table 2), the reduced power generated as NADPH, which is normally used for CO₂ assimilation, might be redirected to other reactions. Here, we hypothesize that the NADPH produced by the chloroplastic electron chain is used by NADP-dependent malate dehydrogenase to produce malate. In good agreement, our organic acid measurements showed an accumulation of malate at ED (Fig. 4E). Accordingly, impairments in stomatal conductance have been associated with changes in organic acid metabolism as observed in both fumarase and succinate dehydrogenase antisense lines (Nunes-Nesi et al., 2007; Araújo et al., 2011a).

Combining physiological and biochemical approaches, we provide evidence that the DAPAT mutation impacts malate levels, partially explaining the stomatal impairments in dapat plants; however, the precise mechanistic link between Lys biosynthesis and malate levels, particularly at the guard cell level, remains to be identified.

We demonstrated that the DAPAT mutation culminates with higher levels of starch at EN and decreased rate of starch breakdown during the night indicating that both accumulation and turnover of starch are altered in dapat plants (Fig. 4A). The reduced capability of dapat plants to degraded starch can
lead to a putative starchless condition, which might culminate in reduced growth (Fig. 2B; Table 1). In good agreement with that, tight regulation between starch turnover and growth has been extensively demonstrated (Sulpice et al., 2009; Andriotis et al., 2012; Ragel et al., 2013) and that fast or incomplete exhaustion of starch culminated in reduced growth (Stitt and Zeeman, 2012). It should be mentioned, however, that the augmentation of protein content during the light period may lead to an increment of 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 (Fig. 2B). Thus, it can be assumed that dapat plants are most likely unable to efficiently degrade starch and this impairment may result in the growth arrest observed in dapat plants. We hypothesize that dapat plants use alternative substrates to sustain mitochondrial respiration and ATP synthesis, since dapat and WT plants presented similar dark respiration (Fig. 3D), provided by higher protein degradation to feed alternative respiration, as previously demonstrated (Araújo et al., 2010; Izumi et al., 2013; Avin-Wittenberg et al., 2015).

The Lys pathway can be assumed to be a respiratory bypass associated with 2-oxoglutarate production that is able to feed the TCA cycle (Araújo et al., 2010; Boex-Fontvieille et al., 2013). Following this assumption, our data provided novel insights into amino acid turnover during the diurnal cycle (Fig. 8). The daily fluctuation of both glutamate and glutamine in dapat plants indicates a strong variation of glutamate content. Intriguingly, the pattern observed in this mutant is clearly different from previous reports in which glutamate normally suffered a small oscillation during the day (Stitt et al., 2002; Gibon et al., 2006). Thus, strong variations in glutamate, glutamine, and proline seem to be associated with reduced aminotransferase activity caused by the DAPAT mutation. It is noteworthy that changes in metabolites observed in dapat plants are rather different from the changes verified in other mutants displaying lack of or low aminotransferase activity such as branched-chain amino acid aminotransferase 3 (hsaa3), tyrosine aminotransferase (tat), and ornithine–δ-aminotransferase (oat) (Funck et al., 2008; Knill et al., 2008; Riewe et al., 2012). When taken together with other Arabidopsis mutants involved in amino acid metabolism and/or related to stress (see Supplementary Fig. S4), our results suggest that the DAPAT gene plays a unique or specific role in cellular metabolism.

Changes in glutamate observed in dapat plants might be explained by the increase in Alanine:2-oxoglutarate aminotransferase (At1g23310) at ED. It is reasonable to assume, therefore, that to maintain basal levels of glutamine, dapat plants make use of glutamate as a precursor of glutamine, proline, and ornithine, which in turn increased at ED (Fig. 8). Alternatively, glutamate and their derivative amino acids decreased at EN. This amino acid reduction during the night suggests that they can be converted into 2-oxoglutarate that goes into the TCA cycle allowing the production of NADH and ATP. Furthermore, in the presence of proline there is an increased activity of both proline dehydrogenase and glutamate dehydrogenase to maintain the production of 2-oxoglutarate, which may be completely oxidized in the TCA cycle to support energy production (Schertl et al., 2014). Thus, glutamate and its derivatives are likely energetic sources supplying organic acids in this mutant. In agreement with this, our transcriptome data indicate an increase in glutamate degradation by GAD1, which is connected to the bypass for glutamate degradation to feed the TCA cycle with succinate mediated by the GABA shunt (Michaeli and Fromm, 2015).

Protein degradation providing substrates to respiration is likely present in dapat mutants. Autophagy is induced in dapat plants (Fig. 7A), and recent studies have reported autophagy is able to provide alternative substrates for respiration (Avin-Wittenberg et al., 2015; Barros et al., 2017), in agreement with drastic reductions in both isoleucine and Lys as well as tryptophan at EN (Fig. 8). By integrating this metabolic data with the transcriptome, which displays increases of IVDH and D2HGDH, related to catabolism of BCAAs and Lys, respectively (Engqvist et al., 2009; Araújo et al., 2010; Peng et al., 2015; Cavalcanti et al., 2017), it is reasonable to assume that alternative respiratory pathways mediated by ET-FETFQO are up-regulated in dapat plants. Furthermore, the maintenance of similar dark respiration rates in dapat plants might also occur through oxidation of malate that is accumulated during the light period. Diurnal oscillation in organic acid levels, especially malate, suggests that these organic acids are used as alternative respiratory substrates providing an important supply mainly under low carbohydrate conditions (Gibon et al., 2009). In this vein, the increased levels of NADH-dependent cME2 coupled with simultaneous increases of citrate synthase, the first commissioned step of the TCA cycle, and the metabolite profiles described above allow us to postulate a pathway for the maintenance of respiration in situations where DAPAT mutation impairs plant growth. Moreover, differential metabolic behaviour between the distinct mutants used here (see Supplementary Figs S4, S5) is highly suggestive of a unique and specific metabolic reprogramming in DAPAT mutant plants as summarized in Fig. 9. It thus seems reasonable to anticipate that the differences in growth and metabolism as depicted by changes in transcripts, proteins, and metabolites are most likely related to energetic factors occurring in response to the LL-DAPAT disruption. Our results thus suggest that the proper functioning of the pathway for Lys biosynthesis is important in fulfilling the energetic requirements to sustain plant growth and development.

We demonstrated here that impaired Lys biosynthesis caused by the DAPAT mutation culminated in constant stress conditions that resulted in an exquisite molecular and, consequently, physiological reprogramming. This reprogramming associated with photosynthetic reduction is in agreement with the dwarf phenotype (Fig. 2A) and that under stress conditions plants make use of alternative substrates for the maintenance of respiration (Araújo et al., 2010, 2011a). Altogether, our results demonstrated that growth reduction observed in the dapat mutant is likely due to an imbalance in storage and breakdown of carbon and nitrogen sources uncoupling growth from primary metabolism.
The specific mechanism in which DAPAT is metabolically involved is rather complicated (Fig. 9). Since Lys seems to be associated with feedback regulation at distinct levels (e.g. transcriptional or enzymatic), cross-pathway metabolic regulation associated with branching Lys metabolism or even other amino acids (Guyer et al., 1995; Azevedo and Arruda, 2010; Ufaz and Galili, 2008) is usually observed. The complex cross-pathway regulation in which Lys metabolism might be inserted seems to be thus a major constraint on biotechnological approaches to enhance Lys in crops and cereal grains, which represents a major nutritional problem for humans and for feeding livestock in developing countries (Galili, 2011; Galili and Amir, 2013; Wang and Galili 2016). The optimization of Lys levels in plants thus requires a comprehensive understanding of the biological processes regulating the homeostasis of this essential amino acid as well as the metabolic consequences of this homeostasis.

In summary, our data provide compelling evidence that the DAPAT mutation leads to energy limitation and culminates with strong alterations in cellular metabolism, and that alternative substrates are used when there is an imbalance in Lys biosynthesis allowing the proper functioning of plant respiration and energy generation. Whilst these data provide a clear connection between mitochondrial metabolism and Lys biosynthesis, future investigation is still required including a deeper focus on growth connections to fully elucidate the precise factors underlying this metabolic phenotype that mimics stress conditions in dapat mutants.
Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Bar chart of functional categories of differentially changed genes in *dapat* mutants (PageMan analysis; Usadel et al., 2006).

Fig. S2. 2D gel maps of rosette of Arabidopsis at two points: (A) end of day and (B) end of night (ED).

Fig. S3. Pie chart comparing the impact of each amino acid on amino acid pools between *dapat* and wild-type along diurnal cycle: (A) at the end of day (ED) and (B) at the end of night (EN).

Fig. S4. Heatmap comparison of GC-MS metabolite profiling between relative values of *dapat* and *dldps2* with the corresponding wild-type Col-0 and WS, respectively.

Fig. S5. Differences among *dapat*, *kin10* overexpression, and *lht1-1* mutants.

Table S1. Up-regulated genes in *dapat* mutant transcriptome.

Table S2. Down-regulated genes in *dapat* mutant transcriptome.

Table S3. List of primers utilized for qPCR.

Table S4. Metabolite profiling in leaves of WT and *dapat* plants.

Table S5. Detailed proteomic data.

Table S6. Metabolite profiling in leaves of *dldps2* and *dapat* as well as their corresponding wild-type Col-0 and WS, respectively.

Table S7. Metabolite profiling in leaves of *dapat*, *lht*, and *kin10* plants.

Table S8. PCA values.

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Author contributions

JHFC, GG, ARF, TA-W, and WLA designed the research; JHFC and MK carried out the research; JASB, CGSQ, IAPL, and TO contributed new reagents/analytical tools; JHFC, MK, JASB, ANN, GG, ARF TAW and WLA analysed the data; JHFC, MK, TAW and WLA wrote the article with comments from all the others.

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