Arabidopsis Seed Development and Germination Is Associated with Temporally Distinct Metabolic Switches*\[W\]

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While the metabolic networks in developing seeds during the period of reserve accumulation have been extensively characterized, much less is known about those present during seed desiccation and subsequent germination. Here we utilized metabolite profiling, in conjunction with selective mRNA and physiological profiling to characterize Arabidopsis (Arabidopsis thaliana) seeds throughout development and germination. Seed maturation was associated with a significant reduction of most sugars, organic acids, and amino acids, suggesting their efficient incorporation into storage reserves. The transition from reserve accumulation to seed desiccation was associated with a major metabolic switch, resulting in the accumulation of distinct sugars, organic acids, nitrogen-rich amino acids, and shikimate-derived metabolites. In contrast, seed vernalization was associated with a decrease in the content of several of the metabolic intermediates accumulated during seed desiccation, implying that these intermediates might support the metabolic reorganization needed for seed germination. Concomitantly, the levels of other metabolites significantly increased during vernalization and were boosted further during germination sensu stricto, implying their importance for germination and seedling establishment. The metabolic switches during seed maturation and germination were also associated with distinct patterns of expression of genes encoding metabolism-associated gene products, as determined by semiquantitative reverse transcription-polymerase chain reaction and analysis of publicly available microarray data. When taken together our results provide a comprehensive picture of the coordinated changes in primary metabolism that underlie seed development and germination in Arabidopsis. They furthermore imply that the metabolic preparation for germination and efficient seedling establishment initiates already during seed desiccation and continues by additional distinct metabolic switches during vernalization and early germination.

Seed maturation is characterized by a switch from maternal to filial metabolic regulation, a process that is dedicated to reserve synthesis and is coupled with the initiation of a nutrient uptake from the canopy (Weber et al., 2005). Maturing seeds of some plant species also gain photosynthetic capacity, which dramatically elevates the internal O\(_2\) levels leading to a partial relief of low oxygen conditions and thus ameliorating energy status of the tissue (Geigenberger, 2003; Rolletschek et al., 2005). At the metabolic level, the relatively O\(_2\)-poor stage of reserve accumulation is initiated by a transient stimulation of fermentative metabolism and continues with a switch from the energy-inefficient invertase to energy-efficient Suc synthase catalyzed route of Suc degradation (Bologa et al., 2003; Rolletschek et al., 2003; Goffman et al., 2005).

Seed germination is associated with degradation and mobilization of the reserves accumulated during maturation (Bewley, 1997; Borisjuk et al., 2004; Penfield et al., 2005). The efficiency of reserve mobilization during germination, and hence of seedling establishment itself, apparently depends on the extent of reserve accumulation during seed maturation. This process is itself heavily influenced by C/N partitioning capacity of the maturing seeds (Eastmond and Rawsthorne, 2000; Eastmond and Graham, 2001; Brocard-Gifford et al., 2003). However, intriguingly, significant reduction in the oil reserve content of Arabidopsis (Arabidopsis thaliana) seeds, as observed in specific mutants, slows down but does not significantly arrest seed germination (Katavic et al., 1995; Focks and Benning, 1998; Lu and Hills, 2002). In combination, these studies suggest that germination and storage reserve mobilization are independently

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regulated (Pritchard et al., 2002). Recent experiments also suggest that seed germination is only slightly affected, if at all, in mutants deficient in lipid reserve mobilization pathways (Penfield et al., 2005). When taken together these observations suggest that germination efficiency is not only affected by reserve accumulation during seed development or their mobilization during seed germination, but also by additional, as yet unknown factors. To elucidate the nature of such factors, we analyzed the metabolite content of Arabidopsis seeds at several different developmental stages, in combination with reverse transcription (RT)-PCR analysis of mRNA levels of selected metabolism-associated genes and bioinformatics analysis of publicly available microarray data. Although previous (Wobus and Weber, 1999; Girke et al., 2000; Baud et al., 2002) and more recent studies (Baud and Graham, 2006) unveiled new insights and important contributions to the understanding of seed maturation metabolism, the data presented here complement and extend these studies by providing a broad overview of changes in primary metabolic processes that occur during Arabidopsis seed development and germination. Given that very few comprehensive metabolite profiling studies have been reported concerning plant development (Sessa et al., 2000; Baxter et al., 2005), they furthermore illustrate that the utility of metabolic profiling extends beyond its much documented use as a diagnostic tool (Fiehn et al., 2000; Sumner et al., 2003; Fernie et al., 2004a).

RESULTS

Arabidopsis Seeds Maintain Comparable PSII Fluorescence through the Entire Period of Reserve Accumulation

Maturing seeds of different species possess photoheterotrophic plastids, which maintain photosynthetic activity during the period of reserve accumulation, contributing both oxygen and metabolic reassimilation of CO2 that is released by reserve biosynthesis (Rolletschek et al., 2003, 2005; Ruuska et al., 2004). Transcript profiling analysis of maturing Arabidopsis seeds suggests that the mRNAs encoding photosynthesis genes peak at the onset of reserve accumulation and then decline during the period of reserve accumulation (Ruuska et al., 2002). Yet, whether the decline in mRNA levels of photosynthesis genes is also correlated with a down-regulation of photosynthetic activity is not clear because Arabidopsis seeds remain green during the entire stage of reserve accumulation (Fig. 1A, 10 ± 1 to 18 ± 1 d after flowering [DAF]). To correlate photosynthetic activity with the metabolic status of the maturing Arabidopsis seeds, we analyzed the photosynthetic activity (as measured by fluorescence kinetics measurements of PSII) every 2 d during the period of reserve accumulation (10 ± 1 to 18 ± 1 DAF).

Figure 1. Photosynthesis in developing Arabidopsis seeds. A, Morphology of developing seeds of Arabidopsis from 4 ± 1 to 20 ± 1 DAF. B, PSII fluorescence measurements (Fv/Fm; Fv = 60 of approximately 100 seeds) of maturing seeds at 10 ± 1, 12 ± 1, 14 ± 1, and 18 ± 1 DAF. Values are average of two repeats. The average value for leaves from the same plant was 0.8 Fv/Fm (data not shown). C, Semiquantitative RT-PCR analyses of mRNAs encoding a light-harvesting complex protein of PSII (LHCII), PSII reaction center protein (PSI), and actin (ACT) as the control, in maturing seeds at 10 ± 1, 14 ± 1, and 17 ± 1 DAF.

Data suggests comparable photosynthesis efficiency until the desiccation period (Fig. 1B), even though the mRNA levels of the representative light-harvesting complex II and PSI genes are declining with maturity (Fig. 1C), in keeping with previous reports (see, for example, Ruuska et al., 2002).

Metabolic Profiling Analysis of Arabidopsis Seeds

We next expanded our analysis to seed primary metabolism to discern metabolic networks during seed maturation and germination. For this purpose, we utilized an established gas chromatography-mass spectrometry (GC-MS) protocol (Roessner et al., 2001) with which we were able to quantify the relative content of over 70 metabolites. Four different stages of seed maturation were analyzed: (1) 10 ± 1 DAF, corresponding to the onset of reserve accumulation (Ruuska et al., 2002); (2) 14 ± 1 DAF, corresponding to the middle stage of reserve accumulation (Ruuska et al., 2002); (3) 17 ± 1 DAF, corresponding to the end of the period of reserve accumulation and the beginning of seed desiccation, in which desiccation-associated proteins are synthesized (Wobus and Weber, 1999); and (4) dry seeds. In addition, we analyzed two different stages of seed germination: (1) vernalization for 72 h at 4°C in the dark; and (2) vernalization for 72 h at 4°C in the dark, followed by an additional growth for 24 h at 21°C in the light (germination sensu stricto, before radical protrusion; seed detailed conditions in “Materials and Methods”). The metabolic analyses were performed twice in two seasons with similar relative patterns of metabolite profiles. The seasonal variations however were quite large, apparently due to environmental
differences, making it difficult to combine the data. Here we present the results of one representative season. The entire separate data of the two seasons is presented as Supplemental Tables S1 to S6. The metabolic data are reported as relative metabolite content as it is commonly used in metabolic profiling studies to ease interpretation of large data sets; however, we have additionally performed calibration of the data and refer the interested reader to the absolute values presented in Supplemental Table S7.

**Alterations in Metabolic Profiles during the Period of Reserve Accumulation**

As shown in Figure 2, the progression of seeds from 10 ± 1 DAF to 14 ± 1 and 17 ± 1 DAF was associated with major decreases in the levels of most amino acids, sugars, polyols, and organic acids, apparently reflecting their rapid utilization in the storage reserve accumulation process. The level of fatty acids that could be detected in the polar fraction of our GC-MS analysis were generally slightly increased from 10 ± 1 to 14 ± 1 DAF and then dropped from 14 ± 1 to 17 ± 1 DAF probably emphasizing the dynamic conversion of sugars into fatty acids and their subsequent incorporation into oil. This process is similarly reflected in the patterns of change in the level of Suc, which, in contrast to the general reduction of sugars, was significantly increased from 10 to 14 ± 1 DAF. The results imply that at this stage the rate of carbon import in the seeds exceeds the rate of Suc cleavage and subsequent entrance of carbon into glycolysis. As opposed to the general reduction in the levels of sugars, the level of raffinose was enhanced during this period, particularly at 14 ± 1 DAF. Metabolites of the tricarboxylic acid (TCA) cycle showed different trends of change from 10 to 17 ± 1 DAF. The levels of four TCA-cycle intermediates, namely citrate, malate, succinate, and particularly 2-oxoglutarate (47-fold reduction) were progressively reduced throughout the period of reserve accumulation (Fig. 2). In contrast, the levels of fumarate and isocitrate increased from 10 ± 1 to 14 ± 1 DAF and decreased from 14 ± 1 to 17 ± 1 DAF, indicating the presence of some variations in mitochondrial metabolism/function occurring at the early (10–14 ± 1 DAF) and late (14–17 ± 1 DAF) stages of reserve accumulation (Fig. 2). It should be noted, however, that the pattern of change in the level of these two metabolites at the early to late stages of reserve accumulation varied markedly between the two seasons. In the second season, isocitrate decreased similarly to the other TCA intermediates, while fumarate increased from 10 ± 1 DAF to 17 ± 1 DAF. The levels of amino acids were also generally decreased from 10 ± 1 DAF to 17 ± 1 DAF (Fig. 2). Most noticeable though was a 184-, 81-, and 38-fold decrease in the respective levels of Gln, Asn, and Lys. These changes apparently signify the incorporation of the free amino acids into storage proteins, coupled with enhanced metabolism of Gln, Asn, and Lys (see “Discussion”).

**Alterations of Metabolic Profiles during the Transition from the Period of Reserve Accumulation to the Period of Seed Desiccation**

The desiccation period (transition from 17 ± 1 DAF to mature seeds) was associated with a major switch in seed metabolism. As opposed to the period of reserve accumulation, which was associated with a general reduction in the levels of most metabolites (Fig. 2), the desiccation period was associated with a general increase in most amino acids, sugars (including raffinose), polyols, organic acids (with the exception of TCA intermediates), as well as in fatty acid-related compounds and slight increase in fatty acids (Fig. 3; Supplemental Tables S2 and S4). This indicates that, as opposed to the accumulation of oil and storage proteins between 10 ± 1 and 17 ± 1 DAF, the desiccation period is associated with a major increase in levels of free metabolites. In detail, there was a dramatic accumulation of the nitrogen-rich amino acids (Asn, Lys, and Arg), the aromatic amino acids (Trp, Phe, and Tyr), Ser, Ala, and the nonproteogenic amino acid γ-amino-butyrate (GABA; Fig. 3). Considering TCA-cycle intermediates, fumarate and succinate were the only metabolites that displayed a major increase in their content, while the other TCA-cycle metabolites declined further during this period. Concerning other metabolites determined in this study, the levels of Suc, Gal, Ara, trehalose, sorbitol/galactitol, gluconate 6P, and glycerate exhibited the greatest increase, while there was a major decrease in the levels Fru 6P and Glc 6P (Fig. 3).

**Principal Component Analysis Suggests That Different Stages of Seed Maturation Are Associated with Distinct Metabolic Profiles**

To get a global view on the metabolic changes that occur during seed development, we also analyzed the data set by a principal component analysis (PCA; Saeed et al., 2003; Scholz et al., 2004; Fig. 4). In this analysis, the samples of a given time point were relatively indiscriminant from one another and the distances between sample types increased across the first component with the number of DAF. Thus, 10 ± 1 and 14 ± 1 DAF samples were situated relatively closely to one another, while 17 ± 1 DAF samples formed a more discrete group on the principal components (Fig. 4, A and C). A similar separation reflects the primary changes in metabolite levels during seed development (Figs. 2 and 3). Furthermore, the 17 ± 1 DAF time point was characterized by an extensive negative trend of changes in metabolites that was partially inverted during desiccation (Fig. 4, A and C). This pattern might have contributed to the separation of 17 ± 1 DAF on the second and third components (Fig. 4B). Mature seeds formed the most separate samples from the point of origin (10 ± 1 DAF) on the first component (Fig. 4, A and C). Compounds that played a considerable contribution to the sample...
discrimination were sugars (Fru and raffinose), sugar phosphates (Glc 6P and Fru 6P), TCA-cycle intermediates, and amino acids (e.g. Gln and Asn). Interestingly, the loadings of component 3, which seems to have a specific impact on the discrimination of time point 17 ± 1 DAF (Fig. 4, B and C), were highly enriched for amino acids in comparison to those of the other components (1 and 2; data not shown). When an independent component analysis (ICA) was applied to the data set very similar trends to those described above were observed (data not shown).

**Figure 2.** Changes in the contents of metabolites during the period of reserve accumulation in Arabidopsis seeds. Values of 14 ± 1 and 17 ± 1 DAF are normalized to the mean response calculated for 10 ± 1 DAF, which was given the value of 1. Value bars facing the right of each section indicate relative increased content as compared to 10 ± 1 DAF. Value bars facing the left of each section indicate the fold-decreased content relative to 10 ± 1 DAF. Values are representative of two independently grown sets of plants and are presented as the mean ± se of three biological repetitions of 30 mg of isolated seeds bulked from at least 10 plants for each time point. For statistical analysis and absolute content (nmol g fresh weight) see Supplemental Tables S1 and S3.

β-ala, Beta Ala; L-AsA, ascorbate; DHA, dehydroascorbate; Fru 6P, Fru 6 phosphate; Glu 6P, Glc 6 phosphate; Myo-ino, myo-inositol; 2-OG, 2-oxoglutarate; PhoA, phosphoric acid; ShikA, shikimate; ThrA, threonate; Treh, trehalose.

**Semiquantitative RT-PCR Analysis of Changes in mRNA Levels of Selected Genes during the Periods of Reserve Accumulation and Desiccation**

We also wished to study patterns of gene expression programs of representative metabolism-associated
genes during seed maturation and germination, using semiquantitative RT-PCR analysis (Fig. 5). The different genes were categorized into three groups based on their expression patterns during seed maturation and germination. The first group consisted of genes whose mRNA levels were relatively high during the initiation of reserve accumulation (10 ± 1 DAF), declined during the periods of reserve accumulation and desiccation (14 ± 1 DAF to dry seeds), and then increased upon the progression of germination (Fig. 5A). This group contained genes encoding photosynthesis-associated proteins (PSI), glycolytic enzymes (fructokinase, Fru bisphosphate aldolase, as well as cytosolic and plastid pyruvate kinases), two succinate dehydrogenase iso-enzymes, and nitrogen metabolism enzymes (GABA transaminase and asparaginase). The second group contained genes whose mRNA levels increased during the periods of reserve accumulation and desiccation (10 ± 1 dry seeds) and remained at relative high levels during seed germination (Fig. 5B). This group included isocitrate lyase of peroxisomal lipid degradation, phosphoenolpyruvate (PEP) carboxykinase of gluco-ogenesis, dihydricolic polyol synthase-2 of Lys biosynthesis, and Thr synthase of Thr biosynthesis. The third group (Fig. 5C) contained genes whose mRNA levels increased during seed maturation and declined during seed germination. This group included trehalose phosphate phosphatase of trehalose metabolism,
alcohol dehydrogenase, a cruciferin storage protein, enzymes of amino acid biosynthesis (Thr synthase, Trp synthase, and Asn synthase), as well as mitochondrial fumarylacetoacetate hydrolase. Fumarylacetoacetate hydrolase is the last enzyme of Tyr catabolism, producing the Kreb’s cycle metabolite fumarate, which accumulates during seed desiccation (Fig. 5C).

Alterations of Metabolic Profiles Occurring during Seed Vernalization and Germination Sensu Stricto

We collected and analyzed samples of imbibed Arabidopsis seeds following seed vernalization (imbibition at 4°C/dark for 72 h) and following transfer to germinative conditions (21°C/light for 24 h). As shown in Figure 6, the vernalization period was associated with changes in the level of different metabolites. While the levels of numerous amino acids were reduced

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**Figure 4.** PCA of metabolite profiles of distinct developmental stages during seed maturation. PCA is presented as the combinations of the first three dimensions, which together comprise 85.6% of the metabolite variance. Each data point represents an independent sample. The analysis of the data was performed using the TMEV software (Saeed et al., 2003). Component 1 explained 52.18% of the variance, component 2 explained 21.23%, and component 3 explained 12.17%. A to C represent components 1 + 2, 2 + 3, and 1 + 3, respectively. The combined percentages of the variance for each plot are given in brackets in each section.

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**Figure 5.** Semiquantitative RT-PCR analysis of mRNAs of selected genes in maturing and germinating Arabidopsis seeds. Total RNA was extracted independently from two different batches of isolated seeds at 10 ± 1, 14 ± 1, 17 ± 1 DAF, dry seeds; seeds after 72 h vernalization (imbibition for 72 h at 4°C in the dark), and seeds exposed to vernalization plus additional 24 h in light at 22°C (germination sensu stricto). At least three reactions were performed with each set of primers, as described in M&M. D, Dry seeds; V, vernalized seeds; G, germination sensu stricto; –RT, negative control with no RT reaction; PSI, PSI reaction center subunit III; FK, fructokinase; FB, Fru bisphosphate aldolase; GABATA, GABA transaminase; ASPA, asparaginase; PKP, pyruvate kinase plastidic; PKC, pyruvate kinase cytosolic; SDH1, succinate dehydrogenase1; SDH2, succinate dehydrogenase2; ICL, isocitrate lyase; PEPCK, PEP carboxykinase; DHPS2, dihydrodipicolinate synthase; THS, Thr synthase; TPP, trehalose-6-P phosphatase; ADH, alcohol dehydrogenase; CRU3-12S, storage protein; TBS2, Trp synthase β-subunit; FAH, fumarylacetoacetate hydrolase; ASN-1, Asn synthase; ACT, actin 2 (used as a control).
to different extents, with GABA, Gly, and Phe exhibiting major reductions over this time period, intriguingly, the levels of Asp, Thr, and Ser significantly increased. Similarly, while the levels of most sugars variously declined, the levels of Fru 6P and Glc 6P were significantly elevated. The levels of dehydroascorbate, 3-phosphoglycerate, and the TCA-cycle intermediates 2-oxoglutarate and isocitrate dramatically increased during this time period. In contrast, the levels of other acids, namely, gluconate 6P, glycerate, and threonate were significantly reduced. The transition of seeds from vernalization to germinative conditions (Fig. 7) was generally associated with an exacerbation of the metabolic changes observed during vernalization (with the exceptions of Arg, Asn, Gly, and Gln and fatty acids, the levels all of which were invariant). This general observation suggests that the transition between vernalization and germination sensu stricto is associated with the activation of initial important metabolic processes needed for seed germination. It is also likely that germination is associated with a follow up of additional metabolic processes, which occur slightly later during germination and therefore could not be observed by our metabolic profiling.

**Changes in Gene Expression Programs Occurring during Vernalization and Germination SensuStricto**

In addition to our semiquantitative RT-PCR analysis of representative genes (Fig. 5) we addressed changes
in gene expression during vernalization and germina-
tion sensu stricto using bioinformatics approach. Using publicly available microarray (AffyWatch, Not-
ttingham Arabidopsis Stock Centre [NASC] Affymetix service; http://Arabidopsis.info/) of imbibed and ger-
minating Arabidopsis seeds, we analyzed two inde-
pendent experiments, both performed on Columbia-0
seeds by Lutz and associates (Nover, Botanisches
Institut, Germany) as follows: (1) an experiment mea-
suring the expression profile of dry seeds and seeds
following 1 or 3 h of imbibition; and (2) an experiment
comparing changes in mRNA levels occurring 24 h
after imbibition, compared to dry seeds in wild-type
plants as a part of a hormonal treatment experiment.
This analysis revealed changes in the mRNA levels of
genes with various functions, but we focused only on
genes associated with primary metabolism. Since many
specific molecular and enzymatic functions operate by
gene families, in such cases we summed the GeneChip
values derived from the different gene families. The

Figure 7. Changes in the contents of metabolites during the transition of Arabidopsis seeds from vernalization to germination sensu stricto. Values of seeds imbibed at 4°C in the dark for 72 h and then transferred to germinative conditions (21°C in the light) for additional 24 h are normalized to the mean response calculated for dry seeds. Value bars facing the right of each section indicate relative increased content as compared to dry seeds. Value bars facing the left of each section indicate the fold-decreased content relative to dry seeds. Values are representative of two independently grown sets of plants and are presented as the mean ± se of three biological repetitions of 30 mg of isolated seeds bulked from at least 10 plants for each time point. For statistical analysis and absolute content (nmol g fresh weight⁻¹) see Supplemental Tables S5 and S6. β-al, beta Ala; L-AsA, ascorbate; DHA, dehydroascorbate; Fru 6P, Fru 6 phosphate; Glu 6P, Glc 6 phosphate; Myo-ino, myo-inositol; 2-OG, 2-oxoglutarate; PhoA, phosphoric acid; ShikA, shikimate; ThrA, threonate; TreA, trehalose.
function of the different genes or gene families were deduced from the AraCyc, Arabidopsis biochemical pathways (http://www.arabidopsis.org/tools/aracyc/), which contains hierarchical information of metabolic pathway including the enzymatic reaction in each pathway and the genes that encode each enzyme. In instances in which we felt that this database may be wrong, manual corrections of gene functional annotations were introduced. Table I presents the data of genes or gene families whose combined mRNA levels were significantly different (either up-regulated or down-regulated) at the 0.05% level between the dry and imbibed seeds in at least one of the experiments (see “Materials and Methods”), while the accession numbers of these genes and their grouping into gene families, whose GeneChip values were summed up, are provided in Supplemental Table S8.

As shown in Table I, imbibing seeds for 1 h caused no significant difference in the mRNA levels of any of the genes present on the GeneChip. Imbibing seeds for 3 h caused significant increase in the mRNA levels of several genes associated with various metabolic functions, including the OPPP pathway (phosphogluconate dehydrogenase), TCA cycle (succinyl-CoA synthetase), fatty acid biosynthesis (3-oxoacyl-[acyl-carrier protein] reductase), nitrogen assimilation (nitrite reductase), polyamine biosynthesis (Arg decarboxylase), ribonucleotide biosynthesis (phosphoribosylformylglycinamidine cyclo-ligase), Lys biosynthesis (dihydropriocinate reductase and dimaminopimelate decarboxylase), and ethylene biosynthesis (1-aminocyclopropane-1-carboxylate synthase). Upon 3 h imbibition, the mRNA levels of two genes encoding enzymes of amino acid degradation as well as one enzyme of trehalose metabolism were significantly reduced, compared to dry seeds. The transition from 3 h imbibition to 24 h imbibitions was generally associated with a general enhancement in the mRNA levels of genes whose increased expression was detected already after 3 h imbibition. These generally signified major increases in mRNA levels of genes associated with biosynthetic processes and reductions in mRNA levels of genes associated with the degradation of amino acids (Gly, Leu, and Tyr). Notably, although the mRNA levels of a number of TCA-cycle enzymes were up-regulated during imbibition, those encoding the TCA-cycle enzyme oxoglutarate dehydrogenase were slightly, but significantly, down-regulated during 24 h imbibition. This may indicate an integration of the GABA shunt with the TCA cycle.

**DISCUSSION**

In this study we complement published observations at the transcript and protein level by performing a combined analysis of the metabolic processes occurring in Arabidopsis seeds during the periods of reserve accumulation, desiccation, vernalization, and germination sensu stricto. Although some reports on the metabolism of seed development have been published previously they have tended to focus on earlier stages of seed development and on specific biochemical pathways largely concentrating on sugar components (Baud et al., 2005; Baud and Graham, 2006; Gomez et al., 2006), fatty acids and oil (Cernac and Benning, 2004; Lin et al., 2004; Vigeolas et al., 2004; Gómez et al., 2005), or specific transcriptional programs (Hobbs et al., 2004; Braybrook et al., 2006). Broader transcriptional studies have also been previously conducted with an emphasis on the stages of development from 5 to 13 DAF (Ruuska et al., 2002). Here it was our intention to provide a metabolomic complement to more global studies of seed development and germination in Arabidopsis. However, discussion is focused toward the more novel aspects of our data set, i.e. the transition from seed maturation to desiccation and further to vernalization and germination sensu stricto. While we observed seasonal variation in the results obtained as well as some differences to published data, this was minor and furthermore a similar pattern of change in the metabolite levels was maintained during seed development and germination in all studies reported. This variation is, however, of interest since cardinal features of metabolic shift would be expected to be highly conserved across all studies. We highlight instances where this is indeed the case in the concluding paragraph.

**Metabolism during Reserve Accumulation**

Our results showed that the period of reserve accumulation (10 ± 1 to 17 ± 1 DAF) is associated with major reductions in the levels of many primary metabolites, including amino acids, sugars, polyls, and organic acids (Fig. 2). The patterns of change in the levels of the measured primary metabolites likely signify their efficient incorporation of sugars and organic acids into fatty acid and amino acid biosynthesis, on route to production of oil and storage proteins. This is specifically reflected by the characteristic changes in the levels of metabolites such as Suc and 3-phosphoglycerate (Fig. 2). These observations also indicate that Suc import between 10 ± 1 and 14 ± 1 DAF exceeds the rate of its incorporation into the glycolytic pathway, and glycolytic rate and fatty acid biosynthesis exceeds oil production.

Even though the levels of all measured amino acids were reduced during the period of reserve accumulation, the levels of Asn, Gln, and Lys were reduced much more prominently than others (Fig. 2). This apparently signifies their extensive metabolism into other amino acids. The metabolic changes occurring during the period of reserve accumulation were also associated with distinct changes in gene expression. The general reduction in sugars, polyls, and organic acids was accompanied by a progressive decline in the expression of genes encoding enzymes in glycolysis and other pathways of carbon metabolism (Fig. 5). Yet, in contrast to the reduction in the levels of amino acids, the mRNA levels of genes encoding enzymes of amino acid biosynthesis were increased during this period.

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Table I. Changes in mRNA levels during seed imbibition for 1, 3, and 24 h

Data was obtained from public Affymetrix GeneChip arrays (ATH1-121501) and processed as described in “Materials and Methods.” Values represent fold changes relative to those in dry seeds, which were given the value of 1. Numbers set in bold type represent statistically significant changes.

<table>
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<th>24 h</th>
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<td>Leu biosynthesis</td>
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Metabolic Switch
Desiccation Stage Is Associated with a Distinct Energy Status and Photosynthesis during Seed Development and Germination

The transition from reserve accumulation to seed desiccation exhibited a continuous decrease in the levels of Fru 6P and Glc 6P (Fig. 3), implying a reduced incorporation into glycolysis. Yet, the period of seed desiccation (occurring from 17 ± 1 DAF to mature seeds) was associated with a significant increase in the levels of several free amino acids as well as a number of organic acids and sugars. Prominent among these changes was that of raffinose, which has previously been shown to increase during late maturation and that was suggested as important in energy storage and membrane protection during desiccation (Baud et al., 2002 and refs. therein). These changes are illustrated schematically in Figure 8A. Similarly, a significant increase in trehalose suggests an important functional role for this metabolite during seed desiccation. Indeed, a knockout mutant of the trehalose phosphate synthase-1 gene fails to develop normally, being arrested just before the period of reserve accumulation (Eastmond and Graham, 2003; Gómez et al., 2005). Since trehalose is known to stabilize proteins (Kaushik and Bhat, 2003), a possible function of this metabolite during the period of reserve accumulation and desiccation may be in the protection of the formation of storage and housekeeping proteins from dehydration.

Among the amino acids the levels of the nitrogen-rich Asn, Arg, and Lys, the nonprotein amino GABA, and the aromatic amino acids Trp, Phe, and Tyr were most dramatically increased at this developmental stage (Fig. 8A). These changes were associated with high mRNA levels of genes encoding enzymes of amino acid biosynthesis, implying that amino acid biosynthesis apparently continues during the desiccation period, and that at least some amino acids significantly accumulate in their free forms rather than being incorporated into storage proteins. These lines of evidences taken in the context of current models of seed metabolism suggest that the seed desiccation process preferentially favors (1) the decrease of the C/N ratio, (2) the accumulation of free amino acids rather than storage proteins, and (3) the production of shikimate-derived secondary metabolites associated with defense (Hull et al., 2000; Glawischnig et al., 2004; Piotrowski et al., 2004) and indole-3-acetic acid biosynthesis (Campanella et al., 2004; Sharaf and Farrag, 2004) that are necessary triggers for seed germination (Normanly, 1997; Slavov et al., 2004). In addition to the accumulation of free amino acids and various carbon metabolites, the period of seed desiccation was also associated with small increases in the levels of various fatty acids, fatty acid-related compounds such as glycerol, as well as intermediates of fatty acid degradation (Fig. 3). These are likely derived from oil degradation, which was previously shown to occur in this period (Chia et al., 2005).

The transition from the period of reserve accumulation to seed desiccation was illustrated using PCA, a complementary statistical tool for the global diagnosis of metabolic changes. Indeed it enables a bird view of the metabolic changes occurring along the period of reserve accumulation and desiccation, were also illustrated using PCA. This could potentially be due to a limitation in the availability of sufficient oxygen for mitochondrial respiration and production of ATP and reducing equivalents. The light reaction of photosynthesis, particularly PSII, enhances the levels of oxygen as well as ATP and redox power during seed maturation (Ruuska et al., 2004; Borisjuk et al., 2005; Rolletschek et al., 2005). Indeed, we show here that PSII activity is kept relatively constant throughout seed maturation (Fig. 1C). Nevertheless, both our own (Fig. 1A) and previous studies (Ruuska et al., 2002) revealed that mRNA levels of photosynthesis genes peak at the onset of reserve accumulation and decline thereafter. These data suggest that photosynthesis supports metabolism during the entire period of reserve synthesis and accumulation and that mitochondrial energy metabolism is down-regulated at this time point and as such provide further evidence for the coordination of respiratory and photosynthetic processes (Raghavendra and Padmasree, 2003; Fernie et al., 2004b).

The Transition from Reserve Accumulation to Seed Desiccation Stage Is Associated with a Distinct Metabolic Switch

The transition from the period of reserve accumulation to seed desiccation exhibited a continuous decrease in the levels of Fru 6P and Glc 6P (Fig. 3), implying a reduced incorporation into glycolysis. Yet, the period of seed desiccation (occurring from 17 ± 1 DAF to mature seeds) was associated with a significant increase in the levels of several free amino acids as well as a number of organic acids and sugars. Prominent among these changes was that of raffinose, which has previously been shown to increase during late maturation and that was suggested as important in energy...
transition from seed maturation to desiccation. Interestingly, the most significant loadings contributing to the discrimination of this developmental stage were mainly characterized by amino acids.

Why do maturing seeds follow a metabolic switch during seed desiccation, changing from accumulation of oil and storage protein to that of free amino acids, sugars, as well as fatty acids and also their degradation products? It has been previously hypothesized that degradation of fatty acids during seed desiccation may provide energy needed for metabolic activity in this period (Chia et al., 2005). Yet, our present finding suggests that metabolism during seed desiccation has an additional function: to render certain metabolites rapidly available to support metabolic recovery during imbibition, before storage reserves degradation and mobilization. An interesting support of this hypothesis is the increased activity of the gluconeogenetic enzyme PEP carboxykinase (catalyzing the synthesis of PEP from oxaloacetate) during seed desiccation, which according to previous publications (Walker et al., 1999) is not coupled with a further major gluconeogenetic conversion of PEP into sugars (Chia et al., 2005) but could rather be utilized for the production of Asp family amino acids, aromatic amino acids, or Ala (Lea and Ireland, 1999).

Functions of the Mitochondria and Glyoxysome during Seed Maturation

Notably, despite the major reduction in the levels of TCA-cycle intermediates during seed maturation and their relatively low levels in mature seeds, the level of fumarate showed a very distinct pattern of change during seed maturation. Indeed it initially increased (10 ± 1 to 14 ± 1 DAF), while dropping during the second stage of reserve accumulation (14 ± 1 to 17 ± 1 DAF), eventually increasing dramatically during seed desiccation (17 ± 1 DAF to mature seeds; Figs. 2 and 3). It should be noted that in a different season, fumarate content increased throughout maturation with the most significant change during desiccation (17 ± 1 DAF to mature seeds; Figs. 2 and 3). It should be noted that in a different season, fumarate content increased throughout maturation with the most significant change during desiccation. The mechanism behind this unprecedented degree of fumarate accumulation in seeds has yet to be elucidated. It is tempting to hypothesize that it may signify a mitochondrial transition between respiratory and anaerobic activity, analogous to the bidirectional fermentative mitochondria occurring in diverse organisms.

Figure 8. Schematic illustration of changes in the levels of metabolites synthesized by metabolic pathways localized to different subcellular organelles. A and B represent changes occurring during seed desiccation (transition from 17 ± 1 DAF to mature seeds; data derived from Fig. 3) and seed vernalization (transition from mature seeds to seeds vernalized for 72 h in the dark; data derived from Fig. 6), respectively. Blue and red letters indicate metabolites whose levels were either decreased or elevated, respectively. Metabolites whose levels were unchanged are marked by bold, black letters. Metabolites whose levels were not measured are marked in italics. Different organs are marked in green (plastids), pink (mitochondria), and purple (glyoxysomes). Arrows represent one or multiple enzymatic steps. AsA, Ascorbate; DHA, dehydroascorbate; FA, fatty acids; Fum, fumarate; Isocit, isocitrate; Mal, malate; 2OG, 2-oxoglutarate; OAA, oxaloacetate; 3PGA, 3-phosphoglycerate; Pyr, pyruvate; Shik, shikimate; Succ, succinate.
Accumulated during Seed Desiccation

Metabolic Switch That Mainly Consumes Metabolites

Imbibition of Mature Seeds Stimulates a Special Metabolic Switch That Mainly Consumes Metabolites Accumulated during Seed Desiccation

While oil and protein reserves are long documented to be mobilized following radical protrusion (Bewley, 1997; Eastmond and Graham, 2001), our results show that active metabolic processes already initiate during seed imbibition (or vernalization). The major metabolic changes observed during this period were significant reductions in the levels of the majority of different metabolites, which accumulated during the period of seed desiccation (Fig. 6; see also schematic illustration in the left section of Fig. 8B). This implies that primary metabolites might be rapidly consumed to support the metabolic switch toward enhancing biosynthetic processes needed for early germination. The accumulation during vernalization of hexose phosphates (Fig. 8B) apparently signifies the entrance of sugars into the glycolytic pathway, while the accumulation of the TCA-cycle intermediates 2-oxoglutarate and isocitrate (Fig. 8B) potentially signifies the initiation of metabolic and respiratory functions of the mitochondria. The drop in Gly and increase in Ser levels during vernalization (Fig. 8B) could prime peroxisomal metabolism for photorespiration.

Despite the general reduction in the levels of amino acids during vernalization, the levels of Asp and Thr were significantly increased during this period (Figs. 7 and 8B) and further boosted strongly during subsequent germination (Fig. 7). This indicates a specific activation of the Thr branch of the Asp-family pathway, which also leads to the synthesis of Met. The importance of the Asp-family pathway metabolism (through Met and S-adenosylmethionine [SAM]) to seedling growth is also supported by previous transcript profiling and proteomics studies (Ravanel et al., 2004), showing that the mRNA level of SAM synthase and the protein level of SAM decarboxylase (that converts SAM into polyamines) increase rapidly during germination.

Our bioinformatics analysis also showed that germination-associated gene expression programs start already during seed imbibition (Table I). This generally includes the activation of genes encoding enzymes in biosynthesis pathways associated with sugar metabolism, TCA cycle, nitrogen assimilation, fatty acid production, and photosynthesis as well as the suppression of degradation pathways of some specific amino acids (see details of specific enzymes in "Results" and in Table I). Induced expression of these genes during imbibition apparently serves as a basis for a boost in biosynthetic processes commenced during subsequent germination (see next section). Notably, the mRNA levels of several genes controlling trehalose biosynthesis (Fig. 5; Table I) were down-regulated during seed imbibition, emphasizing the regulatory properties of trehalose during germination (Schluempf et al., 2004).

Postimbibition Germination Is Associated with a Boost of Biosynthetic Metabolic Processes That Initiate during Imbibition

During postimbibition early germination we observed an increase in the levels of many metabolites, including amino acids, sugars, and organic acids, which apparently reflects the boost of biosynthetic processes. During this period, we also measured a significant increase in the level of dehydroascorbate (Fig. 6). Dehydroascorbate is derived from the antioxidant ascorbate, which is maintained at a constant steady-state level apparently because it is replenished from the hexose phosphate pool. This metabolic program may assist in scavenging of reactive oxygen species resulting from the reoxygenation of the germinating tissues and that might damage proteins and membranes (Job et al., 2005). Recently, Arabidopsis mutants impaired in the biosynthesis of the antioxidant vitamin E were shown to exhibit severe growth defects during germination (Sattler et al., 2004).

In conclusion, in our study we show that early germination events are characterized by the efficient reactivation of metabolic pathways via the availability of key precursors as well as a coordination of energy metabolism. As summarized in the descriptive Figure 8, A and B, we show that specific sets of metabolic primers, distributed across the metabolic network, are synthesized during late maturation. These primers...
successively decrease during seed vernalization concomitantly to increases in specific metabolites of the central pathways but prior to major mobilization of storage reserves. Several conserved features are apparent in both harvests analyzed as well as in the literature, thus confirming a high biological relevance of these changes in the process of seedling development. The results described here also provide a strong complement to studies carried out at the transcript and protein level in the understanding of seed metabolism that is of great interest both from a fundamental and an applied perspective (Alonso-Blanco et al., 2003). The ability to regulate seed development, particularly dormancy, has great agronomic potential. The advent and growing utilization of inducible promoter systems for transgene expression (Zuo et al., 2000; Junker et al., 2004) should allow further dissection of the relative functional importance of elements of the metabolic networks described within this report.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich Israel with the exception of N-methyl-N-(trimethylsilyl)triﬂuoroacetamide (Macherey-Nagel GmbH & Co. KG).

Seed Collection on the Mother Plant

Arabidopsis (Arabidopsis thaliana) seeds (ecotype Wassilewskija) were germinated on soil and grown for two consecutive rounds in the greenhouse during July and August, 2004 (21°C). Flowers were marked, and at given time intervals following anthesis (10 ± 1 to 18 ± 1 DAF), silicles were collected, seeds were dissected, immediately frozen in liquid nitrogen, and stored at −80°C. Ripe fruits were collected at the end of the desiccation period and stored at 4°C. Three to five thousand seeds were harvested for each extraction (30–50 mg).

Seed Germination

Ripe seeds were germinated on moistened ﬁlter paper. Plates were placed at 4°C in the dark and following a period of 72 h transferred to standard plant growth chambers (21°C, approximately 200 µmol m−2 s−1, under a 16/8-h light/dark regime) for additional 24 h. Seeds were washed twice and immediately frozen in liquid nitrogen, following excess water removal.

Fluorescence Measurements

Chl-a fluorescence emission of PSII was measured by pulse amplitude modulated ﬂuorometry using the PAM 101 apparatus (Heinz Walz GmbH). The intrinsic fluorescence, \(F_{\text{m}}\), was elicited by a modulated (1.6 Hz) 650 nm light beam (200 nmol photon m−2 s−1) and the maximal ﬂuorescence, \(F_{\text{v}}\), was elicited by a saturating white-light pulse (1 s, 5,000 µmol photon m−2 s−1). \(F_{\text{m}} - F_{\text{n}}\) variable ﬂuorescence, \(F_{\text{v}}\), and the ratio \(F_{\text{v}}/F_{\text{m}}\) indicates the efficiency of PSII in primary photochemical reaction (Genty et al., 1989). PSI activity was measured by changes in absorption at 830 nm induced by illumination with far-red light (710 nm 9 W m−2) emitted by the PAM-101 PSI attachment; Klughammer and Schreiber, 1994). All measurements were carried out on 100 seeds (clustered on a support to form a group of about 1 cm diameter) taken at different stages of their development.

Extraction, Derivatization, and Analysis of Seed Metabolites Using GC-MS

Metabolite analysis by GC-MS was carried out by a method modiﬁed from that described previously (Roessner et al., 2001). Seeds (approximately 50 mg) were homogenized using precooled mortar and pestle with liquid nitrogen and extracted in a prechilled methanol:chloroform:water extraction solution (1:2.5:1 (v/v)). Standard (0.2 mg mL−1 ribitol in water) was subsequently added as a quantiﬁcation standard. The mixture was extracted for 15 min at 4°C shaking. After centrifugation at 2,200g, 400 µL of water was added to the supernatant. Following vortexing and centrifugation the methanol-water phase was taken and 200 µL reduced to dryness in vacuum. Residue were redissolved and derivatized for 90 min at 37°C (in 40 µL of 20 mg mL−1 methoxyamine hydrochloride in pyridine) followed by a 30-min treatment with 60 µL N-methyl-N-(trimethylsilyl)triﬂuoroacetamide at 37°C. Eight microliters of a retention time standard mixture (0.02% v/v n-dodecane, n-pentadecane, n-nonadecane, n-docosane, n-octacosane, n-dotracosaone, and n-hexatriacontane dissolved in pyridine) was added prior to trimethylsilylation. Sample volumes of 1 µL were then injected onto the GC column with a split ratio of 25:1 using a hot-needle technique. The GC-MS system consisted of an AS 2000 autosampler, a GC-8000 gas chromatograph, and a Voyager quadrupole mass spectrometer (ThermoQuest). The mass spectrometer was tuned according to the manufacturer’s recommendations using tris-(perﬂuorobutyl)-amine (CF43). GC was performed on a 30 m SPB-50 column with 0.25 mm i.d. and 0.25 µm ﬁlm thickness (Supelco). Injection temperature was 230°C, the interface set to 250°C, and the ion source adjusted to 200°C. The temperature program was 5 min isothermal heating at 70°C, followed by a 5°C min−1 oven temperature ramp to 310°C, and a ﬁnal 1 min heating at 310°C. The system was then temperature equilibrated for 6 min at 70°C prior to injection of the next sample. Mass spectra were recorded at two scans per second with a mass-to-charge ratio 50 to 600 scanning range. The chromatograms and mass spectra were evaluated using the MassLab program (ThermoQuest). A retention time and mass spectral library for automatic peak quantiﬁcation of metabolite derivatives was implemented within the MassLab method. Relative and absolute metabolite contents were determined exactly as previously described (Roessner-Tunali et al., 2003) with the exception that quantitative analysis was expanded to include novel metabolites as previously described (Bender-Machado et al., 2004; Schauer et al., 2005). The absolute concentrations of most metabolites were determined by comparison with standard calibration curve response ratios of various concentrations of standard substance solutions, including the internal standard ribitol, which was derivatized concomitantly with tissue samples.

PCA

PCA was performed on the data sets obtained from metabolite proﬁling with the software package TMEV (Saeed et al., 2003) using the default weighted covariance-estimation function. Further ICA analysis was performed using MetaGeneAllyse (Scholz et al., 2004). In this software (available at http://metagenealyse.mpimp-golm.mpg.de) ICA was performed following a PCA preprocessing step to optimize the outcome of the analysis. The data was log10-transformed and normalized to the mean of the entire sample set for each metabolite before the analysis. This transformation reduces the inﬂuence of rare high-measurement values, but does not change the discrimination in the data set.

Semi quantitative RT-PCR

Total RNA was isolated from harvested seeds at 10, 14, and 17 DAF as previously described (Ruska and Ohirogge, 2001), as well as dry seeds, imbibed seeds, and germinating seeds as described above in seed germination. Total RNA (2 µg) treated with DNase RQ-1 (Promega) was reverse transcribed with Superscript II reverse transcriptase (Invitrogen) in a reaction volume of 20 µL. Semi quantitative RNA analysis was performed with 1 µL of cDNA per reaction. Primer sequences are given in Table I. The primers used for the various genes are provided in Supplemental Table S9.

Bioinformatics Data Elaboration

We used two publicly available data sources: (1) AraCyc, Arabidopsis Biochemical Pathways (http://www.arabidopsis.org/tools/aracyc/), which contains hierarchical information of metabolic pathways; and (2) AffyWatch, NASC Affymetrix service (http://arabidopsis.info/), which contains expression data of more than 1,500 arrays. We used experiments from the AtGenExpress project (expression proﬁling of early germinating seeds and hormone treatment of seeds) that measured the expression proﬁle of dry seeds and seeds following 3 or 24 h of imbibition. We used the expression values for each
probe set following MASS.0 analysis that is the standard procedure to measure gene expression levels using Affymetrix technology. To calculate the expression value of each enzymatic reaction we summed the expression value of all the different genes that encode the same enzyme (e.g. the expression level of the Asp kinase enzyme is the sum of the expression values of the four Asp kinase genes that are present on Affymetrix array). For each enzymatic reaction we performed a t test between values of imbibed and dry seeds. The p values were corrected using the Benjamin and Hochberg correction procedure that is the standard procedure when doing multiple tests.

Bioinformatics Analysis

Raw data were collected from the AffyWatch, NASC Affymetrix service (http://Arabidopsis.info/). Expression values for each probe set were calculated following MASS.0 analysis, which is the standard procedure to measure gene expression levels using Affymetrix technology. To calculate the expression value of each enzymatic reaction we summed the expression values of all the different genes that encode the same enzyme (e.g. the expression level of the Asp kinase enzyme is the sum of the expression values of the four Asp kinase genes that are present on Affymetrix array). For each enzymatic reaction, a t test was performed between values of imbibed and dry seeds. The p values were corrected using the Benjamin and Hochberg correction procedure, which is the standard procedure used for multiple tests.

Statistical Analysis

The t tests have been performed using the algorithm embedded into Microsoft Excel. The term significant is used in the text only when the change in question has been confirmed to be significant (t < 0.05) with the t test.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Relative metabolite content during seed development season 1.

Supplemental Table S2. Relative metabolite content during seed desiccation season 1.

Supplemental Table S3. Relative metabolite content during seed development season 2.

Supplemental Table S4. Relative metabolite content during seed desiccation season 2.

Supplemental Table S5. Relative metabolite content during seed germination season 1.

Supplemental Table S6. Relative metabolite content during seed germination season 2.

Supplemental Table S7. Absolute values of measured metabolites.

Supplemental Table S8. Accession numbers and grouping of the gene families.

Supplemental Table S9. Primers list.

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LITERATURE CITED


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