

Targeted Enhancement of Glutamate-to- γ -Aminobutyrate Conversion in Arabidopsis Seeds Affects Carbon-Nitrogen Balance and Storage Reserves in a Development-Dependent Manner^{1[W][OA]}

Aaron Fait*, Adriano Nunes Nesi², Ruthie Angelovici³, Martin Lehmann, Phuong Anh Pham, Luhua Song⁴, Richard P. Haslam, Johnathan A. Napier, Gad Galili, and Alisdair R. Fernie

French Associates Institute for Biotechnology and Agriculture of Dryland, Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Midreshet Ben Gurion 84990, Israel (A.F.); Max Planck Institute for Plant Molecular Physiology, 14476 Potsdam-Golm, Germany (A.N.N., M.L., P.A.P., A.R.F.); Department of Plant Science, Weizmann Institute of Science, Rehovot 76100, Israel (R.A., L.S., G.G.); and Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, United Kingdom (R.P.H., J.A.N.)

In seeds, glutamate decarboxylase (GAD) operates at the metabolic nexus between carbon and nitrogen metabolism by catalyzing the unidirectional decarboxylation of glutamate to form γ -aminobutyric acid (GABA). To elucidate the regulatory role of GAD in seed development, we generated Arabidopsis (*Arabidopsis thaliana*) transgenic plants expressing a truncated GAD from *Petunia hybrida* missing the carboxyl-terminal regulatory Ca²⁺-calmodulin-binding domain under the transcriptional regulation of the seed maturation-specific phaseolin promoter. Dry seeds of the transgenic plants accumulated considerable amounts of GABA, and during desiccation the content of several amino acids increased, although not glutamate or proline. Dry transgenic seeds had higher protein content than wild-type seeds but lower amounts of the intermediates of glycolysis, glycerol and malate. The total fatty acid content of the transgenic seeds was 50% lower than in the wild type, while acyl-coenzyme A accumulated in the transgenic seeds. Labeling experiments revealed altered levels of respiration in the transgenic seeds, and fractionation studies indicated reduced incorporation of label in the sugar and lipid fractions extracted from transgenic seeds. Comparative transcript profiling of the dry seeds supported the metabolic data. Cellular processes up-regulated at the transcript level included the tricarboxylic acid cycle, fatty acid elongation, the shikimate pathway, tryptophan metabolism, nitrogen-carbon remobilization, and programmed cell death. Genes involved in the regulation of germination were similarly up-regulated. Taken together, these results indicate that the GAD-mediated conversion of glutamate to GABA during seed development plays an important role in balancing carbon and nitrogen metabolism and in storage reserve accumulation.

Efficient assimilation of carbon (C) and nitrogen (N) is essential for optimal plant growth, productivity, and yield (Stitt, 1999), particularly in seeds in which the content of essential amino acids is low. Hence, a

comprehensive understanding of seed development and metabolism is central to the enhancement of crop yield and quality. Seed development can be divided into three phases: cell division, maturation (accumulation of food reserves), and desiccation (Weber et al., 2005). The shift from one phase to the other is affected by sugars (Suc, hexoses, trehalose) and abscisic acid. In the early stages of seed development, maternal regulation is maintained via assimilate unloading and supply of nutrients, thereby sustaining cell division. During maturation, seed metabolism changes and storage reserves accumulate in expanding cells. At this time, photosynthetic activity is initiated in the seed, which is believed to improve oxygen supply and the energy state in the seed, thus counteracting increasingly hypoxic conditions (Borisjuk and Rolletschek, 2009). The maturation stage is followed by a phase termed "maturation drying," in which the metabolism of Arabidopsis (*Arabidopsis thaliana*) seeds shifts from a general decrease in unbound metabolites to the accumulation of a set of specific metabolites, including γ -aminobutyric acid (GABA; Fait et al., 2006; Angelovici et al., 2010).

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² Present address: Departamento de Biologia Vegetal, Universidade Federal de Viçosa, 36570-000 Viçosa, MG, Brazil.

³ Present address: Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824.

⁴ Present address: Department of Biochemistry and Molecular Biology, University of Nevada, Reno, Nevada 89557.

* Corresponding author; e-mail fait@bgu.ac.il.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Aaron Fait (fait@bgu.ac.il).

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GABA is a four-C nonprotein amino acid generally abundant in plants whose levels change significantly in response to a wide array of endogenous and exogenous factors, including biotic and abiotic stresses (Bouché et al., 2003; Bouché and Fromm, 2004; Bown et al., 2006), to the diurnal cycle (Masclaux-Daubresse et al., 2002), and to developmental processes, such as seed maturation (Fait et al., 2006), flower fertilization (Palanivelu et al., 2003), and fruit development (Rolin et al., 2000; Carrari et al., 2006; Akihiro et al., 2008; Yin et al., 2010). GABA is derived from Glu via the action of glutamate decarboxylase (GAD), the enzyme responsible for the first step in the GABA shunt metabolic pathway (Fig. 1; Bown and Shelp, 1997). In plant species such as petunia (*Petunia hybrida*; Chen et al., 1994; Arazi et al., 1995), soybean (*Glycine max*; Snedden et al., 1995), tobacco (*Nicotiana tabacum*; Baum et al., 1996), and Arabidopsis (Turano and Fang, 1998; Zik et al., 1998), the GAD polypeptides contain C-terminal extensions, ranging between 30 and 50 amino acids, that are not present in the bacterial GAD enzyme and

function as Ca²⁺-calmodulin-binding domains (Baum et al., 1993). Thus, GAD catalyzes the decarboxylation of Glu to GABA in a calmodulin-dependent manner. Scientists have thus hypothesized both a protective role for GABA during stress events and a signaling function (Shelp et al., 1999; Bouché et al., 2003; Bouché and Fromm, 2004; Chevrot et al., 2006; Fait et al., 2008). While the latter role is under debate, a number of investigations have suggested a primary role of the GABA shunt in central C-N metabolism. The impact of a constitutive and systemically deregulated GABA biosynthesis has been shown in tobacco (Baum et al., 1996; McLean et al., 2003): transgenic plants over-expressing a mutant form of GAD displayed a severely decreased Glu content (Baum et al., 1996), which is generally maintained steady within the cell (Stitt and Fernie, 2003; Forde and Lea, 2007).

It has been suggested that GABA constitutes a readily accessible nontoxic reserve of C and N for amino acid metabolism and tricarboxylic acid (TCA) cycle activity, which is of particular relevance during

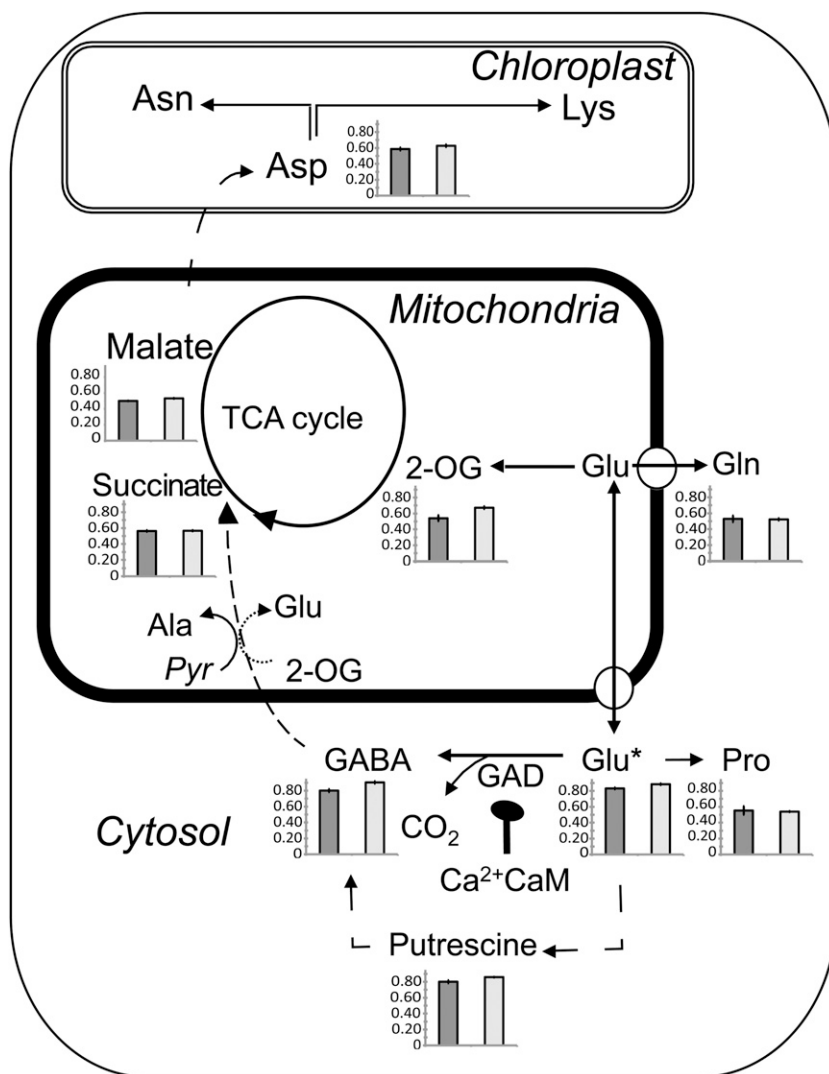


Figure 1. Fate of ¹³C-labeled Glu (Glu*) supplied to stratified (dark gray bars) and 1-DAS (light gray bars) seeds of wild-type Arabidopsis (ecotype Wassilewskija). The relative isotope redistribution of Glu is described in a schematic presentation of the GABA shunt and related major metabolic pathways. Bars represent the C fractional enrichment [¹³C/(¹³C + ¹²C)] of metabolites labeled. C fractional enrichment was calculated by evaluating the labeled portion of the various pools as a proportion of the various pool sizes, following an incubation period of 6 h of stratified and 1-DAS seeds. Values are means ± SD from six determinations per time point.

stress events (Breitkreuz et al., 1999; Solomon and Oliver, 2002; Beuvé et al., 2004; Bouché and Fromm, 2004). However, only recently has direct evidence, integrating metabolite and transcript profiling and stable-isotope labeling, emerged for the metabolic significance of the GABA shunt (Studart-Guimarães et al., 2007). In transgenic plants deficient in succinyl-CoA ligase, the enzyme that catalyzes the conversion of succinyl-CoA to succinate (Studart-Guimarães et al., 2005), succinate production was partly maintained via the GABA shunt (Studart-Guimarães et al., 2007). Earlier molecular studies and radioisotope experiments that demonstrated rapid degradation of Glu to GABA and thereafter to succinate also indicated a metabolic role for GABA in developing and germinating seeds (Vandewalle and Olsson, 1983; Tuin and Shelp, 1994). These results, taken together with the more recent metabolic profiling of developing seeds, suggest that GABA accumulation in the dry seed supports early metabolic reorganization at germination. However, a comprehensive understanding of the interregulation between the GABA shunt and central C-N metabolism is lacking. Moreover, there is a need for additional research to assess the function of GABA during seed development (i.e. at a time when metabolic processes are under tight concerted regulation; Weber et al., 2005; Holdsworth et al., 2008). To address this need, a development-inducible system was used that has previously been successfully applied to study the metabolic regulation of Lys biosynthesis (Zhu and Galili, 2003; Angelovici et al., 2009, 2010). In the system used in this study, the seed-specific phaseolin storage protein promoter drives the expression of a deregulated *GAD* from petunia in developing Arabidopsis seeds. The developmentally induced production of a deregulated *GAD* in maturing seeds provides us with a model system in which the perturbation in central metabolism can be monitored and the consequent effect on concertedly regulated metabolic processes during seed maturation can be assessed. We subjected developing seeds to metabolite profiling and genome-wide gene expression analysis with the aim to investigate the effect of an increasingly deregulated conversion of Glu to GABA on seed metabolism. Our results demonstrated that Glu-to-GABA deregulated conversion has a profound effect on the C-N balance of the seed, despite the existence of metabolite-recycling routes (i.e. GABA transaminase and the TCA cycle). The results are discussed with respect to the current understanding of seed development metabolism and its implications for seed germination.

RESULTS

Stable Isotope-Labeling Experiments Suggest That the GABA Shunt Represents a Considerable Source of C for the TCA Cycle during Early Stages of Seed Germination

Earlier radiolabeling studies and more recent metabolite profiling suggest that GABA plays a role in seed metabolism, accumulating in the dry seed and

decreasing upon germination (Vandewalle and Olsson, 1983; Fait et al., 2006). Although informative, radiolabeling studies are limited in their applicability to monitoring the degree of labeling across a range of metabolites; as a result, stable isotopes coupled with mass spectrometry (MS) or NMR have been increasingly used in the last decade for pathway elucidation and flux analysis (Sauer, 2006, and refs. therein). To elucidate whether the GABA shunt operates as a nexus between C and N metabolism upon seed imbibition prior to seed germination, we evaluated the metabolic fate of Glu in Arabidopsis seeds freshly stratified and 1 d after stratification (DAS; when germination has been initiated); an adapted gas chromatography (GC)-MS-based protocol (Roessner-Tunali et al., 2004) was used in combination with a feeding ^{13}C -substrate. The relative redistribution of ^{13}C was measured by assessing (1) the incorporation of the isotope into each intermediate within the relevant pathways (Fig. 1) and (2) the proportional labeling of ^{13}C and ^{12}C (C fractional enrichment) in metabolites closely associated with Glu. In stratified seeds supplied with [^{13}C]Glu, we detected significant and equivalent incorporation of ^{13}C in GABA and in putrescine, followed by succinate, Asp, and 2-oxoglutarate (2-OG). In 1-DAS seeds, ^{13}C incorporation increased significantly, particularly in GABA and 2-OG, followed by putrescine and Asp (Fig. 1). This finding indicates that the proportion of ^{13}C derived from Glu that entered the GABA pool in early germinating seeds (prior to germination *sensu stricto*) exceeded that entering the pools of Pro, Gln, or 2-OG. The data also indicate the existence in seeds of a tighter relation between Glu-GABA metabolism and the polyamine putrescine than currently realized. Different pathways could potentially sustain the conversion of Glu-GABA to putrescine (1) by the action of putrescine:oxygen oxidoreductase (EC 1.4.3.10) on the aldehyde form of GABA, (2) via the Arg decarboxylase pathway, or (3) via spermidine metabolism. While it is not possible to mathematically deduce the absolute input fluxes into the succinate pool (Baxter et al., 2007), the above data expand on previous work suggesting that the GABA shunt constitutes an important metabolic route sustaining the TCA cycle and early amino acid biosynthesis in germinating seeds.

Expression of a Truncated *GAD* from Petunia Lacking the Calmodulin-Binding Domain Stimulates GABA Accumulation in Dry Seeds

Two types of transgenic Arabidopsis plant (*spGAD* and *spGADΔC*) were generated (Fig. 2A), expressing either the full-length *GAD* or a truncated *GAD* cDNA from petunia, under the control of the bean (*Phaseolus vulgaris*) phaseolin promoter (see "Materials and Methods"), producing a constitutively active, although developmentally induced, *GAD* enzyme. Three and four independent transformants of *spGADΔC* and *spGAD*, respectively, were eventually isolated on antibiotic plates and tested for the segregation of T2

seeds. Thereafter, bulked T3 seeds were tested for segregation to confirm homozygosity and evaluated for GABA content (Fig. 2B). The GABA content in the dry seeds of all isolated *spGAD* lines was similar to that in the wild type. In contrast, the dry seeds of all *spGADΔC* lines were characterized by hyperaccumulation of GABA. Besides the metabolic phenotype of the seed, these plants showed no significant differences in vegetative traits as compared with Wassilewskija plants (data not shown). However, the dimensions of mature T3 seeds of *spGADΔC-2* and *spGADΔC-7*, the two strongest transgenic lines with respect to GABA accumulation in the dry seed, were found to be slightly but significantly smaller than those of the wild type (Fig. 2C, left). The measured elliptic area, based on the width and length of the seeds in the above two transgenic lines, was shown to be 18.8% and 28.5%, respectively, smaller than that of the wild type. The reduction in area was partially reflected in the seed weight, with a statistically significant decrease of 7.3% and 15.5% in *spGADΔC-2* and *spGADΔC-7* as compared with the wild type, respectively (Fig. 2C, right). In germination tests of all genotypes under standard conditions for *Arabidopsis* (seeds of the control and of the transgenics were harvested at the same time and stored

together; see “Materials and Methods”), stratified transgenic seeds displayed only 50% of the germination of wild-type seeds. Staining with tetrazolium confirmed that the transgenic seeds displayed reduced vigor (data not shown).

GABA Content Is Differentially Regulated during the Late Seed Maturation-to-Desiccation Stage, and Its Accumulation Is Indicative of a Shift toward N Metabolism

To assess the overall metabolite response to the maturation-induced gradual accumulation of GABA, we performed a developmental analysis of the metabolic changes in transgenic *spGADΔC* T4 seeds. Siliques of *spGADΔC-2* and *spGADΔC-7* and of the untransformed wild type were successively collected from tagged flowers at 14 and 17 d after flowering (DAF) and at full maturity. Seeds were extracted and analyzed using an established GC-MS-based protocol (Roessner et al., 2001; Fait et al., 2006). Figure 3 shows changes in the metabolite content of seeds of the transgenics relative to the wild type. Only metabolites showing significant ($P < 0.01$) and consistent changes are shown; the complete data set is given in Supplemental

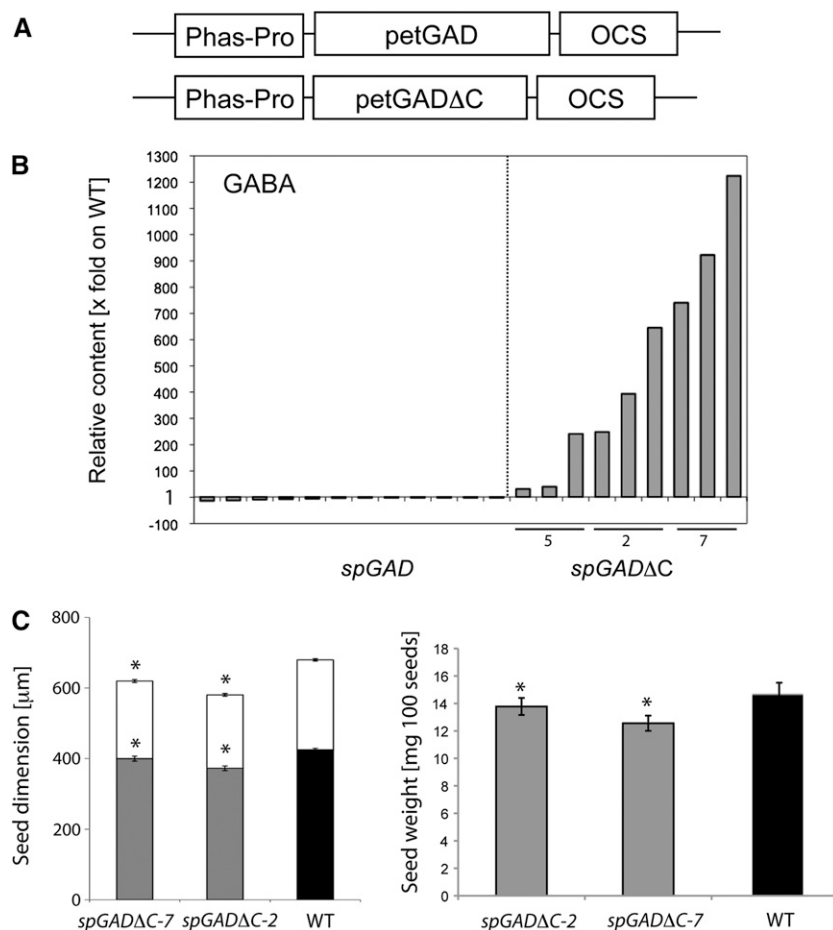


Figure 2. A, Schematic diagram of the chimeric gene utilized in this study. Phas-Pro is the bean phaseolin storage protein promoter, *spGAD* and *spGADΔC* are the coding DNA sequences of the wild type and mutant for GAD from petunia, and OCS is the DNA sequence of the octopine synthase 3' terminator. B, GABA relative content in mature T3 seeds of transgenic plants harboring *spGAD* and *spGADΔC* constructs, collected in three independent bulks from 30 plants each. GABA was measured by a GC-MS-based protocol (Roessner et al., 2001). Values represent fold change relative to the wild type (WT). Transgenic lines *spGADΔC-5*, *spGADΔC-2*, and *spGADΔC-7* are indicated. C, Seed dimensions and weight. The seed dimension values are means \pm SD of 30 individual measurements repeated for three independent bulks of 10 (T3) plants each. The seed weight values are means \pm SD of 100 bulked seeds from 10 plants each and repeated 10 times. Seeds were viewed with a Leica MZ12.5 stereomicroscope (Leica DFC420 digital camera) and measured using Leica Application Suite software. Asterisks represent significant differences ($P < 0.05$) as compared with the wild type.

Table S1. While the phaseolin promoter activity is known to commence during early maturation of the seed (van der Geest and Hall, 1996), our results revealed that GABA gradually accumulated in the transgenic seeds both at late maturation and to a considerably greater extent in the dry seed (Fig. 3). Increased GABA levels were associated with a small but significant ($P < 0.05$) decrease in the content of Glu at 14 DAF and later in the dry seed. Similarly affected were the levels of biosynthetically related amino acids such as Gln and Pro, the latter displaying a marked decrease (one-fourth to one-sixth) in the dry seeds of *spGADΔC* as compared with the wild type. That said, the general effect of the constitutive activity of GAD was a decrease in the content of the vast majority of measured amino acids at 14 DAF (Fig. 3; Supplemental Table S1) and the accumulation of several of these amino acids in the dry seeds of both transgenic lines (Fig. 3; Supplemental Table S1). Increased levels were measured for Asp, Asn, Met, Cys, and Ser, and a particularly significant increase was found for Trp in the dry seeds (Fig. 3; Supplemental Table S1). In line with the results from the stable isotope experiments (Fig. 1), GABA accumulation in the dry seeds was accompanied by a decrease in the content of the polyamine putrescine (Supplemental Table S1), one of the possible GABA precursors (Fait et al., 2008).

In contrast, when estimating the effect of an enhanced Glu-to-GABA conversion on carbohydrate and carboxylic acid metabolism, we found decreases in the concentrations of a number of sugars and organic acids (Fig. 3; Supplemental Table S1). For instance, in the dry transgenic seeds, Fru was decreased to 30% of its content in the dry seed of the wild type (Fig. 3). Gal, maltose, and erythritol followed a similar trend. The glycolysis-derived intermediates of glycerolipid metabolism, glycerate, glycerol, and the related compound myoinositol, were consistently decreased in the transgenic seeds (Fig. 3; Supplemental Table S1). Among the TCA cycle intermediates, succinate, a product of GABA catabolism, accumulated in the dry seeds of the transgenics by 1.5-fold more than in those of the wild type but citrate content was similar in the transgenics and the wild type (Supplemental Table S1). Notably, malate content was one-third in the dry seeds of the transgenics than in those of the wild type (Fig. 3).

¹⁴C-Labeling Experiments Reveal Decreased TCA Cycle Activity But Increased Glu Catabolism in the GABA-Hyperaccumulating Transgenic Lines

We next sought to evaluate the effect during seed development of a constitutively enhanced conversion of Glu to GABA on the activity of the TCA cycle and on glycolysis. For this purpose, we evaluated the relative rates of carbohydrate oxidation in the transformants by measuring the evolution of ¹⁴CO₂ following the incubation of siliques in solutions of positionally labeled molecules. Maturing siliques (14 and 17 DAF)

from the wild type and *spGADΔC-7* were incubated with [^{1-¹⁴C}]Glc, [^{3;4-¹⁴C}]Glc, or [^{U-¹⁴C}]Glc or with [^{U-¹⁴C}]Glu. The ¹⁴CO₂ evolved during the incubation period was collected following 2 and 4 h of incubation. Since carbon dioxide can be released from the C3:4 positions of Glc via enzymes associated with mitochondrial respiration (ap Rees and Beevers, 1960), carbon dioxide evolution from C3:4 positions of Glc provides an indication of the relative rate of the TCA cycle (Nunes-Nesi et al., 2005). In maturing siliques, the evolution of labeled CO₂ from [^{3;4-¹⁴C}]Glc was 50% lower in the representative transgenic line *spGADΔC-7* than in the wild type (Fig. 4). ¹⁴CO₂ can be released from [^{1-¹⁴C}]Glu by the decarboxylation of Glu to GABA (Fig. 5): monitoring the evolution of labeled CO₂ from maturing siliques fed with [^{1-¹⁴C}]Glu confirmed the up-regulation of the GABA shunt (Fig. 4). [^{U-¹⁴C}]Glu feeding gave a comparable increase in CO₂ evolution, suggesting that Glu-derived CO₂ was generated mainly via the activity of the GABA shunt rather than from incorporation into and respiration through the TCA cycle.

To specifically describe the changes in C allocation in the seed, we next supplemented dry seeds with [^{U-¹⁴C}]Glc and measured the incorporation of the radiolabel in the polar neutral, acidic, and basic fractions and in the lipid fraction of the seed extracts. The metabolism of the radiolabeled compound in the transgenic lines was found to be markedly different from that in the wild type (Fig. 5): the proportion of radiolabel incorporated into sugars was significantly lower in both *spGADΔC* lines than in the wild type, a finding that could suggest increased use of added Glc in the transgenic lines to produce compounds other than sugars. The latter scenario is supported by increased incorporation of radiolabel in organic acids and CO₂ evolution in the transgenic seeds (Fig. 5). For seeds fed with [^{1,2-¹⁴C}]acetate, the label incorporation into lipids in the transgenic lines was up to 30% lower than its incorporation in the wild type, which suggested a decrease in C flow toward lipid biosynthesis (Fig. 6), while the amounts of radiolabel metabolized to compounds other than lipids were equivalent to those in the wild type (Supplemental Table S2).

Up-Regulation of the GABA Shunt Leads to the Alteration of Storage Reserve Accumulation and Fatty Acid Metabolism

Accumulation of storage reserves in dry seeds was evaluated in terms of the contents of protein, total fatty acids (TFA), and CoA derivatives in *spGADΔC-2* and *spGADΔC-7* versus the wild type. In keeping with the general effect on amino acids, transgenic seeds displayed considerable accumulation of protein at maturity (Fig. 7A; i.e. 26.8% and 47% higher protein content in *spGADΔC-2* and *spGADΔC-7* than in the wild type, respectively). In contrast, HPLC analysis of TFA revealed markedly reduced levels of TFA in the transgenic seeds, with *spGADΔC-2* and *spGADΔC-7*

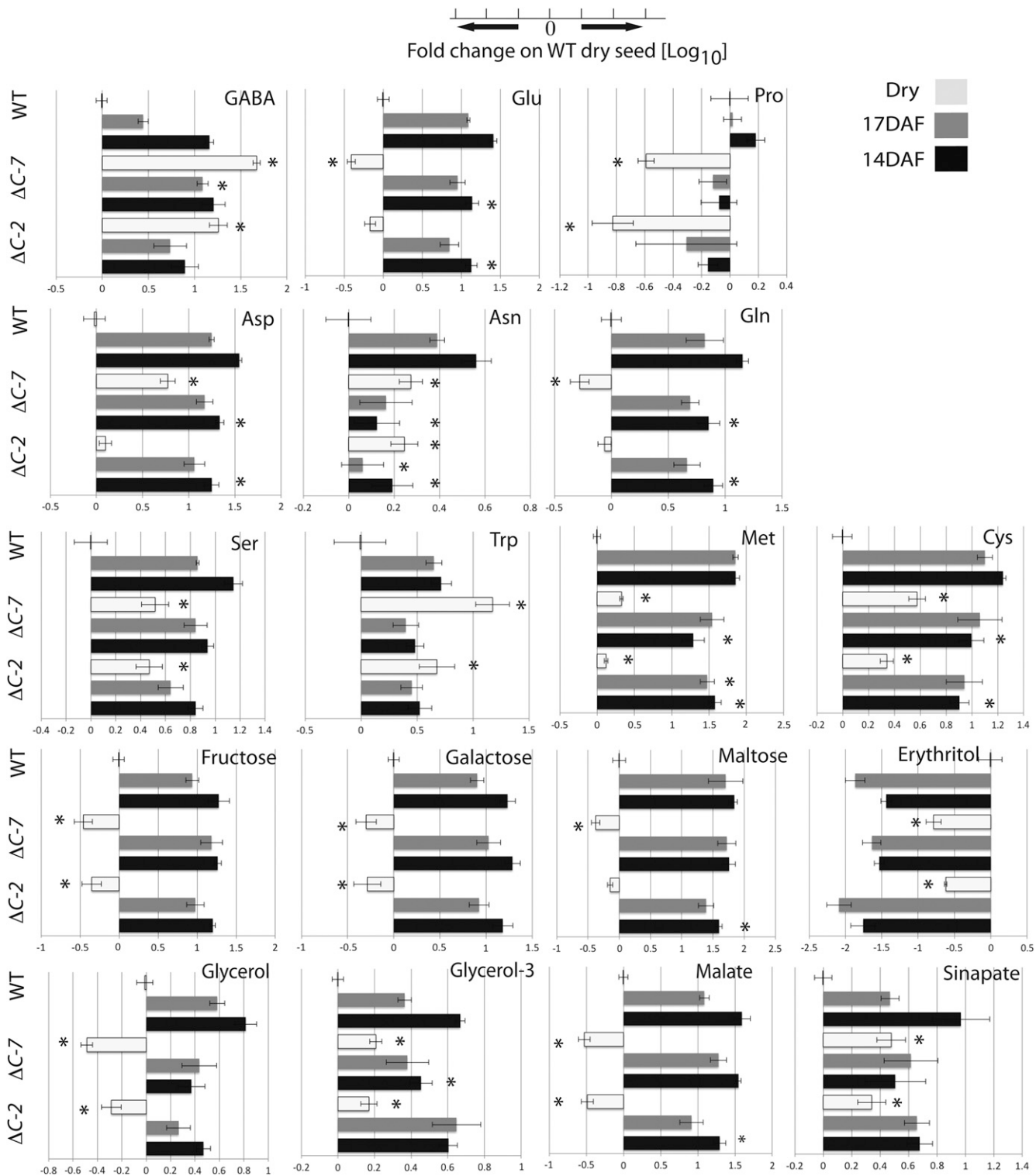
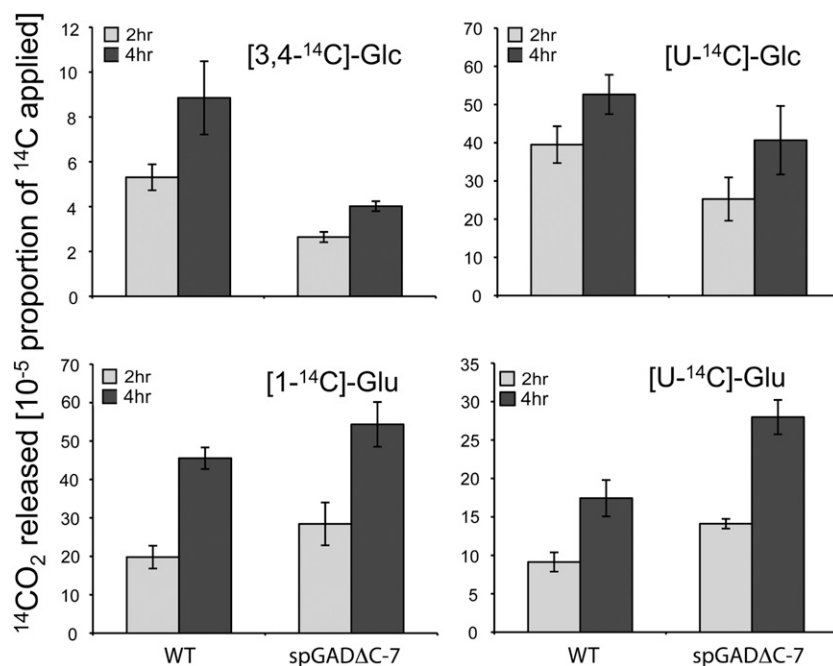


Figure 3. Relative content of metabolites in developing seeds of *spGADΔC-2* ($\Delta C-2$), *spGADΔC-7* ($\Delta C-7$), and the wild type (WT). Relative content of free metabolites was measured in developing seeds at 14 and 17 DAF and in dry seeds as described in “Materials and Methods.” The 14- and 17-DAF stages reflect mid and late maturation, respectively, under the condition of growth used (Fait et al., 2006; Angelovici et al., 2009). Bar graphs indicate the mean fold change of each metabolite at 14 DAF (black bars), 17 DAF (gray bars), and in the dry seeds (white bars) relative to its content in the dry seeds of the wild type. Asterisks denote statistically significant (two-tailed Student’s *t* test; $P < 0.01$) differences between *spGADΔC* and the wild type for the metabolite content at each given developmental stage. Only metabolites that presented significant and consistent changes in content in the transgenics as compared with the wild type are shown. The entire data set is given in Supplemental Table S1.

Figure 4. $^{14}\text{CO}_2$ release from siliques incubated in specifically labeled isotopes. Equivalent samples of maturing siliques collected between 14 and 18 DAF were incubated in $1 \mu\text{Ci mL}^{-1}$ [$1\text{-}^{14}\text{C}$]Glu, [$\text{U-}^{14}\text{C}$]Glu, [$3,4\text{-}^{14}\text{C}$]Glc, or [$\text{U-}^{14}\text{C}$]Glc as described in "Materials and Methods." Values are presented as cumulative $^{14}\text{CO}_2$ release and are means \pm SE of four biological replicates. WT, Wild type.



displaying on average 30% and 70% less TFA than the wild type (Fig. 7B). However, there was a general accumulation of acyl-CoAs in the dry seeds, with a notable 2-fold increase for 16:0, 18:2, 20:1, and 20:3 fatty acids in both transgenics as compared with the wild type (Fig. 8).

Genome-Wide Expression Analysis of *spGADΔC* Transgenics Highlights the Occurrence of Targeted Changes in Genes Involved in Amino Acid, Mitochondrial Electron Transport, and the TCA Cycle and Storage Reserve Metabolism

To understand the role of transcriptional regulation in the changes observed at the metabolite level and to estimate the impact of enhanced GABA production on the gene transcriptional program of the seed, we performed microarray analysis on the dry stored seeds, transgenics versus the wild type, from the same material collected for metabolite profiling using the Affymetrix AtH1 chip. One-way ANOVA of the microarray data indicated that changes in the level of transcripts included 149 genes significantly ($P < 0.001$; false discovery rate [FDR] < 0.05) up-regulated and 55 genes down-regulated in the transgenics as compared with the wild type, showing at least 1.5-fold change. The detailed list of genes showing statistically significant changes in expression following ANOVA between the dry seeds of the transgenic line and the wild type, and the calculated fold changes, are provided in Supplemental Tables S3 and S4. The entire data set is provided in Supplemental Table S5. In summary, genes related to metabolism were abundant, and the vast majority were up-regulated. Moreover, overrepresentation analysis using the tools embedded in the

PageMan software (<http://mapman.mpimpgoelm.mpg.de/general/ora/ora.shtml>) indicated that among the up-regulated genes (Supplemental Table S3) in the transgenic seeds, there was overrepresentation of the following categories: biotic stress (six genes), redox state (three genes), calcium-associated signaling (six genes), and cell wall metabolism in the transgenic seeds (Table I [overrepresentation analysis]; for the list of up-regulated genes, see Supplemental Table S3). Among the genes associated with metabolism, the most markedly affected were Glu dehydrogenase, Gln synthetase (ATGSR2), Ala:glyoxylate aminotransferase, and S-adenosyl Met 1 synthetase as well as several genes associated with aromatic amino acid metabolism (anthranilate synthase α -subunit 1, anthranilate phosphoribosyltransferase, shikimate kinase) and polyamine catabolism (polyamine oxidase; for fold change changes, see Supplemental Table S3). In contrast, the expression of genes associated with Ser catabolism (Ser hydroxymethyltransferase 4 and Trp synthase) was down-regulated (Supplemental Table S4).

The parallel metabolite and transcript analysis indicated that the decrease in the concentrations of a number of TCA cycle intermediates in the seeds of *spGADΔC* lines was also associated with changes in TCA gene expression patterns. As shown in Supplemental Table S3, changes in the level of TCA cycle-associated transcripts included the up-regulation of succinate dehydrogenase and 2-oxoglutarate dehydrogenase. The transcripts of malic enzyme, which is involved in the conversion of malate to pyruvate, the direct precursor of acetyl-CoA, were up-regulated in the transgenic line. CoA metabolism in relation to fatty acid metabolism was indeed altered at the transcript

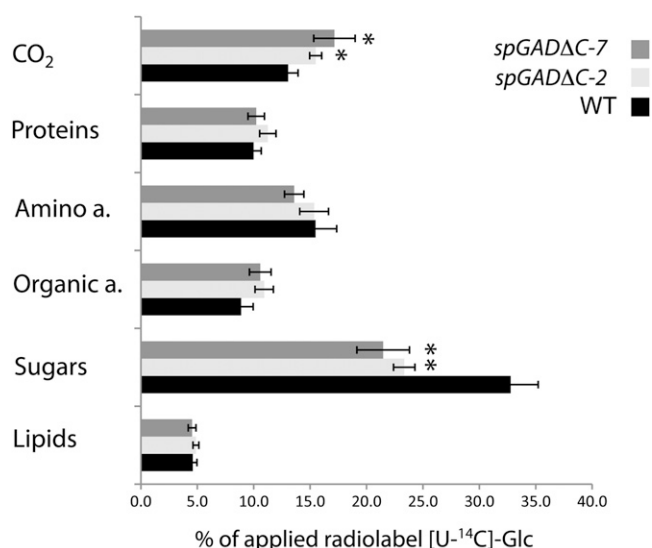


Figure 5. Effect of deregulated Glu decarboxylation during seed maturation on the metabolism of [U-¹⁴C]Glc by stratified seeds. Tissue was fractionated exactly as described in “Materials and Methods.” Seeds were preincubated in 200 μ L of MES-KOH buffer (pH 6.4) containing 2 mM unlabeled precursor. After 1 h of acclimation, 200 μ L of buffer containing specific radiolabel was added. ¹⁴CO₂ was collected using CO₂ traps containing 200 μ L of 10 mM KOH as described by ap Rees and Beevers (1960) and Nunes-Nesi et al. (2005). Collected seeds were frozen immediately in liquid N after washing two times with 1.5 mL of MES-KOH buffer. The material was extracted using combined and modified methods as described in “Materials and Methods.” Values are expressed as percentages of the total radiolabel metabolized and are means \pm SE of four biological replicates. Asterisks indicate values that were judged to be significantly different from the control ($P < 0.05$) when tested by Student’s *t* test. WT, Wild type.

level, as shown in the up-regulation of long-chain fatty acid CoA ligase and long-chain acyl-CoA synthetase and condensing enzyme 3-ketoacyl-CoA synthase 1, which is involved in fatty acid elongation, and by a down-regulation of fatty acid transport (ATP-binding cassette transporter). In seeds of the transgenic plants, we also found a significant up-regulation of the genes (Supplemental Table S3) involved in the degradation of protein and cellular components (Cys protease and RESPONSIVE TO DESICCATION21 [RD21]) and in N remobilization (ATGSR2), which could explain the increased amino acid content in these seeds. Among the genes up-regulated in the transgenic seeds, a small number are associated with the mobilization of glycosyl groups (e.g. UDP-glycosyltransferase/transferase, glycosyl hydrolase family 3 protein). Among other functions, these enzymes are involved in the metabolism of certain disaccharides and polysaccharides. Other up-regulated genes included those involved in the mitochondrial electron transport chain (i.e. alternative oxidase and FAD-binding domain-containing protein [AT1G26380]). With respect to energy, the expression of transcripts of GTP- and ATP-binding proteins was down-regulated (AT5G56500 for the

GTP-binding protein and AT5G57960 and AT1G30960 for the ATP-binding proteins; Supplemental Table S4). Gene Ontology analysis for enrichment in biological processes (<http://david.abcc.ncifcrf.gov/>) indicated a significant enrichment of genes associated with the electron transport process, which included 13 up-regulated genes and six down-regulated genes (Supplemental Tables S3 and S4). Among these, there was a 3.6-fold down-regulation of cytochrome p450 (CYP71A20 and CYP71A19) and a 2-fold down-regulation at the MATERNAL EFFECT EMBRYO ARREST23 electron carrier involved in embryo development and seed dormancy. Arabidopsis NAC domain-containing protein 92 (ANAC092/ATNAC2/ATNAC6) and DELAY-OF-GERMINATION1 (DOG1), associated with the regulation of germination, were up-regulated in the transgenic seeds.

DISCUSSION

In plants, Glu metabolism is pivotal for efficient N incorporation; therefore, its levels are maintained under tight regulation (Stitt and Fernie, 2003; Forde and Lea, 2007). In tobacco leaves, for example, a mutated GAD from petunia was shown to significantly reduce Glu levels (Baum et al., 1996). GAD is the entry enzyme of the GABA shunt and catalyzes the decarboxylation of Glu to GABA (Bown and Shelp, 1997). Recently, GABA was shown to accumulate to high levels in dry seeds of Arabidopsis, a finding implying a developmental role in seeds for this nonproteogenic amino acid (Fait et al., 2006). Labeling experiments conducted in our study directly confirm that GABA is strongly associated with early seed germination metabolic processes, replenishing the TCA cycle and con-

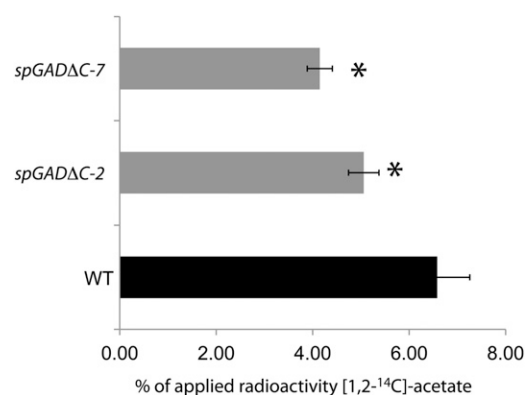


Figure 6. Effect of deregulated Glu decarboxylation during seed maturation on the incorporation of [1,2-¹⁴C]acetate in lipids by stratified seeds. Tissue was fractionated exactly as described in “Materials and Methods.” Values are expressed as percentages of the total radiolabel metabolized and are means \pm SE of four biological replicates. Asterisks indicate values that were judged to be significantly different from the wild type (WT; $P < 0.05$).

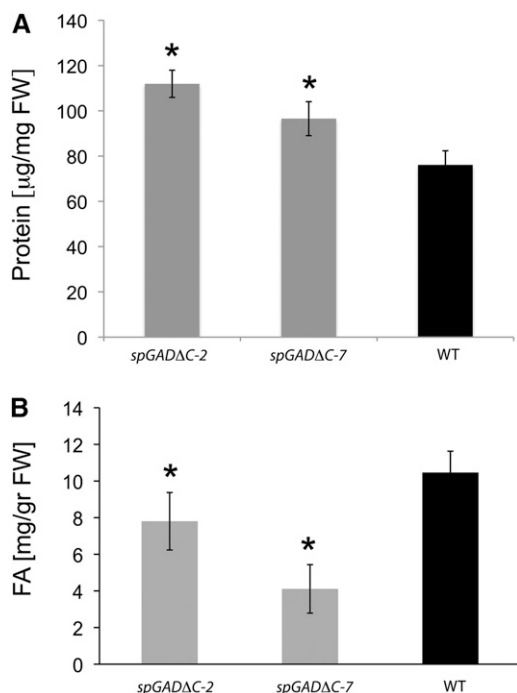


Figure 7. Protein (A) and TFA (B) content in mature seeds of wild-type (WT) and transgenic plants. The effect of up-regulated GABA metabolism on the content of seed proteins and TFAs was measured by standard Bradford protocol and a GC-based protocol as described in “Materials and Methods.” Results are presented as means \pm SE of at least four biological replicates of 20 mg of seeds. FW, Fresh weight.

tributing to amino acid biosynthesis (Fig. 1). These early metabolic events can be of considerable relevance to the imbibing seed, whose level of energy is low and whose storage reserves have not yet been degraded (Lunn and Madsen, 1981; Al-Ani et al., 1985; Borisjuk and Rolletschek, 2009). Regulation of GABA levels probably represents a tuning of the balance between C metabolism and N metabolism. In this study, we investigated the perturbation in seed metabolism following the seed-targeted up-regulation of Glu conversion to GABA during seed maturation. We employed the up-regulation strategy to challenge the seed metabolic network in a development-induced manner with the aim of revealing regulatory aspects of seed metabolism (Angelovici et al., 2009). Our results showed developmental differences in the response of seed metabolism to a deregulated GAD, probably caused by the changing cellular environment between seed maturation and desiccation (Fig. 9). We further showed that enhancing Glu-to-GABA decarboxylation causes a major alteration in the C-N balance and in reserve accumulation, unexpectedly in favor of N. Nonetheless, hyperaccumulation of GABA in the dry seed was not followed by a genome-wide transcriptional response, and the changes in transcript levels were generally in accordance with the metabolic data and included N mobilization, aromatic amino acid metabolism and fatty acid CoA metabolism, electron

transport, and energy balance. Integration of our metabolite and transcript data suggests a tighter link between amino acid metabolism and genes involved in the regulation of germination than was previously perceived.

Up-regulation of N metabolism in the transgenic seeds was associated with decreased levels of malate and of some sugars and sugar alcohols in the dry seed as well as significantly lower TFAs. Taken together, these findings indicate that a finely regulated GABA shunt in maturing seeds is the key to maintaining C-N equilibrium. Moreover, while the link between the TCA cycle and the GABA shunt is well known (Busch and Fromm, 1999; Studart-Guimarães et al., 2007; Bunik and Fernie, 2009), we show here that Glu is metabolized preferentially via GABA (as compared with the alternative Glu-to-2-OG conversion). Our data hence suggest that the Glu-to-GABA conversion represents the main route for Glu incorporation into the TCA cycle. That said, we could not rule out the possibility that a high conversion of 2-OG and its biosynthesis from isocitrate would lead to an underestimation of Glu incorporation into the TCA cycle via 2-OG.

Cellular Conditions Characterizing the Desiccating Seed Lead to the Accumulation of GABA in GAD Transgenic Seeds

A recent review (Angelovici et al., 2010) presents robust evidence that metabolites, transcripts, and the enzymatic machinery accumulate in the dry seed, possibly to ensure the initiation of cellular processes upon water imbibition. There is thus a general shift in seed metabolism during seed desiccation. In this context, our results indicate differential regulation of GABA metabolism at seed maturation (14–17 DAF) and at desiccation (17 DAF to dry seed), which most probably depends on the modulation of GABA catabolism under the changing cellular conditions between maturation and desiccation (Angelovici et al., 2010). The regulation of the GABA shunt is driven by Ca^{2+} , the redox state, and energy constraints (Baum et al., 1996; Busch and Fromm, 1999). Succinic semialdehyde dehydrogenase (SSADH) is the final enzyme of the shunt and as such represents the entry enzyme into the TCA cycle. Indeed, as simplified in the model in Figure 9, in the maturing transgenic seed, the deregulated GAD under the regulation of the phaseolin promoter is active as early as 12 DAF. At this stage, photosynthesis is still taking place in Arabidopsis seeds (Fait et al., 2006; Angelovici et al., 2009) and in the seeds of many other species (Vigeolas et al., 2003; Ruuska et al., 2004; Rolletschek et al., 2005; Tschiersch et al., 2011), with oxygen being evolved at the surface and translocated to the inner part of the seed (Borisjuk and Rolletschek, 2009). This process enables the concomitant production of ATP to sustain metabolic activity (Borisjuk and Rolletschek, 2009). As such, it is likely that the catabolism of GABA via the action of SSADH, supplying succinate to the TCA cycle (Fig. 9, left

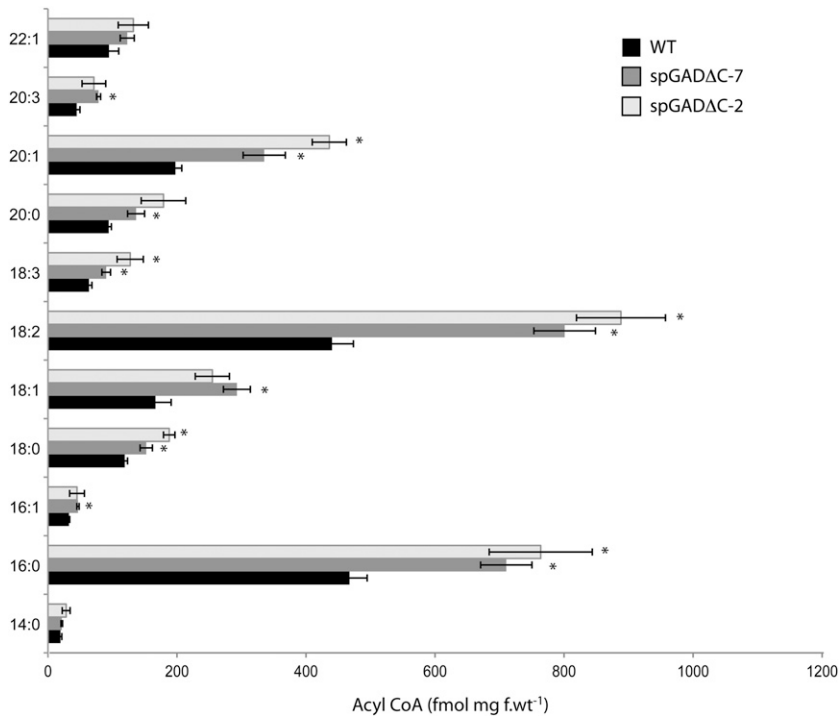


Figure 8. Content of acyl-etheno-CoA derivatives in mature seeds of wild-type (WT) and transgenic plants. Twenty-milligram portions of material were frozen in liquid N and extracted for subsequent quantitative analysis of fluorescent acyl-etheno-CoA derivatives by HPLC (Larson and Graham, 2001; Larson et al., 2002; Sayanova et al., 2007). Values represent means \pm SD of four biological replicates. Asterisks represent significant ($P < 0.05$) differences with respect to the wild type calculated using Student's t test. f.wt, Fresh weight.

scheme) during maturation of the seed, prevents the accumulation of GABA in the transgenic seeds. Nonetheless, the effect of a deregulated Glu-to-GABA conversion was reflected in the decreased levels of the vast majority of the free amino acids measured. We thus conclude that the increased use of Glu to produce GABA prevents the maintenance of amino acid levels.

At late maturation and desiccation, the conditions within the seed become increasingly restrictive, reducing conductance and hence limiting gas exchange; the inner part of the endosperm and embryo becomes more and more hypoxic and lacking in energy (Rolletschek et al., 2005). Such conditions inhibit portions of some metabolic pathways, including sections of the TCA cycle (Tretter and Adam-Vizi, 2000; Rocha et al., 2010), thereby inducing a metabolic shift in the developing seeds (Fait et al., 2006). The changed cellular environment is probably the cause of the accumulation of GABA between late maturation and desiccation (Fig. 9, right scheme). Indeed, regulation of the GABA shunt enzymes was shown previously to be highly dependent on the redox state of the cell: Shelp et al. (1995) demonstrated that GABA shunt activity is associated with a hypoxic environment, and the activity of SSADH was proved to be dependent on favorable redox and energy state (Busch and Fromm, 1999). A similar effect was observed in coffee (*Coffea arabica*) seeds, in which a differential regulation of GABA metabolism was documented across the drying process (Kramer et al., 2010) and GABA accumulation was found to be associated with maximum expression of the dehydrin gene. Although a protective role of GABA during seed desiccation cannot be ruled out, our results suggest that enhanced Glu-to-GABA con-

version is a consequence of a metabolic bottleneck at the entrance of the TCA cycle. GABA-deregulated production occurs in the desiccating seed at the expense of another stress-related amino acid, Pro, which directly competes for the same Glu pools. However, surprisingly, hyperaccumulation of GABA in the dry seeds eventually results in the accumulation of numerous amino acids, including a substantial but unexpected up-regulation of Trp metabolism via the shikimate pathway (Fig. 9), coupled with a significant accumulation of Asp, Asn, Ser, Cys, Met, Trp, and total protein. Generally higher levels of the various proteogenic amino acids were also shown in GABA-enriched rice (*Oryza sativa*) grains expressing a truncated OsGAD2 (Akama et al., 2009). It is possible that GABA-induced amino acid biosynthesis occurs via the TCA cycle, as suggested by our labeling experiments. Alternatively, accumulating GABA could be transaminated by GABA-T to produce Ala from pyruvate, or Gly from glyoxylate to Gly (Clark et al., 2009), thus contributing to amino group incorporation into the C skeleton and partly explaining the depletion of intermediates of C metabolism and fatty acid biosynthesis. This interpretation receives support from the up-regulation of Ala-glyoxylate transaminase. Nonetheless, we found pyruvate content to be higher in the transgenic seeds and pyruvate biosynthesis to be up-regulated (Fig. 9). A third and more plausible hypothesis is that increased amino acid content could be the result of protein degradation, as suggested by our transcript data (see below and Fig. 9), concomitant with increased protein content in the seed. Indirect evidence supporting this hypothesis may be drawn from the induction of protein degradation in response

Table 1. Functional categories overrepresentation analysis

Genes that were significantly up-regulated in the mutant compared with the wild type (Supplemental Table S3) were subjected to a functional categories overrepresentation analysis. Overrepresentation analysis was performed with the PageMan tool (see “Materials and Methods”). Only functional categories with two or more genes are shown.

Main Category	Subcategory	No. of Genes	P^a	Ratio ^b
Miscellaneous		19	0.0001	2.6
Metal handling		5	0.0002	9.7
Amino acid metabolism	Binding, chelation, and storage	4	0.0006	10.9
	Trp anthranilate synthase	2	0.0003	66.9
	Trp	3	0.0004	20.1
Not assigned		34	0.0010	0.6
Protein degradation	Cys protease	4	0.0015	8.0
Lipid metabolism	Fatty acid synthesis and fatty acid elongation: long-chain fatty acid CoA ligase	2	0.0020	28.7
Signaling	Calcium	6	0.0021	4.6
Redox	Glutathione S-transferases	3	0.0030	10.4
Stress	Biotic	6	0.0030	4.2
Secondary metabolism	N miscellaneous alkaloid like	3	0.0032	10.0

^aWilcoxon's P value, the probability of whether the response of the genes in a given group is significantly different from that of other genes in the whole genome set (Usadel et al., 2006; <http://mapman.mpimp-golm.mpg.de/pageman>). ^bRatio between the number of genes from a certain functional category that were found significantly up-regulated in *spGADΔC* compared with the wild type and the expected number of genes when randomly chosen (overrepresentation analysis; <http://mapman.mpimpgolm.mpg.de/general/ora/ora.shtml>).

to accumulating GABA, which was shown in our study (Fig. 9; Supplemental Table S3) and that of Roberts (2007) to activate stress-associated protein degradation processes.

Interaction between Storage Reserve Accumulation and GABA Shunt Regulation

Our results show that in dry transgenic *Arabidopsis* seeds, the increase in amino acids and the depletion of TCA cycle intermediates are accompanied by an increased protein content, a decrease in TFA, and an accumulation of short- to medium-chain fatty acid CoAs. Impaired fatty acid metabolism in the transgenic seeds could be associated with increased GABA production in three different ways. First, a reduction of TCA cycle activity would probably result in impaired production of CoA moieties. Second, the low levels of malate associated with increased GABA accumulation could have a detrimental effect on fatty acid elongation and fatty acid buildup. Among the TCA cycle intermediates measured in our analysis, malate showed the most marked drop in concentration. It has previously been suggested that malate is the preferred substrate for fatty acid elongation not requiring ATP or a reductant (Smith et al., 1992; Pleite et al., 2005), which are limited in desiccating seeds. Confirming the findings of Smith et al. (1992), Kendrick and Ratledge (1992) showed that a structural analog of malate, tartronic acid, which is an inhibitor of malic enzyme, could inhibit the malate-induced stimulation of fatty acyl group desaturation and elongation in the microsomal membranes of *Mucor circinelloides*. In our study, an impaired TCA cycle was also reflected in the alteration of genes associated with mitochondrial electron transport and particularly in

the up-regulation of alternative oxidase genes. In further support of our metabolite and transcript data, extensive analysis of transgenic tomato (*Solanum lycopersicum*) plants deficient in the expression of various enzymes of the TCA cycle showed impaired electron transport and photosynthesis (Nunes-Nesi et al., 2011). A third and more speculative possibility is supported by evidence from radiolabeling experiments in germinating lupines. A direct interaction between fatty acid metabolism and amino acids was shown in yellow lupine (*Lupinus luteus*) seedlings (Borek et al., 2003). It was demonstrated that, in the seedling axis, C atoms from acetate were incorporated mainly into amino acids upon germination. This direct metabolic cross talk might have had a feedback inhibition on the accumulation of fatty acids during seed development. It is intriguing that the same acyl-CoA derivatives found to significantly accumulate in this study were shown to change in a similar direction when a *Brassica napus* aminoalcoholphosphotransferase (AAPT1) was overexpressed in *Arabidopsis* (Qi et al., 2003). AAPT1 governs a reaction downstream of Ser catabolism, converting CDP-ethanolamine + 1,2-diacyl-*sn*-glycerol into CMP + α -phosphatidylethanolamine. The accumulation of Ser in our transgenic lines might have led to the availability of increased substrate and thus mimicked the effect of the overexpression reported by Qi et al. (2003). Last, Asp can be converted to pantothenate via β -Ala and support CoA biosynthesis. Our parallel metabolite and transcript analyses indicated an increase in Asp and pantothenate kinase 1, the latter encoding the enzyme downstream of pantothenate biosynthesis toward CoA production. As such, the increase in acyl-CoAs can be directly linked to the up-regulation in amino acid metabolism induced by the accumulation of GABA. The increased total protein

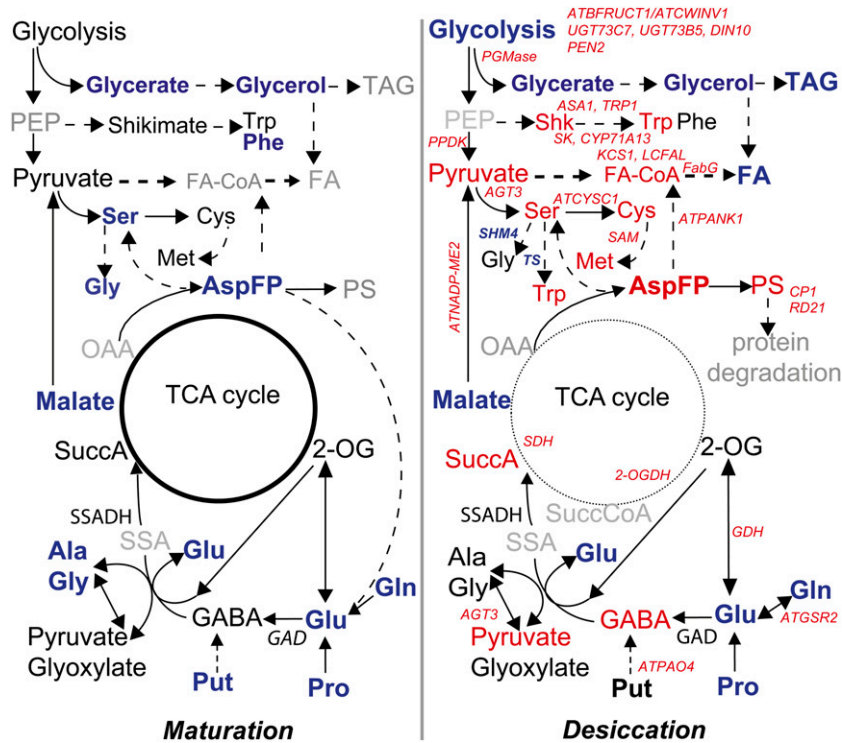


Figure 9. Schematic summary of major metabolic processes altered in maturing (left) and desiccating (right) *spGADΔC* seeds. Deregulated Glu-to-GABA metabolism affects differently the metabolic network at seed maturation and seed desiccation. GABA does not accumulate during seed maturation, but amino acid content is generally reduced. In the dry seed, GABA accumulation is associated with an increased content of amino acids, an increased protein content, and a reduction of TFA. Font colors red and blue indicate increase and decrease, respectively, as compared with the control wild type, and gray indicates nonmeasured compounds. Dashed arrows indicate multiple-step pathways. The thickness of the TCA cycle circular scheme refers to the reduced TCA cycle activity during seed desiccation. Clark et al. (2009) suggest that 2-OG-dependent GABA-T activity is not present in plants. In italics are given the genes associated with metabolic processes whose expression was significantly changed in the transgenics (Supplemental Tables S3 and S4). Abbreviations not used in the text are as follows: AGT3, Ala:glyoxylate aminotransferase 3; ASA1, anthranilate synthase α -subunit 1; AspFP, Asp family pathway; ATBFRUCT1/ATCWINV1, Arabidopsis cell wall invertase 1; ATCYSC1, Cys synthase C1; ATGSR2, Gln synthetase 2; ATNADP-ME2, NADP-malic enzyme 2; ATPANK1, pantothenate kinase 1; ATPAO4, polyamine oxidase 4; CP1, Cys proteinase 1; CYP71A13, indoleacetaldoxime dehydratase; DIN10, dark-inducible 10 hydrolase; FA, fatty acids; FabG, fatty acid biosynthesis, 3-oxoacyl-(acyl-carrier protein) reductase; GDH, Glu dehydrogenase; LCFAL, long-chain fatty acid-CoA ligase/long-chain acyl-CoA synthetase; OAA, oxaloacetate; 2-OGDH, 2-oxoglutarate dehydrogenase E2 component (dihydrolipoamide succinyltransferase); PEN2, penetration 2 hydrolase; PGMase, phosphoglycerate mutase; PPK, pyruvate orthophosphate dikinase; PS, storage protein; SAM1, S-adenosyl-Met synthetase 1; Shk, shikimate; SHM4, Ser hydroxymethyltransferase 4; SK, shikimate kinase; SSA, succinic semialdehyde; SuccA, succinate; SuccCoA, succinyl-CoA; TAG, triacylglycerol; TRP1, anthranilate phosphoribosyltransferase; TS, Trp synthase; UGT73C7 and UGT73B5, UDP-glycosyltransferase.

content in the transgenic seeds can be explained by the higher availability of amino acids. Branched amino acids have been shown to induce the initiation of translation and to stimulate protein synthesis in the skeletal muscles of animals (Yoshizawa, 2004). Unfortunately, we are aware of only a few studies comprehensively attempting to link changes in single amino acids with global metabolism and protein content, particularly in seeds. In rice, transgenic seeds accumulating high levels of Trp did not exhibit substantial changes in the content of other amino acids (Dubouzet et al., 2007). That said, the seminal study of Petrie and Wood (1938) revealed positive associations between levels of amino acids and proteins in illuminated leaves.

It is clear, however, that additional studies are required to elucidate the link between amino acids and the metabolism of storage reserves and to assess their relevance during seed germination. While speculative, the relation between these lines of evidence is supported by the similar regulation shared by key chloroplastic enzymes of starch, lipid, and amino acid synthesis (Geigenberger et al., 2005, and refs. therein).

Finally, in our study, the metabolic changes driven by the hyperaccumulation of GABA during seed maturation were found to be associated with a reduced germination phenotype. The increased expression of *DOG1*, which is one of the major regulators of seed dormancy (Bentsink et al., 2010), and *ANAC092/*

ATNAC2/ATNAC6 in the *spGADΔC* transgenic seeds are probably involved in the altered germination phenotype (Balazadeh et al., 2010) and are indicative of a link between GABA accumulation and the regulation of germination. In addition, genes associated with remobilization processes (*ATGSR2*, *RD21*) and senescence (*SENESCENCE-ASSOCIATED GENE21* [*SAG21*]) were up-regulated. For example, *RD21* is an Arabidopsis Cys protease that was identified as dehydration responsive in Arabidopsis. Its pattern of expression, initiated upon imbibition in radish (*Raphanus sativus*; Kikuchi et al., 2008), hints at a role in the degradation of cellular materials feeding the growing embryo (Rojo et al., 2003). *RD21* has been found to be associated with senescence and with *SAG21*, a gene associated with senescence initiation and responsive to Glc (Weaver et al., 1998); *SAG21* was also increased in the transgenic seeds. Last, the up-regulation of polyamine catabolism (Fig. 9) and Gln synthetase (the plastidial form *GS2*, *ATGSR2*) was found to be linked to the remobilization of N, probably triggered by a decreased content of Glu and Gln (Glass et al., 2002; Moschou et al., 2008).

CONCLUSION

Deregulated Glu-to-GABA catabolism can lead to dramatic alterations in fatty acid metabolism and in the balance between N and C moieties during seed maturation. Unexpectedly, we found that the balance was directed toward a developmentally induced increased N-to-C ratio. Although, according to the scientific literature, up to 95% of seed protein is derived from amino acids that are exported to the seed after degradation of existing protein in the leaves, we showed that altered activity of this pathway during seed maturation could cause metabolic processes specific to the embryo (driven by the phaseolin promoter) to change the C-N balance in storage reserves. The parallel metabolite and transcript profiles indicate an intriguing link between the induction of nonprotein amino acid metabolism and fatty acid assembly, N remobilization, programmed cell death, and the major regulatory processes of germination. The above lines of evidence open new directions for research on the molecular mechanisms involved and their concerted regulation in seed maturation, which will contribute to our current understanding of seed biology.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich Israel, with the exception of *N*-methyl-*N*-[trimethylsilyl] trifluoroacetamide, which was obtained from Macherey-Nagel.

Production of Transgenic Plants

A strategy identical to the one described by Karchi et al. (1994) was used to transform wild-type Arabidopsis (*Arabidopsis thaliana*) with a chimeric gene

encoding a GAD isolated from petunia (*Petunia hybrida*; Baum et al., 1993) fused to a promoter derived from the bean (*Phaseolus vulgaris*) phaseolin storage protein (Phas). cDNA coding either the 58-kD petunia calmodulin-binding GAD (Baum et al., 1993) or a mutant GAD lacking 27 amino acids at the C terminus (Arazi et al., 1995) was introduced downstream of the Phas promoter and upstream of a 3' untranslated region of the octopine synthase gene from *Agrobacterium tumefaciens*, essentially as described by Karchi et al. (1994). Transgenic Arabidopsis plants harboring the chimeric DNA constructs were prepared by inflorescence-dipping transformation (Clough and Bent, 1998). Seeds obtained from the transformed plants were considered as T1 seeds. These seeds were collected and surface sterilized for 10 min in diluted bleach solution containing a few drops of Tween 20, followed by three rinses with sterile distilled water. The seeds were then plated on a selective medium containing kanamycin and carbenicillin. The resistant T1 seeds were grown in soil to raise T1 plants, which were also grown to maturity. The T2 seeds were collected. Using the same procedure, we obtained the T3 and T4 generations.

Plant Growth and Seed Collection

Collection of seeds and siliques was performed essentially as described previously (Fait et al., 2006; Angelovici et al., 2008). Arabidopsis Ws seeds of the wild type and *spGAD* and *spGADΔC* transgenic lines were germinated on soil and grown for two consecutive rounds in a greenhouse (21°C) under the same light regime with a minimum of 250 μmol photons m⁻² s⁻¹. Flowers were tagged on the day of anthesis and at 14 ± 1 and 17 ± 1 DAF. Mature siliques were collected and cryolyophilized to dryness, and seeds were then dissected from the dried siliques and immediately frozen in liquid N. Mature dry seeds were collected at the end of the desiccation period, allowed to dry in paper bags for 4 weeks at room temperature, and stored at 4°C until further analysis. Seed viability was assayed by germinating seeds on agar plates, following 72 h of stratification. The tetrazolium assay was performed exactly as described by Wharton (1955).

GC-MS Analysis of Seed Metabolites

Siliques representing each developmental stage were collected as described above from at least 30 individual plants divided into at least four groups unless otherwise stated. Approximately 10 mg of seeds was utilized for each extraction. Metabolites were extracted and analyzed with a GC-MS-based protocol optimized for Arabidopsis (Liseč et al., 2006) by adjusting the extraction protocol to seed material as described by Fait et al. (2006). Relative metabolite content was calculated as described by Roessner et al. (2001) following peak identification using TagFinder (Luedemann et al., 2008). Substances were identified by comparison with mass spectral tags represented in query database (Kopka et al., 2005; Erban et al., 2007). The protein concentration of ethanolic seed extracts was determined by the assay of Bradford (1976).

Measurements of Total Protein and TFAs, and HPLC Analysis of Acyl-Ethano-CoA Derivatives

Twenty-milligram portions of material were frozen in liquid N and extracted for subsequent quantitative analysis of fluorescent acyl-ethano-CoA derivatives by HPLC. Analysis of acyl-CoA was performed using an Agilent 1100 LC system [Phenomenex LUNA 150 3 2 mm C18(2) column]; the methodology and gradient conditions have been described previously (Larson and Graham, 2001; Larson et al., 2002; Sayanova et al., 2007). The concentration of protein extracted in boiling ethanol was quantified using the assay of Bradford (1976). Fatty acid methyl esters were quantified by GC with pentadecanoic acid (15:0) as the internal standard (Browse et al., 1986).

Labeling of Siliques and Seed Material

For radiolabeling of siliques and seeds, 1 μCi mL⁻¹ solutions of the following radiolabeled precursors were used: [U-¹⁴C]Glc (specific activity, 11.1 GBq mmol⁻¹), [U-¹⁴C]acetate (specific activity, 2.07 GBq mmol⁻¹), [U-¹⁴C]Glu (specific activity, 9.36 GBq mmol⁻¹), and [C1-¹⁴C]Glu (specific activity, 2.22 GBq mmol⁻¹). To measure the release of ¹⁴CO₂ during the maturation period, flowers were tagged at anthesis, and siliques were collected between 14 and 18 DAF from at least 10 plants per genotype. Intact siliques were submerged in a solution of 200 μL of MES-KOH buffer (pH 6.4) containing 2 mM nonlabeled precursor. After 1 h of acclimation, 200 μL of buffer containing the specific

radiolabel was added. To measure the release of $^{14}\text{CO}_2$ from imbibed seeds, 10 mg of dried seeds was stratified (1 mL of double-distilled water, 4°C, darkness) for 72 h. Four hours prior to harvesting, the water was replaced with 200 μL of MES-KOH buffer (pH 6.4) containing 2 mM nonlabeled precursor. After 1 h of acclimation, 200 μL of buffer containing the specific radiolabel was added. CO_2 was collected 2 and 4 h following incubation by using CO_2 traps containing 200 μL of 10 mM KOH, as described by ap Rees and Bevers (1960) and Nunes-Nesi et al. (2005).

Fractionation of ^{14}C -Labeled Seed Extracts

Collected seeds were frozen immediately in liquid N after washing twice with 1.5 mL of MES-KOH buffer. The material was extracted using a modification of the method described by Roessner et al. (2001), Liseč et al. (2006), and Bligh and Dyer (1959). To inhibit enzyme activity, the seeds were ground with 950 μL of a methanol-chloroform mixture in a ratio of 2:1. After shaking the homogenate for 15 min at room temperature, the extract was centrifuged for 10 min at 14,000 rpm. The procedure was repeated for a second time, and each supernatant was transferred to a 4-mL glass vial. The supernatant was mixed with 625 μL of chloroform and 625 μL of water. For phase separation of the apolar and polar phases, the mixture was centrifuged for 30 min at 4,000 rpm. To analyze the lipid fraction, 800 μL of the lower apolar phase was dried on a filter paper with a N sample concentrator to prevent the quenching effects of the chloroform. For the fractionation of the upper polar phase, a 1,140- μL aliquot was taken and dried under vacuum overnight. To prevent contamination by the interphase, the remainder of the polar phase was discarded. After drying, the almost invisible pellet was suspended in 2 mL of water. Afterward, it was fractionated into a neutral fraction containing the sugars, an acidic fraction containing the organic acids, and a basic fraction containing the amino acids, as described by Quick et al. (1989) and Geigenberger et al. (1997). To this end, 1 mL of the aqueous solution was filtered over in-house-made columns containing specific anion- and cation-exchange resins. The columns were washed three times, each time with 1 mL of double-distilled water, and the solutions that passed through the columns were combined to give the neutral fraction. To extract the acidic fraction, the anion-exchange column was washed three times, each time with 1 mL of 4 M formic acid. To obtain the basic fraction, the cation-exchange column was washed three times, each time with 1 mL of 1 M NH_4OH . For sugar determination, an aliquot of 200 μL of the neutral fraction was treated with 4 units mL^{-1} hexokinase (25°C, 4 h). In parallel, another aliquot of the same fraction was treated with 1 unit mL^{-1} Glc oxidase and 32 units mL^{-1} peroxidase (25°C, 6 h). After heat inactivation of the enzymes (95°C, 5 min), the solution was also fractionated into neutral, anionic, and cationic fractions.

The pellet remaining after the extraction of the methanol-chloroform-soluble fraction was dried and resuspended in 0.5 mL of double-distilled water. An aliquot of 200 μL was treated with 10 units of amyloglucosidase (37°C, overnight) followed by 10 units of pronase (37°C, overnight). The enzymes were inactivated by heat treatment (95°C, 5 min), and the mixture was filtered and fractionated into a neutral fraction containing starch, an acidic fraction containing the cell wall fragments, and a basic fraction containing the proteins. Fractionation on ion-exchange columns was performed as described before (Runquist and Kruger, 1999).

^{13}C Feeding Experiments

For ^{13}C feeding experiments, seeds (10 mg for each of six replicates) were stratified in 10 mM MES-KOH for 72 h at 4°C in the dark and then transferred to continuous light at 21°C for 24 h. Six hours prior to the end of the stratification period and at the end of the 24-h germination period (i.e. 1 DAS), imbibed seeds were fed with a substrate according to a modified method of Morcuende et al. (1998) in a solution containing 10 mM MES-KOH (pH 6.5) and 15 mM [^{13}C]Glu, and the incubation was left to proceed for 6 h. At the end of this time, seeds were removed, washed carefully with 10 mM MES-KOH (pH 6.5), allowed to stand briefly on filter paper to remove the solution, weighed, frozen in liquid N, and stored at -80°C until further analysis. Samples were then extracted and evaluated exactly as described by Roessner-Tunali et al. (2004). Label redistribution was determined as described by Giegé et al. (2003) and Tieman et al. (2006).

RNA Extraction and Microarray Analysis

All experiments analyzing RNA expression levels were carried out using two replicates of seed materials, obtained from two independent seed lots,

from plants grown under controlled-environment conditions. Total RNA was extracted from stored dry seeds as described previously (Angelovici et al., 2009). Total RNA was treated with DNase RQ-1 (Promega); thereafter, RNA was amplified by using two-cycle Affymetrix labeling according to the standard Affymetrix protocol. Hybridization, labeling, scanning, and data extraction were performed according to the standard Affymetrix protocols. Transcriptome analysis was carried out with Partek Genome Suite software (www.partek.com). Preprocessing was carried out using the Robust Microarray Averaging algorithm (Irizarry et al., 2003). One-way ANOVA was performed. Overrepresentation/underrepresentation analysis was performed by PageMan (<http://mapman.mpimp-golm.mpg.de/general/ora/ora.shtml>; Usadel et al., 2006). Enriched categories were identified using the Database for Annotation, Visualization, and Integrated Discovery version 6.7 Web-accessible program (<http://david.abcc.ncifcrf.gov/>). Interpretation of the gene expression data set with respect to metabolic pathways and other functional categories was aided by visualizing changes in gene expression via the MapMan software (Usadel et al., 2005).

Statistical Analysis

Statistical analysis was performed on the data sets obtained from metabolite profiling with the software package TMEV (Saeed et al., 2003). Prior to the analysis, data were log transformed. In addition, to test statistical significance between specific time points, *t* tests were performed using the algorithm incorporated into Microsoft Excel with Bonferroni correction of the critical *P* value for multiple comparisons.

Supplemental Data

Supplemental Table S1. Log₁₀-transformed mean fold change in the metabolite content of developing seeds of *spGADΔC* transgenics and the wild type relative to that in wild-type dry seeds.

Supplemental Table S2. The incorporation of [$^{1,2-14}\text{C}$]acetate in the polar neutral, acidic, and basic fractions and in the lipid fraction of the seed extracts.

Supplemental Table S3. Calculated ratios of average expression levels of genes exhibiting significantly up-regulated expression in *spGADΔC-2* as compared with the wild type in dry seeds (*P* < 0.05 after FDR).

Supplemental Table S4. Calculated ratios of average expression levels of genes exhibiting significantly down-regulated expression in *spGADΔC-2* as compared with the wild type in dry seeds (*P* < 0.05 after FDR).

Supplemental Table S5. Full data set of genome-wide gene expression profiles of dry seed of *spGADΔC-2* and the wild type.

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LITERATURE CITED

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