RUNNING HEAD: A Deregulated Glu Metabolism Alters C-N Balance in Seeds

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Targeted enhancement of glutamate to γ-aminobutyrate conversion in Arabidopsis seeds affects C-N balance and storage reserves in a development-dependent manner

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ABSTRACT

In seeds, glutamate decarboxylase (GAD) operates at the metabolic nexus between carbon and nitrogen metabolism by catalysing the unidirectional decarboxylation of Glu to form gamma-aminobutyric acid (GABA). To elucidate the regulatory role of GAD in seed development, we generated Arabidopsis thaliana transgenic plants expressing a truncated GAD from Petunia hybrida missing the C-terminal regulatory Ca²⁺-calmodulin (CaM) 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, though not Glu or Pro. Dry transgenic seeds had higher protein content than the wild-type seeds, but lower amounts of the intermediates of glycolysis, glycerol and malate. The total fatty acid (TFA) content of the transgenic seeds was 50% lower than in that of the wild-type, while acyl CoAs 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 upregulated at the transcript level included the tricarboxylic acid cycle, fatty acid elongation, the shikimate pathway and Trp metabolism, N-C remobilization and programmed cell death. Genes involved in the regulation of germination were similarly upregulated. Taken together these results indicate that the GAD-mediated conversion of Glu to GABA during seed development plays an important role in balancing carbon and nitrogen metabolism and in storage reserve accumulation.
INTRODUCTION

Efficient assimilation of carbon and nitrogen 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 (sucrose, hexoses, trehalose) and abscisic acid (ABA). 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 seeds shifts from a general decrease in unbound metabolites to the accumulation of a set of specific metabolites, including gamma-aminobutyrate (GABA) (Fait et al., 2006; Angelovici et al., 2010).

GABA is a four-carbon non-protein 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 (Chen et al., 2004), soybean (Snedden et al., 1995), tobacco (Baum et al., 1996), petunia (Arazi et al., 1995), and Arabidopsis thaliana (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 (CaM)-binding domains (Baum et al., 1993). Thus, GAD catalyses the decarboxylation of Glu to GABA in a CaM-dependent manner. Scientists have thus hypothesized both a protective role for
GABA during stress events and a signalling 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 systemic deregulated GABA biosynthesis has been shown in tobacco (Baum et al., 1996; McLean et al., 2003): transgenic plants overexpressing 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 non-toxic reserve of C and N for amino acid metabolism and tricarboxylic acid (TCA) cycle activity, which is of particular relevance during stress events (Breitkreuz et al., 1999; Solomon and Oliver, 2002; Beuve et al., 2004; Bouchè and Fromm, 2004). However, only recently has direct evidence – integrating metabolite and transcript profiling and stable-isotope labelling – 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 catalyses 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 inter-regulation 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 metabolic regulation of Lys biosynthesis (Zhu and Galili, 2003; Angelovici et al., 2009; 2010). In the system used in the current study, the seed-specific phaseolin storage protein promoter drives the expression of a deregulated GAD from petunia (Petunia hybrida) 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 the 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 our current understanding of seed development metabolism and its implications for seed germination.

RESULTS

Stable Isotope Labelling Experiments Suggest that the GABA shunt Represents a Considerable Source of Carbon for the TCA Cycle during Early Stages of Seed Germination

Earlier radiolabelling 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, radiolabelling studies are limited in their applicability to monitoring the degree of labelling across a range of metabolites: as a result, stable isotopes coupled with MS or NMR have been increasingly used in the last decade for pathway elucidation and flux analysis (Sauer, 2006, and references 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 day after stratification (1DAS, when germination has been initiated); an adapted 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 (i) the incorporation of the isotope into each intermediate within the relevant pathways (Fig. 1) and (ii) the proportional labelling of $^{13}$C and $^{12}$C (carbon fractional enrichment) in metabolites closely associated with Glu. In stratified seeds supplied with $[^{13}]$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 1DAS 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 strictu) 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: (i) by the action of putrescine:oxygen oxidoreductase (EC 1.4.3.10) on the aldehyde form of GABA, (ii) via the arginine decarboxylase pathway, or (iii) 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 works 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 bean *Phaseolus vulgaris* phaseolin promoter (see M&M), producing a constitutively active – though developmentally induced – GAD enzyme. Three and four independent transformants of spGADΔC and spGAD, respectively, were eventually isolated on antibiotic plates and tested for 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 hyper-accumulation of GABA. Besides the metabolic phenotype of the seed, these plants showed no significant differences in vegetative traits as compared with Wassilewskija (Ws) 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
germination (seeds of the control and of the transgenics were harvested at the same
time and stored together, see M&M), 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 towards 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 days after flowering (DAF), and at full maturity. Seeds were extracted
and analysed using an established GC-MS-based protocol (Roessner et al., 2001;
Fait et al., 2006). Figure 3A and 3B show changes in 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 Table 1. While the phaseolin promoter activity is known to commence
during early maturation of the seed (Apolonia et al., 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
14DAF 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 14DAF (Fig. 3,
Supplemental Table 1), and the accumulation of several of these amino acids in the
dry seeds of both transgenic lines (Fig. 3, Supplemental Table 1). 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 1). 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 1), 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 1). For instance, in the dry transgenic seeds, fructose was decreased to 30 % of its content in the dry seed of the wild type (Fig. 3). Galactose, maltose and erythritol followed a similar trend. The glycolysis-derived intermediates of glycerolipid metabolism, glycerate, glycerol and the related compound myo-inositol were consistently decreased in the transgenic seeds (Fig. 3, Supplemental Table 1). 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 1). Notably, malate content was one-third in the dry seeds of the transgenics than in those of the wild type (Fig. 3).

14C Labelling Experiments Reveal Decreased TCA Cycle Activity but Increased Glu Catabolism in the GABA Hyper-Accumulating 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 incubation of siliques in solutions of positionally labelled molecules. Maturing siliques (14 and 17DAF) from the wild type and from 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 at following two and four hours 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 labelled CO2 from [3:4-14C]Glc was 50% lower in the representative transgenic line spGADΔC-7 than that 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 labelled
CO$_2$ from maturing siliques fed with [1-$^{14}$C]Glu confirmed the upregulation of the GABA shunt (Fig. 4). [U-$^{14}$C]Glu feeding gave a comparable increase in CO$_2$ evolution, suggesting that Glu-derived CO$_2$ 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-$^{14}$C]Glc and measured the incorporation of the radiolabel in the polar neutral, acidic, and basic and in the lipid fractions of the seed extracts. The metabolism of the radiolabelled 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 spGAD$_{\Delta}$C lines than that 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 CO$_2$ evolution in the transgenic seeds (Fig. 5). For seeds fed with [1,2-$^{14}$C]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 2).

**Upregulation 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 spGAD$_{\Delta}$C-2 and spGAD$_{\Delta}$C-7 vs 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 spGAD$_{\Delta}$C-2 and spGAD$_{\Delta}$C-7 than in the wild type, respectively. In contrast, HPLC analysis of TFA revealed markedly reduced levels of TFA in the transgenic seeds, with spGAD$_{\Delta}$C-2 and spGAD$_{\Delta}$C-7 displaying on average 30% and 70% less of TFA than the wild type (Fig. 7B). However, there was a general accumulation of acyl-CoAs in the dry seeds, with a notable twofold increase for 16:0, 18:2, 20:1 and 20:3 fatty acids in both transgenics as compared to the wild type (Fig. 8).
Genome-Wide Expression Analysis of \textit{spGAD}\Delta C 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 vs. the wild type – from the same material collected for metabolite profiling, using the affimetrix 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) upregulated and 55 downregulated in the transgenics as compared to the wild type, showing at least 1.5-fold change. The detailed list of genes showing statistically significant changes in expression following ANOVA analysis between the dry seeds of the transgenic line and the wild type, and calculated fold changes are provided in Supplemental Tables 3 and 4. The entire dataset is provided in Supplemental Table 5. In summary, genes related to metabolism were abundant, and the vast majority was upregulated. Moreover, overrepresentation analysis using the tools embedded in the PageMan software (http://mapman.mpimp-golm.mpg.de/general/ora/ora.shtml) indicated that among the upregulated genes (Supplemental Table 3) in the transgenic seeds there was over representation of the following categories: biotic stress (six genes), redox-state- (three genes), calcium-associated signalling (six genes), and cell wall metabolism in the transgenic seeds (Table 1, overrepresentation analysis; Supplemental Table 3 list of upregulated genes). Among the genes associated with metabolism, the most markedly affected were glutamate dehydrogenase (GDH), glutamine synthetase (ATGSR2), alanine:glyoxylate aminotransferase (AGT3), and S-adenosyl methionine1 synthetase (SAM1) as well as several genes associated with aromatic amino acid metabolism (ASA1, TRP1, SK) and polyamine catabolism [polyamine oxidase (PAO)] (Supplemental Table 3 - fold change changes). In contrast, the expression of genes associated with serine catabolism [serine hydroxymethyltransferase 4 (SHM4) and tryptophan synthase (TS)] was downregulated (Supplemental Table 4).

The parallel metabolite and transcript analysis indicated that the decrease in the concentrations of a number of TCA cycle intermediates in the seeds of \textit{spGAD}\Delta C
lines was also associated with changes in TCA gene expression patterns. As shown in Supplemental Table 3, changes in the level of TCA-cycle-associated transcripts included the upregulation of succinate dehydrogenase (SDH), 2-oxoglutarate dehydrogenase (2-OGDH). The transcripts of malic enzyme (AtNADP-ME2), which is involved in the conversion of malate to pyruvate, the direct precursor of acetyl-CoA, were upregulated in the transgenic line. CoA metabolism in relation to fatty acid metabolism was indeed altered at the transcript level, as shown in the upregulation of long-chain fatty acid CoA ligase and long-chain acyl-CoA synthetase and condensing enzyme KCS1 (3-ketoacyl-CoA synthase 1) which is involved in fatty acid elongation and by a downregulation of fatty acid transport (ABC transporter). In the seeds of the transgenic plants we also found a significant upregulation of the genes (Supplemental Table 3) involved in the degradation of protein and cellular components (Cys protease and RD21) and in N remobilization (ATGSR2), which could explain the increased amino acid content in these seeds. Among the genes upregulated in the transgenic seeds, a small number is 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 upregulated genes included those involved in the mitochondrial electron transport chain, i.e., alternative oxidase (AOX1) and FAD-binding domain-containing protein (AT1G26380). With respect to energy, expression of transcripts of GTP and ATP binding proteins were downregulated (AT5G56500, AT5G57960 and AT1G30960, respectively, Supplemental Table 4). Gene ontology analysis for enrichment in biological processes (http://david.abcc.ncifcrf.gov/) indicated a significant enrichment of genes associated with electron transport process, which included 13 upregulated and six downregulated genes (Supplemental Table 3 and Supplemental Table 4). Among these, there was a 3.6-fold downregulation of cytochrome p450 (CYP71A20 and CYP71A19) and a twofold downregulation at MATERNAL EFFECT EMBRYO ARREST 23 electron carrier involved in embryo development and seed dormancy. ANAC092/ATNAC2/ATNAC6 and DOG1 associated with the regulation of germination were upregulated in the transgenic seeds.
DISCUSSION

In plants glutamate metabolism is pivotal for efficient N incorporation, and its levels are therefore 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 in high levels in dry seeds of Arabidopsis, a finding implying a developmental role in seeds for this non-proteogenic amino acid (Fait et al., 2006). Labelling experiments conducted in the current study directly confirm that GABA is strongly associated with early seed germination metabolic processes, replenishing the TCA cycle and contributing 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 the present study, we investigated the perturbation in seed metabolism following the seed-targeted upregulation of Glu conversion to GABA during seed maturation. We employed the upregulation strategy to challenge the seed metabolic network in a development-induced manner with the aim to reveal 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 favour of N. Nonetheless, hyper-accumulation 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 germination than was previously perceived.

Upregulation 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-Guimaraes et al., 2007; Bunić and Fernie, 2009), we show here that Glu is metabolised preferentially via GABA (as compared to 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 it couldn’t be ruled 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 the 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 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 (14DAF-17DAF) and at desiccation (17DAF 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 days after flowering. 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 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 decrease of the levels of the vast majority of the free amino acids measured. We thus conclude that the increased use of Glu to produce GABA prevents 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 previously shown to be highly dependent on the redox state of the cell: Shelp et al. (1995) demonstrated that GABA shunt activity is associated with hypoxic environment, and the activity of SSADH was proved to be dependent on favourable redox and energy state (Busch et al., 1999). A similar effect was observed in coffee 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 conversion 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, hyper-accumulation of GABA in the dry seeds eventually results in the accumulation of numerous amino acids, including a substantial but unexpected upregulation 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 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 labelling experiments. Alternatively, accumulating GABA could be transaminated by GABA-T to produce Ala from pyruvate, or
glyoxylate to glycine (Clark et al., 2009), thus contributing to amino group incorporation into C skeleton, and partly explaining the depletion of intermediates of the C metabolism and fatty acid biosynthesis. This interpretation receives support from the upregulation of alanine-glyoxylate transaminase (AGT3). Nonetheless, we found pyruvate content to be higher in the transgenic seeds and pyruvate biosynthesis to be upregulated (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 to accumulating GABA, which was shown in our study (Supplemental Table 3, Fig. 9) 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 build-up. 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 (1997) showed that a structural analogue 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 upregulation of alternative oxidase genes. In further support of our metabolite and transcript data,
extensive analysis of transgenic tomato 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* L.) 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 in 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 serine catabolism, converting CDP-ethanolamine + 1,2-diacyl-sn-glycerol into CMP + α-phosphatidylethanolamine. The accumulation of serine 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 panthothenate via beta-Ala and support CoA biosynthesis. Our parallel metabolite and transcript analyses indicated an increase in Asp and ATPANK1 (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 upregulation in amino acid metabolism induced by the accumulation of GABA. The increased total protein content in the transgenic seeds can be explained by the higher availability of amino acids. Branched amino acids have been shown to induce initiation of translation and to stimulate protein synthesis in the skeletal muscles of animals (Yoshizawa, 2003). 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, however, clear 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 references therein).

Finally, in our study the metabolic changes driven by the hyper-accumulation of GABA during seed maturation were found to be associated with a reduced germination phenotype. The increased expression of *delay-of-germination 1* (*DOG1*), which is one of the major regulators of seed dormancy (Bentsink et al., 2010), and *ANAC092/ATNAC2/ATNAC6* (Arabidopsis NAC domain containing protein 92) 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, RD-21*) and senescence (*SAG21*) were upregulated. For example, *RD21* (Responsive to desiccation-21) is an Arabidopsis cysteine protease, which 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* (SENESCENCE-ASSOCIATED GENE 21), a gene associated with senescence initiation and responsive to glucose (Weaver et al., 1998); *SAG21* was also increased in the transgenic seeds. Last, the upregulation of polyamine catabolism (Fig. 9) and glutamine 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 the dramatic alteration in fatty acid metabolism and in the balance between N and C moieties during seed maturation. Unexpectedly, we found that the balance was directed towards 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 non-protein 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 Ltd. (Jerusalem, Israel) with the exception of N-methyl-N-[trimethylsilyl] trifluoroacetamide, which was obtained from Macherey-Nagel GmbH & Co. KG (Düren, Germany).

Production of Transgenic Plants
A strategy identical to the one described in Karchi et al. (1994) was used to transform wild-type Arabidopsis with a chimeric gene encoding a glutamate decarboxylase isolated from Petunia hybrida (Baum et al., 1993) fused to a promoter derived from the bean phaseolin storage protein (Phas). Complementary DNA coding either the 58-kDa petunia CaM-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 in Karchi et al. (1994). Transgenic Arabidopsis plants harbouring 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 (Angelovici et al., 2008; Fait et al., 2006). *Arabidopsis thaliana* wassilewskija (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 cryo-lyophilized to dryness, and seeds were then dissected from the dried siliques and immediately frozen in liquid nitrogen. Mature dry seeds were collected at the end of the desiccation period, allowed to dry in paper bags for four 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 in 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 in at least four groups unless otherwise stated. Approximately 10 mg of seeds were utilized for each extraction. Metabolites were extracted and analyzed with a GC-MS-based protocol optimized for Arabidopsis (Lisec et al., 2006) by adjusting the extraction protocol to seed material as described in Fait et al. (2006). Relative metabolite content was calculated as described in Roessner et al. (2001) following peak identification using TagFinder (Luedemann et al., 2008). Substances were identified by comparison to mass spectral tags represented in query database (Erban et al., 2007; Hummel et al., 2007). The protein concentration of ethanolic seed extracts was determined by the assay of Bradford (1976).

Measurements of Total Protein and Total Fatty Acids and HPLC Analysis of Acyl-Etheno-CoA Derivatives

Twenty-milligram portions of material were frozen in liquid nitrogen, and extracted for subsequent quantitative analysis of fluorescent acyl-etheno-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 previously described (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).

**Labelling of Siliques and Seed Material**

For radiolabelling of siliques and seeds, 1 µCi ml⁻¹ solutions of the following radiolabelled precursors were used: \([U-^{14}C]glucose\) (specific activity 11.1 GBq mmol⁻¹); \([U-^{14}C]acetate\) (specific activity 2.07 GBq mmol⁻¹); \([U-^{14}C]glutamate\) (specific activity of 9.36 GBq mmol⁻¹); and \([C1-^{14}C]glutamate\) (specific activity of 2.22 GBq mmol⁻¹). To measure release of \(^{14}\text{CO}_2\) 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 of non-labeled precursor. After 1 h of acclimation, 200 µl of buffer containing the specific radiolabel was added. To measure release of \(^{14}\text{CO}_2\) from imbibed seeds, 10 mg of dried seeds were stratified (1 ml of doubly distilled H₂O, 4°C, darkness) for 72 h. Four hours prior to harvesting, the water was replaced with 200 µl MES-KOH buffer (pH 6.4) containing 2 mM of non-labeled precursor. After 1 h of acclimation, 200 µl of buffer containing the specific radiolabel was added. CO₂ was collected 2 and 4 h following incubation by using CO₂ traps containing 200 µl of 10 mM KOH, as described in ap Rees and Beevers (1960) and Nunes-Nesi et al. (2005).

**Fractionation of \(^{14}\text{C}-\text{Labelled Seed Extracts}\)**

Collected seeds were frozen immediately in liquid nitrogen after washing twice with 1.5 ml of MES-KOH buffer. The material was extracted using a modification of the method described in Roessner et al. (2001), Lisec 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 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 4000 rpm. To analyse the lipid fraction, 800 µl of the lower apolar phase
was dried on a filter paper with a nitrogen sample concentrator to prevent the quenching effects of the chloroform. For the fractionation of the upper polar phase, a 1140 µl aliquot was taken and dried under vacuum overnight. To prevent contamination by the inter-phase, the remainder of the polar phase was discarded. After drying, the almost invisible pellet was suspended in 2 ml of water. Afterwards 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 in 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 doubly 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 with three times, each time with 1 ml of 1 M NH₄OH. For sugar determination an aliquot of 200 µl of the neutral fraction was treated with 4 U ml⁻¹ of hexokinase (25 °C, 4 h). In parallel, another aliquot of the same fraction was treated with 1 U ml⁻¹ of glucose oxidase and 32 U ml⁻¹ of 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 fraction.

The pellet remaining after the extraction of the methanol-chloroform-soluble fraction was dried and resuspended in 0.5 ml of doubly distilled water. An aliquot of 200 µl was treated with 10 U of amyloglucosidase (37 °C, overnight), followed by 10 U of pronase (37 °C, overnight). The enzymes were inactivated by heat treatment (95 °C, 5 min), and 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).

**¹³C Feeding Experiments**

For ¹³C 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 [U-^{13}C]glutamate, 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 nitrogen, 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 in Giege 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 (Ruuska and Ohlrogge, 2001; Angelovici et al., 2008). Total RNA was treated with DNAase RQ-1 (Promega); thereafter, RNA was amplified by using two-cycle Affymetrix labelling 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 Page-Man (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 (DAVID) v6.7 web-accessible program (http://david.abcc.ncifcrf.gov/). Interpretation of the gene expression dataset 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 (Microsoft Corporation, Seattle, WA, USA) with Bonferroni correction of the critical p-value for multiple comparisons.

Supplemental Material

Supplemental Table 1. Log10 transformed mean fold change in the metabolite content of developing seeds of spGAΔDC transgenics and the wild type relative to that in wild type dry seeds.

Supplemental Table 2. The incorporation of [1,2-14C]-acetate in the polar neutral, acidic, basic and in the lipid fractions of the seed extracts.

Supplemental Table 3. Calculated ratios of average expression levels of genes exhibiting significantly upregulated expression in the spGADΔC-2 as compared to the WT in dry seeds (p<0.05 after FDR).

Supplemental Table 4. Calculated ratios of average expression levels of genes exhibiting significantly downregulated expression in the spGADΔC-2 as compared to the WT in dry seeds (p<0.05 after FDR).

Supplemental Table 5. Full dataset of genome wide gene expression profile of dry seed of spGADΔC-2 line and of the wild type. RNA levels were determined using two replicates of seed materials, obtained from two independent seed lots, from plants grown under controlled-environment conditions. 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).

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Table I. Functional Categories overrepresentation analysis. Genes that were significantly upregulated in the mutant compared to the wild type (Supplemental Table 3) were subjected to a functional categories overrepresented analysis. Overrepresentation analysis was performed with the PageMan tool (see M&M). Only functional categories with two or more genes are shown.

<table>
<thead>
<tr>
<th>Main category</th>
<th>Subcategory</th>
<th>No. of genes</th>
<th>p value*</th>
<th>Ratio**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miscellaneous</td>
<td></td>
<td>19</td>
<td>0.0001</td>
<td>2.6</td>
</tr>
<tr>
<td>Metal handling</td>
<td></td>
<td>5</td>
<td>0.0002</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>Binding, chelation and storage</td>
<td>4</td>
<td>0.0006</td>
<td>10.9</td>
</tr>
<tr>
<td>Amino acid metabolism</td>
<td>Tryptophan anthranilate synthase</td>
<td>2</td>
<td>0.0003</td>
<td>66.9</td>
</tr>
<tr>
<td></td>
<td>Tryptophan</td>
<td>3</td>
<td>0.0004</td>
<td>20.1</td>
</tr>
<tr>
<td>Not assigned</td>
<td></td>
<td>34</td>
<td>0.0010</td>
<td>0.6</td>
</tr>
<tr>
<td>Protein degradation</td>
<td>Cysteine protease</td>
<td>4</td>
<td>0.0015</td>
<td>8.0</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>Fatty acid (FA) synthesis and FA elongation: long chain FA CoA ligase</td>
<td>2</td>
<td>0.0020</td>
<td>28.7</td>
</tr>
<tr>
<td>Signalling</td>
<td>Calcium</td>
<td>6</td>
<td>0.0021</td>
<td>4.6</td>
</tr>
<tr>
<td>Redox</td>
<td>Glutathione S transferases</td>
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<td>0.0030</td>
<td>10.4</td>
</tr>
<tr>
<td>Stress</td>
<td>Biotic</td>
<td>6</td>
<td>0.0030</td>
<td>4.2</td>
</tr>
<tr>
<td>Secondary metabolism</td>
<td>N miscellaneous alkaloid-like</td>
<td>3</td>
<td>0.0032</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Wilcoxon’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).

** Ratio, represents the ratio between the number of genes from a certain functional category that were found significantly upregulated in the \( spGAD_{ΔC} \) compared to the wild type and the expected number of genes when randomly chosen (overrepresentation analysis, http://mapman.mpimp-golm.mpg.de/general/ora/ora.shtml).
Figure 1. Fate of 13C-labelled Glu (Glu*) supplied to stratified (dark grey) and 1DAS (light grey) seeds of wild-type A. thaliana (ecotype Wassilewskija, Ws). The relative isotope redistribution of Glu is described onto a schematic presentation of the GABA shunt and related major metabolic pathways. Bars represent the carbon fractional enrichment, CFE [13C/(13C+12C)] of metabolites labeled. CFE was calculated by evaluating the labelled portion of the various pools as a proportion of the various pool sizes, following an incubation period of 6 h of stratified (dark grey bars) and 1 DAS seeds (light grey bars). Values are means ± SD from six determinations per time point.

Figure 2. (A) Schematic diagram of the chimeric gene utilized in the present study. Phas-Pro, the bean phaseolin storage protein promoter; spGAD and spGΔDC, the coding DNA sequence of the WT and mutant for of glutamate decarboxylase from Petunia; Ocs, the DNA sequence of the octopine synthase 3’ terminator. (B) GABA relative content in mature T3 seeds of transgenic plants harbouring spGAD and spGΔDC 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 WT. Transgenic lines spGΔDΔC-5, spGΔDΔC-2 and spGΔDΔC-7 are indicated. (C) Seed dimensions and weight. The seed dimension values are mean ± SD of 30 individual measurements repeated for three independent bulks of 10 (T3) plants each. The seed weight values are mean ± SD of bulked 100 seeds from 10 plants each and repeated ten times. Seeds have been viewed under a Leica MZ12.5 stereomicroscope (Leica DFC420 digital camera) and measured using the Leica Application Suite software. Asterisk represents significant differences (p<0.05) as compared to the WT.

Figure 3. Relative content of metabolites in developing seeds of spGΔDΔC -2 (ΔC-2), spGΔDΔC -7 (ΔC-7) and WT. Relative content of free metabolites was measured in developing seeds at 14, 17 DAF and in the dry seeds as described in Materials and Methods. Fourteen and 17 DAF stages reflect mid and late maturation respectively under the condition of growth used (see Fait et al., 2006 and Angelovici et al., 2009). Bar graphs indicate the mean fold change of each metabolite at 14DAF (black bars), 17DAF (grey bars) and in the dry seeds (white bars) relative to its content in the dry seeds of the WT. Asterisks denote a statistically significant (two tailed Student t-test,
p<0.01) difference between the spGADΔC and the WT for the metabolite content at the given developmental stage. Only metabolites which presented a significant and consistent change in content in the transgenics as compared to the WT are shown. The entire dataset is given in Supplemental Table 1.

Figure 4. 14CO2 release from siliques incubated in specifically labeled isotopes. Equivalent samples of maturing siliques collected between 14 to 18DAF were incubated in 1 μCi ml-1 of [1-14C]- and [U-14C]-glutamate and [3,4-14C]- and [U-14C]-glucose as described in the Materials and Methods. Values are presented as cumulative 14CO2 release, and are mean ± SE of four biological replicates.

Figure 5. Effect of deregulated Glu decarboxylation during seed maturation on metabolism of [U-14C]Glc by stratified seeds. Tissue was fractionated exactly as described in the Materials and Methods. Seeds were preincubated in 200 μl MES-KOH buffer (pH 6.4) containing 2 mM of unlabeled precursor. After 1 h of acclimation 200 μl buffer containing specific radio label was added. 14CO2 was collected using CO2 traps containing 200μl of 10 KOH as described in ap Rees and Beevers (1960) and Nunes-Nesi et al. (2005). Collected seeds were frozen immediately in liquid nitrogen after washing two times with 1.5 ml MES-KOH buffer. The material was extracted using combined and modified methods as described in the Materials and Methods. Values are expressed as percentages of the total radiolabel metabolized and are means ± SE of four biological replicates. Asterisks demarcate values that were judged to be significantly different from the control (P < 0.05) when tested by Student t-test procedure.

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 the Materials and Methods. Values are expressed as percentages of the total radiolabel metabolized and are means ± SE of four biological replicates. Asterisks demarcate values that were judged to be significantly different from the wild type (p < 0.05).

Figure 7. Protein (A) and total fatty acid (B) content in mature seeds of WT and transgenic plants. The effect of upregulated GABA metabolism on the content of seed proteins and total fatty acids was measured by standard Bradford protocol and GC based protocol as described in the materials and methods. Results are presented as mean ± SE of at least four biological replicates of 20mg seeds.
Figure 8. Content of Acyl-etheno-CoA derivatives in mature seeds of WT and transgenic plants. Twenty-milligram portions of material were frozen in liquid nitrogen, 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 mean ± SD of four biological replicates. *, represents significant (p<0.05) differences in respect to the WT calculated using the Student t-test procedure.

Figure 9. Schematic summary of major metabolic processes altered in maturing (left) and desiccating spGAΔDC seeds (right). De-regulated Glu to GABA metabolism affects differently the metabolic network at seed maturation and seed desiccation. GABA does not accumulate during seed maturation but amino acids content is generally reduced. In the dry seed GABA accumulation is associated with increased content of amino acids, increased protein content and with a reduction of TFA. Font color red and blue indicate increase or decrease, respectively, as compared to the control (WT), grey indicate non-measured compounds. Dashed arrows indicate multiple-step pathways. Thickness of TCA cycle circular scheme refer 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 respective metabolic processes, whose expression was significantly changed in the transgensics (Supplemental Table 3,4). Abbreviations: 2-OG, 2-oxoglutarate, SuccA, succinate, SSA, succinic semialdehyde; SuccCoA, succinyl-CoA; Shk, shikimate; AspFP, aspartic acid family pathway; PS, storage protein, TAG, triacylglycerol, FA, fatty acids; OAA oxaloacetate. ASA1, anthranilate synthase alpha subunit 1; ATCYSC1, cysteine synthase C1; ATNADP-ME2, NADP-malic enzyme 2; TRP1, anthranilate phosphoribosyltransferase; CP1 cysteine proteinase1; RD21, responsive to dehydration 21, cysteine-type peptidase; PGMase, phosphoglycerate mutase; LCFAL, long-chain-fatty-acid--CoA ligase / long-chain acyl-CoA synthetase; SDH, succinate dehydrogenase; 2-OGDH, 2-oxoglutarate dehydrogenase E2 component (dihydrolipoamide succinyltransferase); GDH, glutamate dehydrogenase; ATGSR2, glutamine synthetase 2; SAM1, S-adenosylmethionine synthetase 1; SK, Shikimate kinase; ATBFRUCT1/ATCWINV1, arabidopsis thaliana cell wall invertase 1; UGT73C7, UGT73B5, UDP-glycosyltransferase; DIN10, dark inducible10, hydrolase; PEN2, penetration 2 hydrolase; ATPAO4, polyamine oxidase 4; AGT3 alanine:glyoxylate
aminotransferase 3; ATPANK1, pantothenate kinase 1, PPDK pyruvate orthophosphate dikinase; FabG, fatty acid biosynthesis, 3-oxoacyl-(acyl-carrier protein) reductase; SHM4 serine hydroxymethyltransferase 4, TS, tryptophan synthase; CYP71A13, indoleacetaldoxime dehydratase.
Figure 1. Fate of $^{13}$C-labelled Glu (Glu*) supplied to stratified (dark grey) and 1DAS (light grey) seeds of wild-type A. thaliana (ecotype Wassilewskija, Ws). The relative isotope redistribution of Glu is described onto a schematic presentation of the GABA shunt and related major metabolic pathways. Bars represent the carbon fractional enrichment, CFE [13C/(13C+12C)] of metabolites labelled. CFE was calculated by evaluating the labelled portion of the various pools as a proportion of the various pool sizes, following an incubation period of 6 h of stratified (dark grey bars) and 1 DAS seeds (light grey bars). Values are means ± SD from six determinations per time point.
Figure 2. (A) Schematic diagram of the chimeric gene utilized in the present study. Phas-Pro, the bean phaseolin storage protein promoter; spGAD and spGADΔC, the coding DNA sequence of the WT and mutant for of glutamate decarboxylase from Petunia; Ocs, 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 WT. Transgenic lines spGADΔC-5, spGADΔC-2 and spGADΔC-7 are indicated. (C) Seed dimensions and weight. The seed dimension values are mean±SD of 30 individual measurements repeated for three independent bulks of 10 (T3) plants each. The seed weight values are mean±SD of bulked 100 seeds from 10 plants each and repeated ten times. Seeds have been viewed under a Leica MZ12.5 stereomicroscope (Leica DFC420 digital camera) and measured using the Leica Application Suite software. Asterisk represent significant differences (p<0.05) as compared to the WT.
Figure 3. Relative content of metabolites in developing seeds of spGADΔC-2 (ΔC-2), spGADΔC-7 (ΔC-7) and WT. Relative content of free metabolites was measured in developing seeds at 14, 17 DAF and in the dry seeds as described in Materials and Methods. Fourteen and 17 DAF stages reflect mid and late maturation respectively under the condition of growth used (see Fait et al., 2006 and Angelovici et al., 2009). Bar graphs indicate the mean fold change of each metabolite at 14DAF (black bars), 17DAF (grey bars) and in the dry seeds (white bars) relative to its content in the dry seeds of the WT. Asterisks denote a statistically significant (two tailed Student t-test, p<0.01) difference between the spGADΔC and the WT for the metabolite content at the given developmental stage. Only metabolites which presented a significant and consistent change in content in the transgenics as compared to the WT are shown. The entire dataset is given in Supplemental Table 1.
Figure 4. $^{14}$CO$_2$ release from siliques incubated in specifically labelled isotopes. Equivalent samples of maturing siliques collected between 14 to 18DAF were incubated in 1 μCi ml$^{-1}$ of [1-$^{14}$C]- and [U-$^{14}$C]-glutamate and [3,4-$^{14}$C]- and [U-$^{14}$C]-glucose as described in the Materials and Methods. Values are presented as cumulative $^{14}$CO$_2$ release, and are mean ± SE of four biological replicates.
Figure 5. Effect of deregulated Glu decarboxylation during seed maturation on metabolism of [U-14C]Glc by stratified seeds. Tissue was fractionated exactly as described in the Materials and Methods. Seeds were preincubated in 200 µl MES-KOH buffer (pH 6.4) containing 2 mM of unlabeled precursor. After 1 h of acclimation 200 µl buffer containing specific radio label was added. \(^{14}\)CO\(_2\) was collected using CO\(_2\) traps containing 200µl of 10 KOH as described in ap Rees and Beevers (1960) and Nunes-Nesi et al. (2005). Collected seeds were frozen immediately in liquid nitrogen after washing two times with 1.5 ml MES-KOH buffer. The material was extracted using combined and modified methods as described in the Materials and Methods. Values are expressed as percentages of the total radiolabel metabolized and are means ± SE of four biological replicates. Asterisks demarcate values that were judged to be significantly different from the control (P < 0.05) when tested by Student t-test procedure.
Figure 6. Effect of deregulated Glu decarboxylation during seed maturation on the incorporation of [1,2-$^{14}$C]-acetate in lipids by stratified seeds. Tissue was fractionated exactly as described in the Materials and Methods. Values are expressed as percentages of the total radiolabel metabolized and are means ± SE of four biological replicates. Asterisks demarcate values that were judged to be significantly different from the wild type (P < 0.05).
Figure 7. Protein (A) and total fatty acid (B) content in mature seeds of WT and transgenic plants. The effect of upregulated GABA metabolism on the content of seed proteins and total fatty acids was measured by standard Bradford protocol and GC based protocol as described in the materials and methods. Results are presented as mean ± SE of at least four biological replicates of 20mg seeds.
Figure 8. Content of Acyl-etheno-CoA Derivatives in mature seeds of WT and transgenic plants. Twenty-milligram portions of material were frozen in liquid nitrogen, 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 mean ± SD of four biological replicates. *, represents significant (p<0.05) differences in respect to the WT calculated using the Student t-test procedure.
Figure 9. Schematic summary of major metabolic processes altered in maturing (left) and desiccating spGADAC seeds (right). De-regulated Glu to GABA metabolism affects differently the metabolic network at seed maturation and seed desiccation. GABA does not accumulate during seed maturation but amino acids content is generally reduced. In the dry seed GABA accumulation is associated with increased content of amino acids, increased protein content and with a reduction of TFA. Font color red and blue indicate increase or decrease, respectively, as compared to the control (WT), grey indicate non-measured compounds. Dashed arrows indicate multiple step pathways. Thickness of TCA cycle circular scheme refer 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 respective metabolic processes, whose expression was significantly changed in the transgensics (Supplemental Table 3,4). Abbreviations: 2-OG, 2-oxoglutarate, SuccA, succinate, SSA, succinic semialdehyde; SuccCoA, succinyl-CoA; Shk, shikimate; AspFP, aspartic acid family pathway; PS, storage protein, TAG, triacylglycerol, FA, fatty acids; OAA oxaloacetate. ASA1, anthranilate synthase alpha subunit 1; ATCYSC1, cysteine synthase C1; ATNADP-ME2, NADP-malic enzyme 2; TRP1, anthranilate phosphoribosyltransferase; CP1 cysteine proteinase 1; RD21, responsive to dehydration 21, cysteine-type peptidase; PGMase, phosphoglycerate mutase; LCFAL, long-chain-fatty-acid-CoA ligase / long-chain acyl-CoA synthetase; SDH, succinate dehydrogenase; 2-OGDH, 2-oxoglutarate dehydrogenase E2 component; GDH, glutamate dehydrogenase; ATGSR2, glutamine synthetase 2; SAM1, S-adenosylmethionine synthetase 1; SK, Shikimate kinase; ATBFRUCT1/ATCWINV1, arabidopsis thaliana cell wall invertase 1; UGT73C7, UGT73B5, UDP-glycosyltransferase; DIN10, dark inducible 10, hydrolase; PEN2, penetration 2 hydrolase; ATPAO4, polyamine oxidase 4; AGT3 alanine:glyoxylate aminotransferase; CYP71A13, indoleacetaldoxime dehydratase; SHM4 serine hydroxymethyltransferase 4, TS, tryptophan synthase; CYP71A13, indoleacetaldoxime dehydratase.