Ectopic Expression of an Amino Acid Transporter (VfAAP1) in Seeds of *Vicia narbonensis* and Pea Increases Storage Proteins[1]

Hardy Rolletschek, Felicia Hosein[2], Manoela Miranda[3], Ute Heim[4], Klaus-Peter Götz, Armin Schlereth[5], Ljudmilla Borisjuk, Isolde Saalbach, Ulrich Wobus, and Hans Weber*

Institut für Pflanzengenetik und Kulturpflanzenforschung, D–06466 Gatersleben, Germany (H.R., F.H., M.M., U.H., A.S., L.B., I.S., U.W., H.W.); and Fachgebiet Pflanzenbau in den Tropen und Subtropen, Humboldt Universität zu Berlin, D–14195 Berlin, Germany (K.-P.G.)

Storage protein synthesis is dependent on available nitrogen in the seed, which may be controlled by amino acid import via specific transporters. To analyze their rate-limiting role for seed protein synthesis, a *Vicia faba* amino acid permease, VfAAP1, has been ectopically expressed in pea (*Pisum sativum*) and *Vicia narbonensis* seeds under the control of the legumin B4 promoter. In mature seeds, starch is unchanged but total nitrogen is 10% to 25% higher, which affects mainly globulin, vicilin, and legumin, rather than albumin synthesis. Transgenic seeds in vitro take up more [^{14}C]-glutamine, indicating increased sink strength for amino acids. In addition, more [^{14}C] is partitioned into proteins. Levels of total free amino acids in growing seeds are unchanged but with a shift toward higher relative abundance of asparagine, aspartate, glutamine, and glutamate. Hexoses are decreased, whereas metabolites of glycolysis and the tricarboxylic acid cycle are unchanged or slightly lower. Phospho*enol*pyruvate carboxylase activity and the phospho*enol*pyruvate carboxylase-to-pyruvate kinase ratios are higher in seeds of one and three lines, indicating increased anaplerotic fluxes. Increases of individual seed size by 20% to 30% and of vegetative biomass indicate growth responses probably due to improved nitrogen status. However, seed yield per plant was not altered. Root application of [^{15}N] ammonia results in significantly higher label in transgenic seeds, as well as in stems and pods, and indicates stimulation of nitrogen root uptake. In summary, VfAAP1 expression increases seed sink strength for nitrogen, improves plant nitrogen status, and leads to higher seed protein. We conclude that seed protein synthesis is nitrogen limited and that seed uptake activity for nitrogen is rate limiting for storage protein synthesis.

Legume seeds are a major source of plant-derived proteins and economically important for worldwide feed and food. Vicia and pea (*Pisum sativum*) seeds contain globulin storage proteins, hexameric legumins, and trimeric vicilins/convicilins, which together account for the majority of seed protein. The remainder consists of albumins, including lectins, lipoxygenases, proteinase inhibitors, late embryogenesis abundant proteins, and many other soluble proteins (Casey et al., 1993). Storage protein accumulation in legumes occurs in the embryo during maturation. Gln and/or Asn are translocated through the phloem (Miflin and Lea, 1977) and are symplastically unloaded into the seed coat where they are metabolized and reconstructed (Rochat and Boutin, 1991; Lanfermeijer et al., 1992). Mainly Gln, Ala, and Thr are released from the pea seed coat (Lanfermeijer et al., 1992) and, at maturation, Asn is also unloaded (Rochat and Boutin, 1991). Efflux of amino acids (and Suc) from pea seed coats is passive with linear kinetics, probably mediated by nonselective pores (DeJong et al., 1996, 1997). Amino acid uptake into soybean (*Glycine max*) and pea embryos is partially passive, especially during the early stages (Bennett and Spanswick, 1983; DeJong et al., 1997). A saturable system, attributed to H^+-amino acid cotransport, becomes important later on. The saturable system is induced by nitrogen starvation, indicating control by assimilate availability (Bennett and Spanswick, 1983; Lanfermeijer et al., 1990).

Storage parenchyma cells of the cotyledons import Asn and Gln (Miflin and Lea, 1977). The synthesis of other amino acids requires carbon skeletons, which are formed in the cytosol via the anaplerotic pathway of phospho*enol*pyruvate (PEP) carboxylase (Turpin and Weger, 1990). PEP carboxylase has been investigated in seeds of pea, soybean, wheat (*Triticum aestivum*), and Vicia (Hedley et al., 1975; Flinn, 1985; Smith et al., 1989; Gonzalez et al., 1998; Golombek et al., 1999). The

[1] This work was supported by the Deutsche Forschungsgemeinschaft (Schwerpunktprogramm 1108, Transport Processes in Plants) and by the Bundesministerium für Bildung und Forschung (grant no. 03I0637).

[2] Present address: Biology Department, University of the West Indies, Trinidad.

[3] Present address: Biology Department, University of Victoria, Victoria, British Columbia, Canada V8W 5N5.

[4] Present address: Sungene, BASF Plant Science, D–06466 Gatersleben, Germany.

[5] Present address: Max-Planck-Institut für Molekulare Pflanzenphysiologyie, D–14476 Golm, Germany.

* Corresponding author; e-mail weber@ipk-gatersleben.de; fax 49–39482–5500.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.056523.

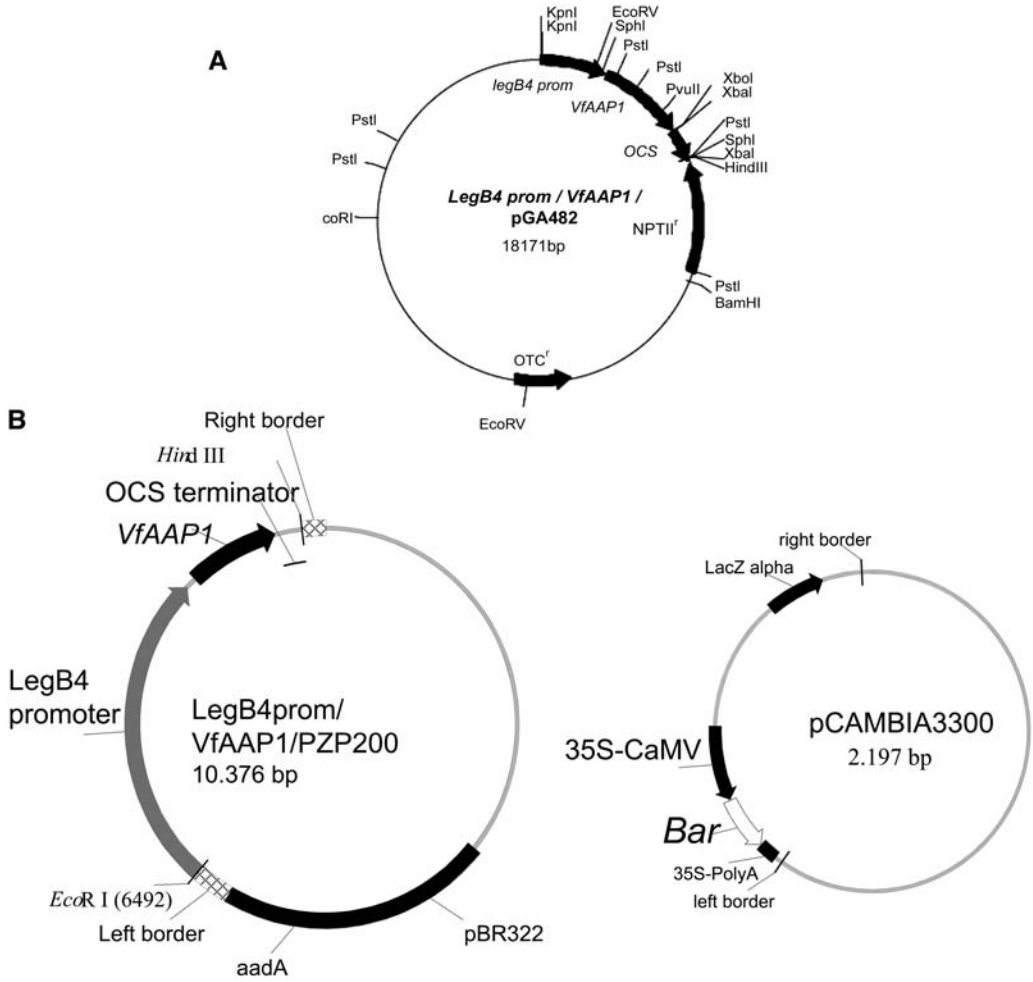

Figure 1. A, Vector construct (LeB4 promoter/VfAAP1/pGA482) for transformation of *V. narbonensis*. B, Vector constructs for pea transformation. Cotransformation has been applied with the selection marker (pCAMBIA3300) and the VfAAP1 (LeB4 promoter/VfAAP1/pZP200) on different plasmids.

enzyme refixes HCO_3^- liberated by respiration and, together with PEP, yields oxalacetate that can either be converted into Asp or into malate and other intermediates of the citric acid cycle. PEP carboxylase can control the anaplerotic carbon flow and potentially improves seed carbon economy (Rolletschek et al., 2004).

Storage protein synthesis is regulated at different levels. The most important are availability and partitioning of assimilates and nitrogen compounds and the genetic properties of cultivars. Increased storage protein content in maize (*Zea mays*) kernels is associated with a higher capacity to deliver Asn by nitrate reductase and Asn synthase (Lohaus et al., 1998). Within the seeds, nutrients like sugars and nitrogen confer regulatory control on storage activities (Weber et al., 1997, 1998a, 2005; Borisjuk et al., 2004). Storage protein accumulation in pea depends strongly on nitrogen availability in the seed (Lhuillier-Soundélé et al., 1999; Golombek et al., 2001; Miranda et al., 2001; Salon et al., 2001). Also, in maize and barley (*Hordeum vulgare*), endosperm-specific synthesis of storage proteins is under nutritional control and dependent on nitrogen availability (Balconi et al., 1991; Müller and Knudsen, 1993). Therefore, nitrogen uptake and partitioning activities could be important in regulating storage protein synthesis, and maturing legume embryos develop high-sink strength for nitrogenous assimilates. Because its uptake from the soil, as well as nitrogen fixation, decreases during seed filling, the high demand can potentially induce nitrogen remobilization and premature senescence (Salon et al., 2001). At maturation, 60% to 85% of plant nitrogen has been relocated in the seeds (Peoples and Gifford, 1990). The ability of the embryo to attract and import amino acids can be due to high storage protein synthesis, i.e. high demand, or to active uptake via membrane-localized transporters. An 11S and 7S globulin null mutant of soybean strongly accumulates amino acids in the seeds (Takahashi et al., 2003), suggesting efficient

uptake of amino acids independent of globulin biosynthesis.

Plant amino acid transporters of the amino acid permease (AAP) subfamily are integral membrane proteins and catalyze H^+-coupled amino acid uptake. AAPs are encoded by multigene families with eight members known from Arabidopsis (*Arabidopsis thaliana*), all with low selectivity with respect to amino acid side chains when expressed in yeast (*Saccharomyces cerevisiae*). Differential expression in various cell types indicates that AAPs are required for specific functions with putative roles in phloem uptake and interorgan transport (Okumoto et al., 2002). AtAAP1 is expressed seed-specifically and may control storage protein synthesis (Hirner et al., 1998). Two AAP isoforms have been described in pea. PsAAP1 is strongly expressed in vegetative organs and embryonic transfer cells, whereas PsAAP2 transcripts could not be detected (Tegeder et al., 2000). Seven AAP isoforms are known from *Vicia faba*. VfAAP1, orthologous to PsAAP2, is expressed in embryonic storage parenchyma cells at early maturation but not in transfer cells. Transcripts of VfAAP4, the ortholog to PsAAP1, could not be detected. VfAAP3 is expressed in different sink organs, but only to low levels in seeds (Miranda et al., 2001). Four other isoforms (VfAAP2, VfAAPa, VfAAPb, and VfAAPc) differ substantially by expression pattern (Montamat et al., 1999).

Much work has been concentrated on the molecular characterization of AAPs (Montamat et al., 1999; Tegeder et al., 2000; Miranda et al., 2001; Okumoto et al., 2002). However, there is a lack of knowledge as to how far amino acid transport activity in the seed can be rate limiting for storage protein accumulation and what the physiological and biochemical consequences of increased seed sink strength are for nitrogen. To answer these questions, the well-characterized VfAAP1 (Miranda et al., 2001) has been ectopically expressed in maturing embryos of *Vicia narbonensis* and pea followed by a characterization of transgenic seeds.

RESULTS

V. narbonensis Seeds Expressing VfAAP1

A full-length cDNA of the *V. faba* AAP (VfAAP1; Miranda et al., 2001) was fused to the legumin B4 promoter (LeB4), which confers seed-specific expression (Bäumlein et al., 1992). The cDNA construct was cloned into pGA 482 (An et al., 1987; Fig. 1A) and introduced into *V. narbonensis* using an Agrobacterium-mediated protocol (Pickardt et al., 1991). A total of 15 F_0 lines were regenerated. To obtain stable lines, the plants were allowed to self-pollinate and PCR-positive plants were further propagated. Seeds from 3 independent transgenic lines (AAP-10, AAP-12, and AAP-21) of the F_4 to F_6 generation were chosen for further analysis. The expression of VfAAP1 was checked by northern analysis using embryos of lines AAP-10, AAP-12, and AAP-21 at 24, 32, and 39 d after pollination (DAP; Fig. 2A). VfAAP1 mRNA levels increased strongly from 24 to 39 DAP reflecting the activity profile of the LeB4 promoter. The line AAP-12 showed the strongest expression. In the wild-type control, only a faint band was visible with no differences during development. This signal probably corresponds to the *V. narbonensis* endogenous amino acid transporter.

To analyze whether the transgenic seeds have an altered composition, we analyzed dry mature embryos for the concentrations of total carbon and nitrogen, globulins, albumins, and starch. Compared to wild-type seeds, the VfAAP1-expressing lines contained 10% to 25% more total nitrogen on a per gram basis (Fig. 2B). Total carbon was not different (Fig. 2C). Storage protein composition was also analyzed after

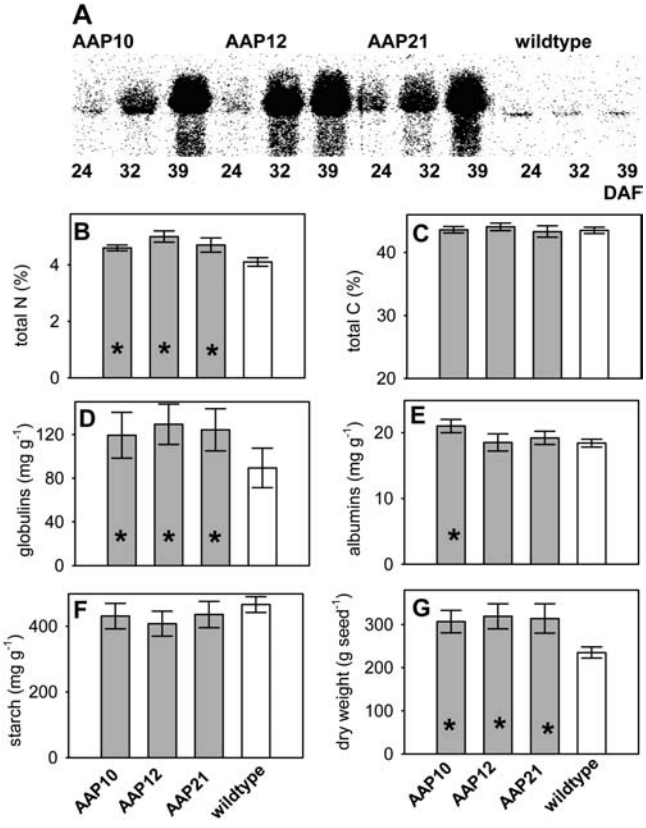

Figure 2. Characterization of transgenic *V. narbonensis* embryos expressing VfAAP1. A, Northern gel-blot analysis of seeds from lines AAP-10, AAP-12, AAP-21, and the untransformed wild type at 24, 32, and 39 DAP. B, Percentage of total nitrogen in mature dry embryos. C, Percentage of total carbon in mature dry embryos. D, Extractable globulins in dry embryos. E, Extractable albumins in mature dry embryos. F, Starch in mature dry embryos. G, Dry weight per mature seed; $n = 50$. The data are presented as means ± SD of four to six individual seeds per line. Asterisk, Significant differences according to Student's t test, $P < 0.05$.

Table I. *Levels of free sugars in mature dry embryos of V. narbonensis expressing VfAAP1 and the wild type*

25 DAP; means ± SD; $n = 4$ to 6.

Sugars[a]	AAP-10	AAP-12	AAP-21	Wild Type
Suc	116 ± 10	146 ± 20	154 ± 28	155 ± 42
Glc	0.048 ± 0.025	0.082 ± 0.08	0.036 ± 0.017	0.071 ± 0.07
Fru	0.083 ± 0.07	0.07 ± 0.04	0.017 ± 0.016	0.121 ± 0.07
Rha	15 ± 3	20 ± 5	21 ± 5	26 ± 6
Gal	7 ± 1	8 ± 2	9 ± 1	9 ± 2
Raffinose	23 ± 2	31 ± 2	31 ± 2	27 ± 6
Stachyose	63 ± 4	83 ± 7	83 ± 4	69 ± 13
Verbascose	36 ± 3	40 ± 4	43 ± 10	35 ± 9

[a]Millimoles per gram fresh weight.

extraction of dry embryo powder with aqueous buffers. Total globulins, containing the major 7S and 11S storage proteins, were significantly increased by approximately 30% in the transgenic lines as compared to the wild type (Fig. 2D). The albumins, which include the sum of water-soluble storage and nonstorage proteins, increased by about 15% in seeds of AAP-10 but were not different in seeds of AAP-12 and AAP-21 (Fig. 2E). Starch content was not significantly altered, although mean values were slightly lower (Fig. 2F). Soluble sugars in dry seeds also were not altered (Table I). Remarkably, the seeds from all lines had increased individual dry weights by 20% to 30% (Fig. 2G). Yield-related parameters were determined using a set of 10 mature plants of line AAP-12. As mentioned, individual seed weight was increased by approximately 20% (Fig. 3A). Values of seeds per plant and seeds per pod showed a trend toward lower levels, although not significantly ($P > 0.05$; Fig. 3, B and C). Also, seed yield per plant was not significantly changed (Fig. 3D; there is some trend toward higher levels). Interestingly the AAP-12 plants had a higher total and vegetative biomass (Fig. 3, E and F). Given the fact that the seed biomass was not altered, the harvest index was lower for AAP-12, 0.35 compared to 0.42 for the control (harvest index = ratio of grain yield to above-ground biomass).

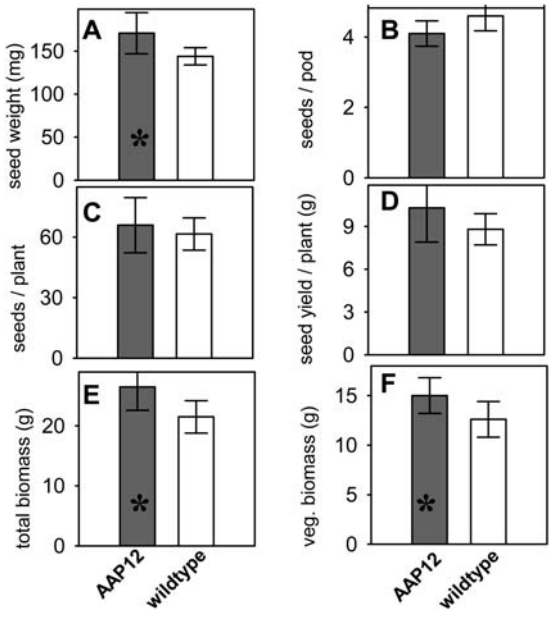

Figure 3. Yield-related parameters. A set of 10 mature plants each of AAP-12 and wild type has been analyzed. A, Individual mature seed weight. B, Seeds per pod. C, Seed number per plant. D, Seed yield per plant. E, Total biomass above ground. F, Vegetative biomass. The data are presented as means ± SD of 10 plants each of AAP-12 and wild type. Asterisk, Significant differences according to Student's *t* test, $P < 0.05$.

In summary, the ectopic expression of VfAAP1 in *V. narbonensis* characteristically changed seed composition and concentration, leading to increased seed nitrogen and seed protein content on a per gram basis as well as of individual seed dry weight. Taken together, these 2 effects indicate that, on the individual seed basis, the crude protein content is increased by as much as 40% to 50%.

Globulin Synthesis Is Stimulated in AAP1-Expressing *V. narbonensis* Seeds

To analyze the effect of VfAAP1 expression on globulin synthesis, we performed northern analysis on growing seeds (30 DAP) of lines AAP-10 and AAP-12. In wild-type seeds (Fig. 4A, top, lanes 1 and 2), only a faint band is visible, whereas in AAP-10 and AAP-12 seeds (Fig. 4A, top, lanes 3–5 and lanes 6–8, respectively), VfAAP1 is highly expressed. The same blot was rehybridized with a legumin B probe (Wobus et al., 1986), which revealed increased transcript levels in the transgenic seeds (Fig. 4A, middle). Legumins and vicilins of mature seeds of all three AAP lines were analyzed by a radial immunodiffusion technique. Compared to wild-type seeds, the legumin and vicilin concentration is significantly higher for all 3 lines ($P < 0.03$; Fig. 4B), whereas the vicilin-to-legumin ratio is not significantly altered. Taken together, the results indicate that VfAAP1 expression

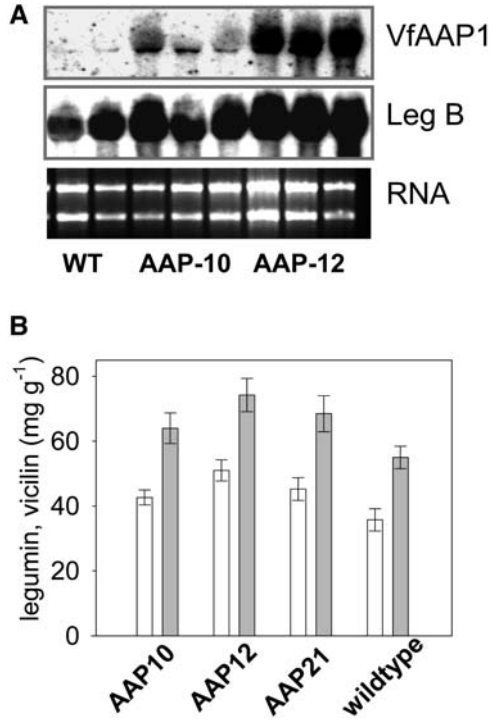

Figure 4. Effect of VfAAP1 expression on vicilin/legumin synthesis. A, Northern analysis; VfAAP1 expression in growing seeds (30 DAP) in lines AAP-10, AAP-12, and wild type (top), legumin B expression (middle), RNA ethidium bromide stain (bottom). B, Concentration of vicilin (gray columns) and legumin (white columns) determined by radial immunodiffusion assay. The data are presented as means ± SD of five experiments. Significant differences according to Student's *t* test, $P < 0.05$.

stimulates the synthesis of globulins, especially vicilins and legumins.

Pea Seeds Expressing VfAAP1

The LeB4-VfAAP1 fusion gene was cloned into the binary vector PZP 200 and introduced into pea. A cotransformation method was applied, with the selection marker gene (*bar* gene) on a second vector (pCAMBIA 3300; Fig. 1B). Integration of the transgenes was checked by PCR in the F_0 generation of a total of 21 independent transformants. The efficiency of VfAAP1 cotransformation was 38%. The F_0 plants were allowed to self-pollinate and 10 to 20 of the F_1 seedlings of each cotransformed line were again tested by PCR. Using segregation analysis for both genes (VfAAP1 and *bar*), the noncoupled single insert lines were determined. From all progeny of a single insert line, with the out-segregated *bar* gene, the homozygous state of VfAAP1 was tested in the next generation. Thus, application of cotransformation of both the *bar* gene and VfAAP1 allowed the selection of transgenic plants without the resistance (*bar*) gene. The homozygous line 14/10 having a single insert was chosen for further analysis. As a control, we used line 14/3 derived from the same heterozygous parents but with out-segregated VfAAP1.

For both lines 14/10 (containing the VfAAP1 transgene) and 14/3 (with out-segregated transgenes), VfAAP1 expression was analyzed by northern analysis using embryos at approximately 28 DAP. VfAAP1 mRNA levels were detectable at high levels in embryos of line 14/10 (Fig. 5A, lanes 1–3) and not in line 14/3 (Fig. 5A, lanes 4 and 5). To analyze whether transgenic seeds have an altered composition, we analyzed dry mature embryos for the concentration of total carbon and nitrogen, globulins, albumins, and starch. Seeds of line 14/10 have 20% more total nitrogen (Fig. 5B). Total carbon was increased significantly by approximately 4% (Fig. 5C). Total globulins were significantly increased by 43% in the 14/10 seeds as compared to 14/3 (Fig. 5D). Albumins and starch were not different (Fig. 5, E and F). Seed weight of 14/10 was slightly, but significantly, higher by approximately 5% (Fig. 5G). Seeds of a second transgenic pea line (line 18) containing two copies of VfAAP1 also have significantly increased nitrogen and globulin content (data not shown).

The results show that expression of VfAAP1 in pea embryos also increased both seed nitrogen concentra-

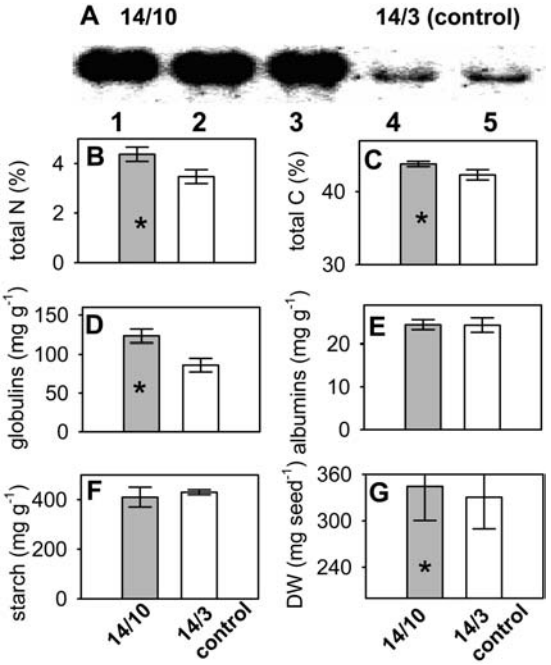

Figure 5. Characterization of transgenic pea embryos expressing VfAAP1. A, Northern gel-blot analysis of seeds from transgenic line 14/10 (lanes 1–3) and the control 14/3 (lanes 4 and 5) at 28 DAP. B, Percentage of total nitrogen in dry embryos. C, Percentage of total carbon in dry embryos. D, Extractable globulins in dry embryos. E, Extractable albumins in dry embryos. F, Starch in dry embryos. G, Dry weight per seed; $n = 50$. The data are presented as means ± SD of four to six individual seeds per line. Asterisk, Significant differences according to Student's *t* test, $P < 0.05$.

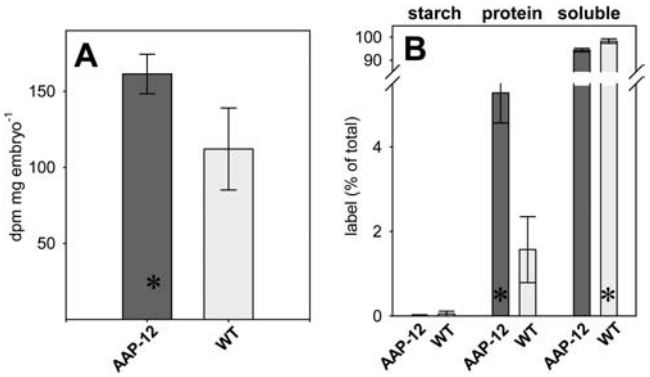

Figure 6. Uptake and partitioning of [^{14}C]Gln by isolated embryos of *V. narbonensis* expressing VfAAP1. Isolated embryos of the *V. narbonensis* lines AAP-12 and wild type at 28 DAP were pulse labeled for 6 h with [^{14}C]Gln and processed subsequently. A, Content of [^{14}C] label (dpm) per embryo. B, Partitioning of [^{14}C] label into the starch, protein, and soluble fraction given as a percentage of uptake. The data are presented as means ± SD of four to six individual seeds per line. Asterisk, Significant differences according to Student's *t* test, $P < 0.05$.

tion, especially globulins, as well as individual seed dry weight similar to the VfAAP1-expressing *V. narbonensis* plants (Fig. 2).

AAP1-Expressing Seeds Take Up More Amino Acids

To analyze the uptake and incorporation of amino acids, seeds of the *V. narbonensis* line AAP-12 (28 DAP) and the wild type were pulse labeled for 6 h with [^{14}C]Gln. Transgenic embryos took up approximately 50% more [^{14}C] label on a per gram basis compared to the wild type (Fig. 6A), indicating that the engineered AAP-12 seeds have a higher rate of amino acid import. To further assess the fate of the [^{14}C] label, we analyzed the partitioning into fractions of starch, protein, and soluble compounds. [^{14}C] label from Gln was incorporated into starch only in very low amounts, with no significant difference between AAP-12 and wild-type embryos. Remarkably, AAP-12 embryos partitioned 4-fold more label into the protein fraction. In wild-type seeds, significantly higher levels of the label appeared in the soluble fraction (Fig. 6B).

Our data suggest that the VfAAP1 is physiologically active in maturing AAP-12 embryos and most probably increases amino acid uptake rates. In addition, a higher rate of label from Gln is incorporated into the protein fraction.

Mature *V. narbonensis* seeds expressing VfAAP1 are generally increased in seed weight (Figs. 2G and 5G), but otherwise have no seed phenotype (Fig. 7). This indicates higher sink strength of the VfAAP1-expressing seeds. We analyzed the accumulation of assimilates in growing embryos and total nitrogen and carbon accumulation throughout development. During the final stages, both nitrogen and carbon content of VfAAP1-expressing cotyledons was significantly increased on a per embryo level. Differences appeared after approximately 23 DAP, with increasing rates up to 40 DAP relative to wild-type seeds (data not shown), indicating that VfAAP1 expression leads to increased uptake rates of assimilate into seeds.

Amino Acid Pools in Growing Seeds

Single amino acids were measured in embryos of AAP-10, AAP-12, AAP-21, and wild-type seeds at 23 to 25 DAP. There were no significant changes in the overall pool of free amino acids in seeds of either of the lines (Table II). Smaller, but not significant, increases were observed for Ala and Glu. Comparing the relative levels of the major free amino acids in seeds of the line with the strongest phenotype, AAP-12, and the wild type, from 21 to 25 DAP, again did not show changes in overall levels. However, the relative amounts are different from 25 up to 31 DAP. There was a relative increase of Gln and Asn, whereas Arg was relatively decreased compared to the wild type (Fig. 8).

Enzymes and Profiles of Intermediary Metabolites

Because seeds import mainly amides, which have a high nitrogen-to-carbon ratio, the biosynthesis of other amino acids requires the provision of carbon skeletons from glycolytic and tricarboxylic acid cycle products. To analyze changes within the carbon metabolite pattern within VfAAP1-expressing *V. narbonensis* seeds, steady-state levels were measured for Suc, Glc, and Fru for key intermediates of glycolysis and the tricarboxylic acid cycle as well as for ATP and ADP at 23 to 25 DAP (Table III). In general, there were only minor changes within the levels of intermediary metabolites between transgenic and wild-type seed, less than 2-fold. The largest differences occurred in the

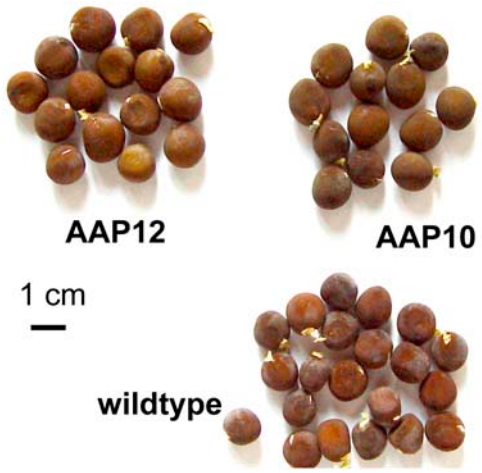

Figure 7. Phenotype of mature dry seeds overexpressing VfAAP1 (AAP-12, AAP-10) and the wild type.

Table II. *Steady-state levels of free amino acids in growing embryos of V. narbonensis expressing VfAAP1 and the wild type*

25 DAP; means ± SD; $n = 4$ to 6.

Amino Acids[a]	AAP-10	AAP-12	AAP-21	Wild Type
Asp	3.53 ± 0.42	3.1 ± 1.23	3.29 ± 0.37	3.32 ± 0.33
Glu	7.33 ± 1.38	7.9 ± 1.4	8.4 ± 1.39	6.84 ± 1.22
Ser	9 ± 2.11	10.14 ± 0.86	11.5 ± 1.95	10.9 ± 2.1
Asn	5.66 ± 2.18	8.06 ± 2.56	5.8 ± 0.93	5.66 ± 1.72
Gly	0.89 ± 0.27	0.67 ± 0.32	0.7 ± 0.28	0.64 ± 0.13
Gln	1.02 ± 0.61	2.4 ± 1.09	1.53 ± 0.8	1.88 ± 1.2
His	0.15 ± 0.03	0.21 ± 0.05	0.2 ± 0.06	0.18 ± 0.05
Thr	2.37 ± 0.27	1.95 ± 0.62	2.14 ± 0.26	2 ± 0.4
Ala	5.1 ± 1.03	12 ± 2.5	9.41 ± 5.5	7.85 ± 4.3
Arg	25.05 ± 9.6	29.06 ± 3.8	28.1 ± 2.07	29.7 ± 7.2
Tyr	0.43 ± 0.26	0.35 ± 0.1	0.58 ± 0.22	0.69 ± 0.21
Val	1.67 ± 0.18	1.54 ± 0.17	1.74 ± 0.27	1.64 ± 0.31
Met	0.03 ± 0.001	0.039 ± 0.009	0.043 ± 0.011	0.033 ± 0.012
Ile	0.48 ± 0.1	0.59 ± 0.15	0.59 ± 0.12	0.54 ± 0.22
Leu	0.54 ± 0.1	0.61 ± 0.15	0.64 ± 0.14	0.53 ± 0.19
Lys	0.11 ± 0.068	0.042 ± 0.022	0.193 ± 0.05	0.07 ± 0.03
Phe	0.17 ± 0.05	0.25 ± 0.03	0.23 ± 0.1	0.21 ± 0.12
Total amino acids	63.53 ± 6.3	78.1 ± 7.6	75.1 ± 3.9	72.68 ± 10.1

[a]Micromoles per gram fresh weight.

AAP-12 seeds, the line with the strongest phenotype. The general trends were as follows. Levels of free hexoses were lower, whereas the pool of hexose phosphates and nucleotide sugars was not strongly influenced. Pool sizes of the C2/C3 metabolites of the lower glycolysis, PEP to acetyl-CoA, were unchanged, but pyruvate was decreased in seeds of AAP-12 and AAP-21. Intermediates of the tricarboxylic acid cycle were either unchanged (malate and succinate) or decreased (citrate and iso-citrate in AAP-12 and AAP-21 seeds). Shikimate and chorismate were not affected. There was a trend toward higher ATP-to-ADP ratios.

In addition, we measured enzymes related to carbon metabolism (Suc synthase, ADP Glc pyrophosphorylase, PEP carboxylase, PEP phosphatase, Glc-6-P dehydrogenase, pyruvate kinase, and citrate synthase, as well as Asp aminotransferase) in growing embryos of all 3 transgenic lines and the wild type (Table IV). Again, only minor differences could be detected. ADP-Glc pyrophosphorylase activity was significantly lower in all 3 lines, Suc synthase and Glc-6-P dehydrogenase were lower in AAP-21 seeds, and pyruvate kinase and citrate synthase were lower in AAP-10 and AAP-12 seeds. PEP carboxylase was higher in AAP-21 seeds.

Dry Matter and Nitrogen Accumulation on the Whole-Plant Level

Dry weight as well as total nitrogen distribution has been measured at 33 DAP (first flower) in leaves, stems, pods, and seeds of AAP-12 plants. Leaves and stems of AAP-12 plants have higher dry matter content, whereas for pods and seeds there was no difference (Fig. 9A). At 33 DAP, leaves and stems of AAP-12 absolutely contained more nitrogen than wild-type plants (Fig. 9B). Thus, at 33 DAP, the AAP-12 plants have a higher vegetative biomass and a higher absolute nitrogen content. This preferentially concerns leaves and stems. However, the nitrogen concentration is not different.

Uptake and Allocation of [^{15}N] Applied to Roots

Six millimolars of [^{15}N]-NH_4 (in 200 mL water) were applied to the roots of AAP-12 and wild-type plants at 29 DAP. Four days later, the plants were harvested and the [^{15}N] label was measured in the different organs. Label uptake was 100% higher for the AAP-12 plants. The stems, pods, and, most pronounced, seeds of AAP-12 contained absolutely more label compared to the wild type (Fig. 9C). In AAP-12 plants, 71% of the label taken up was present in the seeds compared to 64% in the wild type. The concentration of label was higher in seeds and pods of AAP-12, but was not different in leaves and stems (Fig. 9D). The results indicate that AAP-12 plants take up more nitrogen, which at 33 DAP is partitioned into seeds and vegetative organs.

DISCUSSION

VfAAP1 Expression Increased Amino Acid Uptake into Seeds

Seed storage protein synthesis is dependent on the available nitrogen in the seed. Thus, protein accumu-

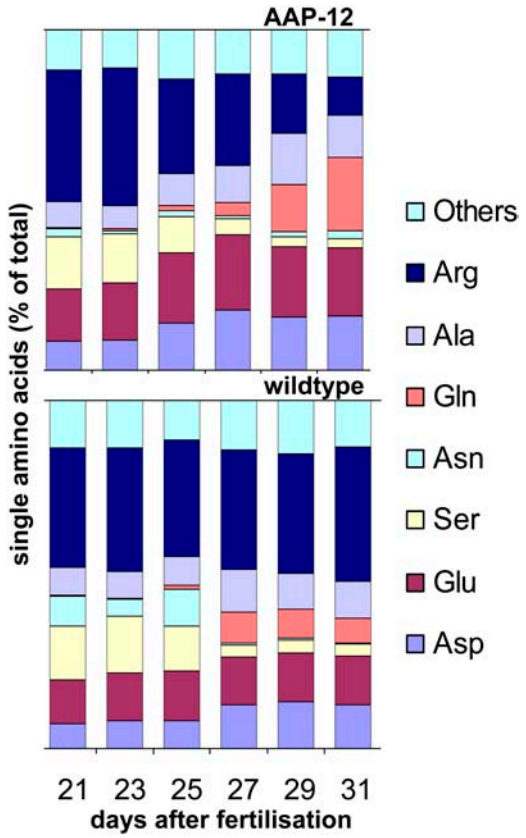

Figure 8. Proportion of the major free amino acids present in growing embryos of line AAP-12 and wild-type embryos. Samples were taken from 21 to 31 DAP.

lation could be controlled by the capacity of the seed itself to import amino acids via specific transporters. Previous results showed that in *V. faba* seeds, sink strength for nitrogen is acquired during maturation and is associated with amino acid transport (Golombek et al., 2001). VfAAP1 functions as an uptake system for amino acids at the level of the storage parenchyma (Miranda et al., 2001). Thus, uptake activity via AAPs into the parenchyma cells could control storage protein accumulation and thus provide a rate-limiting function. To test this assumption, ectopic expression of VfAAP1 in pea and *V. narbonensis* has been performed. The used LeB4 promoter targeted transgene expression to the storage parenchyma cells of the embryo from the early-to-late maturation stage when endogenous storage proteins are synthesized. VfAAP1-expressing Vicia seeds in vitro take up more [^{14}C]Gln (Fig. 6A), indicating increased seed sink strength for amino acids. The label derived from Gln is mainly incorporated into proteins rather than starch. At 28 DAP, the AAP-12 embryos incorporated approximately 4-fold more label into protein, which indicates that storage protein synthesis is stimulated. In both mature transgenic pea and *V. narbonensis* seeds, total nitrogen is increased by 10% to 25% at the per gram level. The levels of free amino acids in dry seeds are low, <3% of total nitrogen content (data not shown). Therefore, total nitrogen directly reflects protein content (Rolletschek et al., 2002). We conclude that VfAAP1 expression increased amino acid uptake into growing seeds, resulting in higher storage protein content.

Improving Seed Nitrogen Uptake Stimulates Globulin Synthesis

In both transgenic pea and Vicia seeds, the protein increase affects globulins rather than albumins (Figs. 2 and 5). The stimulation occurs probably on a transcriptional level and comprises the 2 major classes of globulins, 7S vicilins and 11S legumins (Fig. 4). This is in contrast to AGP-antisense seeds, where increased nitrogen is mainly due to higher albumin levels (Rolletschek et al., 2002). The AGP-antisense seeds have significantly lower starch and, because the relative cell volume occupied by starch is decreased, the cytoplasmic compartment in which protein synthesis occurs is larger. In cold-acclimatized Arabidopsis leaves, a shift toward increased cytoplasmic volumes also leads to increased protein content (Strand et al., 1999). In VfAAP1-expressing seeds, starch is not affected. Stimulation of globulin biosynthesis may be caused by increased expression of storage protein genes due to nutritional effects. Accordingly, feeding Gln to *V. faba* seeds in vitro increased both vicilin (Miranda et al., 2001) and legumin B mRNA (Weber et al., 1998b), and a promoter controlling a barley hordein gene was shown to be activated by nitrogen assimilates (Müller and Knudsen, 1993). Although steady-state levels of free amino acids are unchanged in VfAAP-expressing seeds, this does not exclude an increase within specific compartments. Alternatively, stimulation of globulin synthesis could be due to increased levels of only one or a few amino acids. The AAP1 seeds are characterized by a shift toward a higher relative abundance of Asn and Gln (Fig. 8). Another possibility could be that higher influx rates due to VfAAP1 expression triggers globulin synthesis.

Our analysis revealed that the amino acid pool does not increase dramatically in the AAP seeds. Based on this, we can deduce that VfAAP1 overexpression does not cause accumulation of amino acids; rather, imported nitrogen is readily incorporated into proteins. However, when storage protein synthesis is affected, seeds indeed accumulate amino acids as has been shown for a soybean mutant defective in 7S and 11S globulin synthesis (Takahashi et al., 2003). Seeds therefore possess an uptake mechanism to actively import amino acids, which are subsequently incorporated into storage proteins. In the AAP1 seeds, the higher uptake of nitrogen is readily translated into higher globulin concentration. This confirms earlier findings that seed storage protein synthesis is a function of available nitrogen (Barratt, 1982; Balconi et al., 1991;

Table III. *Steady-state levels of free sugars and metabolites in growing embryos of V. narbonensis expressing VfAAP1 and the wild type*

23 to 25 DAP; means ± SD; bold values indicate significant differences according to Student's *t* test of normalized (log-transformed) data; $P < 0.05$; $n = 5$ to 7.

Sugars and Metabolites	AAP-10	AAP-12	AAP-21	Wild Type
Suc[a]	106.7 ± 5.4	94.6 ± 7.9	109.3 ± 10.9	105.3 ± 7.05
Glc[a]	**3.66 ± 1.57**	**4.57 ± 1.13**	**2.92 ± 0.58**	7.58 ± 1.06
Fru[a]	**2.85 ± 1.03**	4.74 ± 0.83	**3.85 ± 1.45**	5.47 ± 1.31
ATP[b]	**240 ± 13**	183 ± 12	206 ± 25	194 ± 14
ADP[b]	**88 ± 8**	**57 ± 11**	65 ± 9	74 ± 8.4
ATP/ADP	2.73	3.21	3.17	2.62
Glc-6-P[c]	480 ± 61	325 ± 14	371 ± 52	393 ± 92
Glc-1-P[c]	121 ± 21	98 ± 9.4	99 ± 9	113 ± 14
Fru-6-P[c]	109 ± 11	57 ± 6.9	69 ± 11	75 ± 8.2
Fru-1, 6-bisP[c]	103 ± 35	107 ± 34	104 ± 34	71 ± 18
3-PGA[c]	117 ± 28	75 ± 10.3	77 ± 5.3	63.4 ± 7.6
UDP-Glc[c]	54 ± 12	43 ± 10	85 ± 38	64 ± 16
ADP-Glc[c]	**123 ± 13**	54 ± 36	68 ± 11	64 ± 18
PEP[c]	148 ± 59	74 ± 19	155 ± 57	129 ± 52
Pyruvate[c]	804 ± 321	**500 ± 117**	**558 ± 104**	834 ± 155
Acetyl-CoA[c]	123 ± 32	114 ± 9.7	131 ± 12	150 ± 20
Malate[c]	180 ± 26	198 ± 58	188 ± 49	211 ± 23
Citrate[c]	361 ± 56	**262 ± 19**	**280 ± 14**	356 ± 42
Iso-citrate[c]	219 ± 56	**174 ± 8.5**	**161 ± 3.3**	270 ± 60
Succinate[c]	358 ± 67	440 ± 57	407 ± 71	399 ± 39
Shikimate[c]	737 ± 128	658 ± 30	644 ± 160	678 ± 125
Chorismate[c]	244 ± 69	169 ± 31	175 ± 41	160 ± 21

[a]Micromoles per gram fresh weight. [b]Nanomoles per gram fresh weight. [c]Relative units.

Lhuillier-Soundélé et al., 1999). It further suggests that nitrogen import into the seed is a rate-limiting step.

Seed Expression of VfAAP1 Does Not Significantly Affect Metabolite Levels

Assuming that preferentially Asn/Gln are taken up into the seed, which have a high nitrogen-to-carbon ratio, there is a need for carbon skeletons in order to synthesize other amino acids. This must be met by increased provision of keto acids and by an increased carbon flux via PEP carboxylase into tricarboxylic acid cycle intermediates (Turpin and Weger, 1990). Therefore, in the AAP seeds, anaplerotic fluxes must be increased. Accordingly, PEP carboxylase activity was found to be higher (however, only significant for AAP-21 seeds; Table II) and, moreover, the PEP carboxylase-to-pyruvate kinase ratios are higher for all 3 AAP lines, indicating increased flux into the Glu/Asp family (Rontein et al., 2002). Glu and Asp are relatively increased in AAP-12 seeds (Fig. 6). Such changes can be expected when anaplerotic fluxes are increased (Rontein et al., 2002). Flux analysis with Brassica embryos shows that 25% to 30% of total carbon flux is directed into protein and that mitochondrial carbon metabolism is oriented primarily to amino acid synthesis (Schwender et al., 2004). The pool sizes of metabolites in the AAP seeds, at 23 to 25 DAP, are not much different from the wild type (Table III). The largest differences are observed for the strongest line, AAP-12. These have been summarized in a pathway scheme for better clarity (Fig. 10). Free hexoses are lower, whereas the pool sizes of the C2/C3 metabolites of glycolysis and that of the tricarboxylic acid cycle are either unchanged or show a trend toward slightly lower levels, however, without clear consistency between the 3 AAP lines (Table III; Fig. 10).

The AAP seeds resemble somewhat the PPC seeds, which express a Corynebacterium PEP carboxylase and have increased anaplerotic fluxes (Rolletschek et al., 2004). However, the PPC seeds are characterized by a clear shift in the ratio of phosphorylated-to-nonphosphorylated products and increased tricarboxylic acid cycle products due to the expression of the unregulated bacterial PEP carboxylase. Similar changes in metabolite profiles are not observed in the AAP seeds (Table III) and cannot be expected because increased levels of organic acids would down-regulate the endogenous PEP carboxylase by a feedback mechanism (Golombek et al., 1999). However, at least from the higher protein synthesis in the AAP seeds, we must expect an increased anaplerotic flux. From the fact that PPC seeds have higher protein

Table IV. *Maximum catalytic activity of enzymes measured in growing embryos of VfAAP1-expressing seeds and the wild type*

28 DAP; means ± SD; bold values indicate significant differences according to Student's *t* test of normalized (log-transformed) data; $P < 0.05$; $n = 5$ to 7.

Enzymes	AAP-10	APP-12	APP-21	Wild Type
ADP Glc pyrophosphorylase	**0.32 ± 0.06**	**0.44 ± 0.04**	**0.33 ± 0.03**	0.49 ± 0.01
Suc synthase[a]	2.77 ± 0.03	2.6 ± 0.19	**2.38 ± 0.24**	3.28 ± 0.75
PEP carboxylase	0.65 ± 0.02	0.67 ± 0.07	**0.74 ± 0.04**	0.64 ± 0.15
PEP phosphatase	0.4 ± 0.01	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.01
Asp-Aminotransferase	16.2 ± 0.4	17.3 ± 0.8	17.4 ± 0.5	17.1 ± 0.9
Glc-6-P dehydrogenase	0.29 ± 0.043	0.3 ± 0.057	**0.25 ± 0.024**	0.31 ± 0.018
Pyruvate kinase	**1.25 ± 0.14**	**1.43 ± 0.24**	1.78 ± 0.25	1.69 ± 0.15
Citrate synthase	**0.26 ± 0.024**	**0.29 ± 0.015**	0.29 ± 0.04	0.32 ± 0.023

[a]Micromoles per gram fresh weight min^{-1}.

content, we concluded that provision of carbon acceptors is generally rate limiting for protein synthesis (Rolletschek et al., 2004). This suggests that, in the AAP seeds, which have an increased demand, the availability of keto acids may also become limiting. At least some of the tricarboxylic acid cycle products are lower as citrate and iso-citrate in AAP-12 and AAP-21 seeds.

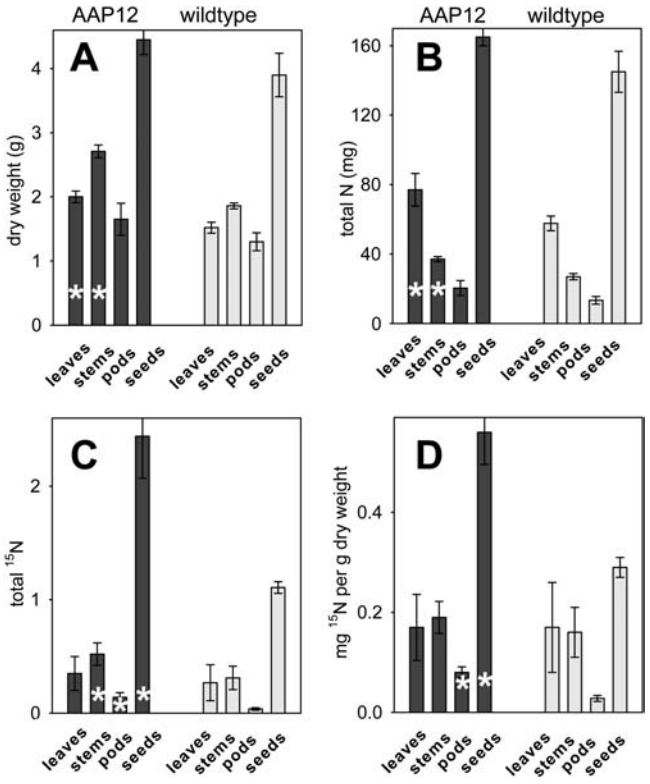

Figure 9. Dry matter distribution between organs and allocation of [^{15}N] tracer in plants of line AAP-12 and wild type. Labeling has been done at 29 DAP (first flower). Plants were held in soil. [^{15}N]-NH_4Cl (200 mL of 6 mM) was applied onto the pots to the root system at 29 DAP (first flower). Four days after application, all plant organs were harvested, dried at 60°C, and analyzed. A, Biomass (dry weight). B, Total nitrogen. C, Total content of [^{15}N] label in different organs. D, Concentration of [^{15}N] label in different organs. Asterisk, Significant differences according to Student's *t* test, $P < 0.05$, $n = 5$.

Seed Expression of VfAAP1 Leads to Growth Responses

Both the PPC seeds with higher anaplerotic fluxes as well as the AAP seeds with increased amino acid import are characterized by higher protein accumulation. This suggests a coregulation with respect to nitrogen and carbon import into the seed and a mutual dependence of nitrogen uptake and carbon fluxes. Interestingly, VfAAP1-expressing Vicia and pea seeds are 20% to 30% larger. Thus, on the per seed level, not only the protein, but also the total carbon content and starch, are increased, indicating that higher seed sink strength for nitrogen also causes higher influx of carbon. How this is achieved is unclear at the moment and will be analyzed in the future by [^{13}C]- and [^{15}N]-labeling studies.

It is known that the level of sink demand can feed back through translocation and assimilation rates (Peoples and Gifford, 1990). Whole-plant growth depends on both source supply and sink demand (Minchin et al., 2002). Enhancing sink strength can increase utilization of photosynthesis products and may enhance photosynthetic output and growth (Paul and Foyer, 2001). Accordingly, AAP plants have an increased vegetative and seed biomass, at least under greenhouse conditions (Figs. 3 and 9). In rice and wheat plants overexpressing a modified ADP-Glc pyrophosphorylase, increased seed growth is directly associated with an overall increase of plant growth (Smidansky et al., 2002a, 2002b), but with no change in the harvest index (Sinclair et al., 2004). For AAP plants, however, it is difficult to speculate on yield-related parameters unless field trials have been performed. At least under greenhouse conditions the harvest index is lower for the AAP-12 line (Fig. 3).

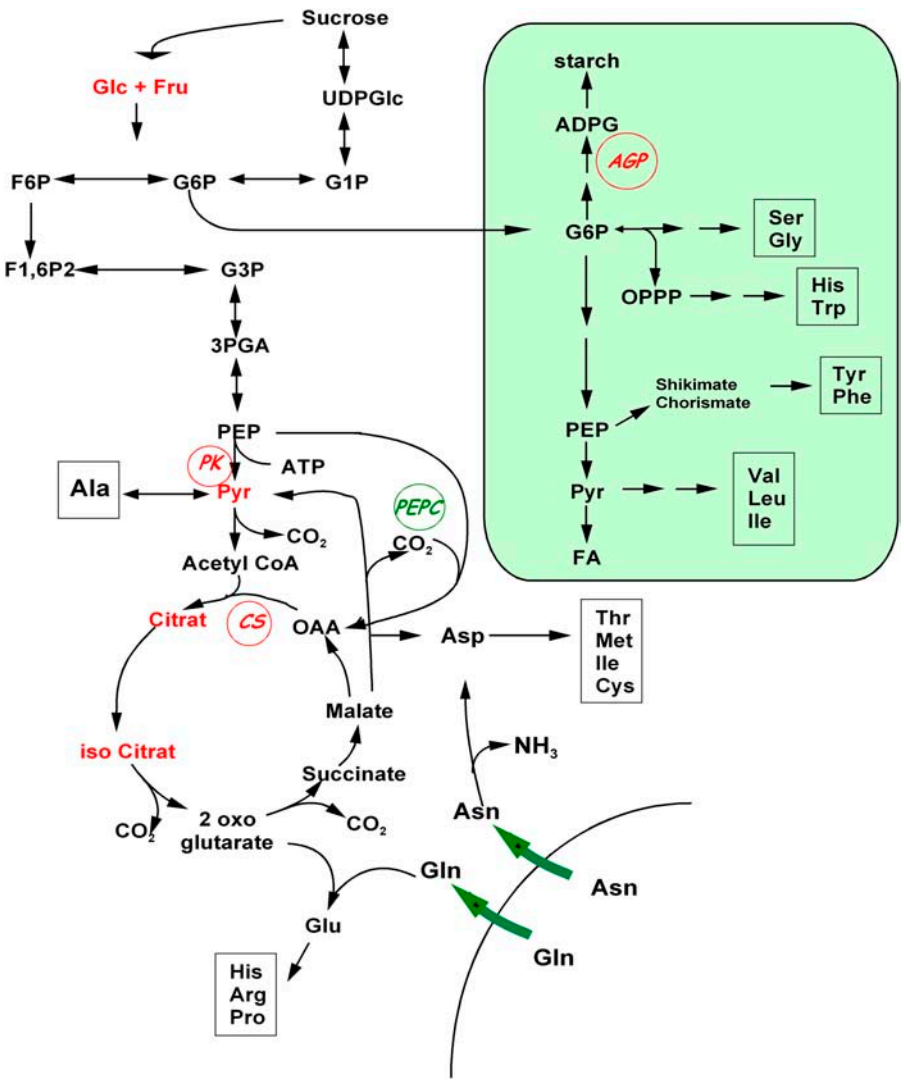

Figure 10. Schematic view of the proposed pathway of carbohydrates and amino acids in growing embryos. Suc imported into seeds is cleaved by Suc synthase or invertase. Hexose phosphates are either metabolized in the glycolytic pathway to pyruvate and/or imported as Glc-6-P into the amyloplasts toward starch synthesis. It is proposed that Asn and Gln are taken up from the apoplast. PEP carboxylase catalyzes the conversion of PEP and CO_2 to oxaloacetate. 2-Oxo-glutarate and oxalacetate are used as the main carbon acceptors for amino acid biosynthesis. Changes in carbohydrate metabolites, amino acids, and enzymes are indicated by color code for embryos of the line AAP-12. Values, which are decreased in transgenic embryos compared to the wild type, appear in red. Metabolites, which are increased, are shown in green, based on the data from Table III.

Seed protein accumulation is not only controlled by seed sink strength but also by nitrogen acquisition, assimilation, and partitioning within the plant. Maize cultivars with higher seed protein content assimilate more nitrogen in the leaves (Lohaus et al., 1998; Lohaus and Moellers, 2000). Leaf-specific antisense inhibition of StAAP1 in potato reduces amino acid levels in tubers (Koch et al., 2003), showing that phloem loading and long-distance transport determine nitrogen content in sink tissues.

Expressing VfAAP1 apparently brings about a higher capacity for amino acid uptake into seeds. However, how far this increased capacity can be realized, e.g. translated into higher seed protein, depends on other factors like nitrogen uptake from the soil, nitrogen fixation and allocation, as well as remobilization from vegetative tissues. Application of [^{15}N]-labeled ammonia to the roots of AAP-12 plants at 29 DAP shows significantly higher label accumulation in AAP-12 seeds compared to the wild type. Nitrogen stored in vegetative organs during early-to-mid-development can become available to the seed at late stages via remobilization. This indicates that the higher demand of nitrogen in the transgenic seeds could be covered by altered allocation and/or by stimulation of nitrogen uptake via the roots, probably via whole-plant signaling of nitrogen demand (Gansel et al., 2001). Studies with Arabidopsis (Lejay et al., 1999) have shown that the nitrogen status of the whole plant can control uptake of mineral nitrogen via long-distance signaling (Tillard et al., 1998).

In conclusion, VfAAP1 overexpression in pea and Vicia seeds increased amino acid uptake and led to higher protein content and individual seed size. Thus, seed protein synthesis is nitrogen limited and amino acid transport activity into storage parenchyma cells of the cotyledons is rate limiting.

MATERIALS AND METHODS

Plant Material

Vicia narbonensis and pea (*Pisum sativum*) plants were grown in 2-L pots in growth chambers under a light/dark regime of 16-h light (20°C) and 8-h dark (18°C). Plants were fertilized once a week with nitrate and ammonium to keep nonlimiting nitrogen conditions. For the isolation of embryos, pods were tagged according to days after pollination, collected in the middle of the light phase, and processed further. For metabolite measurements and enzyme assays, seeds were harvested and embryos were immediately isolated and snap frozen in liquid nitrogen.

Plant Transformation

The *Vicia faba* amino acid transporter VfAAP1 (Miranda et al., 2001) was cloned under the control of the LeB4 promoter (Bäumlein et al., 1992) into pGA 482 (An et al., 1987) as seen in Figure 1A. Transformation of *V. narbonensis* via Agrobacterium-mediated gene transfer was done according to Pickardt et al. (1991).

Transformation of pea cv Eiffel was performed after Schroeder et al. (1993) and modified as described in Giersberg et al. (2004). The LeB4 promoter-VfAAP1 fusion was cloned into PZP200 (Hajdukiewicz et al., 1994) as seen in Figure 1B. A cotransformation procedure was applied for pea with the selection marker (*bar* gene) on a second vector, pCAMBIA3300, as shown in Figure 1B.

RNA Isolation and Hybridization Techniques

Nucleic acids were isolated and northern hybridization was performed as described in Heim et al. (1993). The VfAAP1 cDNA fragment was used as a probe after labeling with ^{32}P-dCTP as described in Miranda et al. (2001).

Extraction and Determination of Starch, Protein, Total Carbon, and Nitrogen

After ethanol extraction, the starch-containing insoluble material was solubilized in 1 N KOH for 1 h at 95°C and neutralized with 5 N HCl. Starch was hydrolyzed with amyloglucosidase and determined enzymatically. To determine albumin and globulin fractions of extractable proteins, powdered samples were extracted in acetate buffer (50 mM acetate, 1 mM KCl, 10% [v/v] dimethyl sulfoxide, 0.5% [v/v] butanol; pH 4.5) and, subsequently, in phosphate buffer (100 mM KH_2PO_4, 100 mM Na_2HPO_4, 500 mM KCl; pH 7). Proteins were measured with bovine serum albumin as standard. Relative content of total carbon and nitrogen in dried, powdered samples of cotyledons was measured using an elemental analyzer (Vario EL; Elementaranalysensysteme, Hanau, Germany). Legumin/vicilin concentrations were determined by a radial immunodiffusion technique (Mancini et al., 1965). Antibodies as well as purified legumin and vicilin protein fractions used for standardization were kindly provided by R. Manteuffel (Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany).

Extraction and Determination of Metabolic Intermediates

Frozen plant material was weighed, homogenized by mortar and pestle, and extracted with trichloroacetic acid. Soluble sugars and free amino acids were measured as described in Rolletschek et al. (2002). Adenine nucleotides were determined after derivatization by HPLC, following the procedure of Haink and Deussen (2003) and modified as follows: 20 μL extract were incubated with 100 μL chloroacetaldehyde and 880 μL buffer (62 mM citrate, 76 mM KH_2PO_4, pH 5.5) at 80°C for 40 min. Derivatized samples were immediately cooled to 4°C until analysis. Chromatographic separation within 4 min and fluorescence detection was done as described by Haink and Deussen (2003). Glycolysis and citrate cycle intermediates were measured by ion chromatography-electrospray ionization-mass spectrometry, as described previously (Rolletschek et al., 2004). Chromatographic conditions were modified as follows. A binary gradient at a constant flow rate of 0.5 mL/min was applied using 100 mM sodium hydroxide (eluent B) and distilled water (eluent A). The gradient was produced by the linear concentration changes; these were initiated with 20% B, raised to 34% B during the first 7 min, 70% B in the next 7 min, and 100% B in the final 1 min. After holding at 100% B for 4 min, levels were returned to 20% B over a 2-min period and equilibrated for 10 min.

[^{14}C]- and [^{15}N]-Labeling Experiments

Free amino acid uptake of developing seeds was monitored in vitro by incubating intact seeds of 28 DAP in a solution containing 100 mM Suc, 5 mM Gln, 5 mM Asn, and 10 mM MES buffer, pH 7.8. Ten seeds were incubated in 40 mL nutrient solution containing 40 μL [U-^{14}C]Gln (Amersham-Buchler, Braunschweig, Germany). After 6 h of incubation, embryos were dissected and homogenized in 2 mL ethanol and fractionated as described in Rolletschek et al. (2002). Label uptake was measured by liquid scintillation counting (Rotiszint, Roth, Germany).

Two hundred milliliters of 6 mM [^{15}N]-NH_4Cl (Chemotrade, Leipzig, Germany) were applied to the roots of AAP-12 plants at 29 DAP. Sampling of all seeds was done 4 d later. Samples were dried for 2 d at 60°C and ground to pass through a 0.5-mm sieve. For the determination of atomic percent of ^{15}N, the remaining solution of NH_4Cl following titration (Kjeldahl nitrogen analysis) was evaporated. The remaining solution was adjusted to a nitrogen concentration of approximately 500 μg/mL and the enrichment of [^{15}N] was determined by emission spectrometry (Isonitromat 5200; Statron, Fürstenwalde, Germany; see also Götz and Herzog, 2000).

Enzyme Assays

Cotyledons (approximately 28 DAP) were homogenized on ice in 5 volumes of buffer (100 mM MOPS, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) together with 100 mg polyvinylpolypyrrolidone. Homogenates were centrifuged for 10 min at 4°C and 10,000*g*, and supernatants were snap frozen in N_2 in 100-μL aliquots. Enzyme activities were measured as described in Dey and Harborne (1990).

Statistics

Statistical analysis was done using either a Student's *t* test or a Mann-Whitney Rank Sum Test, using Sigma Stat software (Jandel Scientific, Erkrath, Germany).

ACKNOWLEDGMENTS

We are grateful to Petra Hoffmeister, Angela Schwarz, and Katrin Blaschek for excellent technical assistance. We thank Jochen Kumlehn for critical discussions.

Received November 13, 2004; returned for revision January 31, 2005; accepted February 1, 2005.

LITERATURE CITED

An G, Ebert PR, Mitra A, Ha SB (1987) Binary vectors. Plant Mol Biol Man **A3:** 1–19

Balconi CE, Rizzi E, Manzocchi L, Soave C, Motto M (1991) Analysis of in vivo and in vitro grown endosperms of high and low protein strains of maize. Plant Sci **73:** 9–18

Barratt DHP (1982) Changes during development in the nitrogen, uncombined amino acid and carbohydrate contents of cotyledons from different cultivars and lines of field bean (*Vicia faba*). Ann Bot (Lond) **49:** 761–768

Bäumlein H, Nagy I, Villarroel R, Inzé D, Wobus U (1992) Cis-analysis of a seed protein gene promoter: the conservative RY repeat CATGCATG within the legumin box is essential for tissue-specific expression of a legumin gene. Plant J **2:** 233–239

Bennett AB, Spanswick RM (1983) Derepression of amino acid-H^+ cotransport in developing soybean embryos. Plant Physiol **72:** 781–786

Borisjuk L, Rolletschek H, Radchuk R, Weschke W, Wobus U, Weber H (2004) Seed development and differentiation: a role for metabolic regulation. Plant Biol **6:** 375–386

Casey R, Domoney C, Smith AM (1993) Biochemistry and molecular biology of seed proteins. *In* R Casey, RD Davies, eds, Peas: Genetics, Molecular Biology and Biotechnology. CAB International, Wallingford, UK, pp 121–163

DeJong A, Koerselman-Kooij JW, Schuurmans JAMJ, Borstlap AC (1996) Characterization of the uptake of sucrose and glucose by isolated seed coat halves of developing pea seeds. Evidence that a sugar facilitator with diffusional kinetics is involved in seed coat unloading. Planta **199:** 486–492

DeJong A, Koerselman-Kooij JW, Schuurmans JAMJ, Borstlap AC (1997) The mechanism of amino acid efflux from seed coats of developing seeds as revealed by uptake experiments. Plant Physiol **114:** 731–736

Dey PM, Harborne JB (1990) Methods in plant biochemistry. *In* PJ Lea, ed, Enzymes of Primary Metabolism, Vol. 3. Academic Press, London

Flinn AM (1985) Carbon dioxide fixation in developing seeds. *In* PD Hebblethwaite, MC Heath, TCK Dawkins, eds, The Pea Crop: A Basis for Improvement. Butterworths, London, pp 349–358

Gansel X, Muños S, Tillard P, Gojon A (2001) Differential regulation of the NO_3^- and NH_4^+ transporter genes AtNrt2.1 and AtAmt1.1 in Arabidopsis: relation with long distance and local controls by N status of the plant. Plant J **26:** 143–155

Giersberg M, Saalbach I, Bäumlein H (2004) Gene farming in pea under field conditions. *In* R Fischer, S Schillberg, eds, Molecular Farming. Wiley-VCH Verlag GmbH, Weinhein, Germany, pp 183–190

Golombek S, Heim U, Horstmann C, Wobus U, Weber H (1999) Phosphoenolpyruvate carboxylase in developing seeds of *Vicia faba* L. Gene expression and metabolic regulation. Planta **208:** 66–72

Golombek S, Rolletschek H, Wobus U, Weber H (2001) Control of storage protein accumulation during legume seed development. J Plant Physiol **158:** 457–464

Gonzalez MC, Osuna L, Echevarria C, Vidal J, Cejudo FJ (1998) Expression and localization of PEP carboxylase in developing and germinating wheat grains. Plant Physiol **116:** 1249–1258

Götz K-P, Herzog H (2000) Distribution and utilisation of [^{15}N] in cowpeas injected into the stem under influence of water deficit. Isotopes Environ Health Stud **36:** 111–121

Haink G, Deussen A (2003) Liquid chromatography method for the analysis of adenosine compounds. J Chromatogr B **784:** 189–193

Hajdukiewicz P, Svab Z, Maliga P (1994) The small versatile *pPZP* family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol **25:** 989–994

Hedley CL, Harvey DM, Keely RJ (1975) Role of PEP carboxylase during seed development in *Pisum sativum.* Nature **258:** 352–354

Heim U, Weber H, Bäumlein H, Wobus U (1993) A sucrose-synthase gene of *Vicia faba*. Expression pattern in developing seeds in relation to starch synthesis and metabolic regulation. Planta **191:** 394–401

Hirner B, Fischer WN, Renstsch D, Kwart M, Frommer WB (1998) Developmental control of H^+/amino acid permease gene expression during seed development of Arabidopsis. Plant J **14:** 535–544

Koch W, Kwart M, Laubner M, Heineke D, Stransky H, Frommer WB, Tegeder M (2003) Reduced amino acid content in transgenic potato tubers due to antisense inhibition of the leaf H^+/amino acid symporter StAAP1. Plant J **33:** 211–220

Lanfermeijer FC, Koerselman-Kooij JW, Borstlap AC (1990) Changing kinetics of L-valine uptake by immature pea cotyledons during development. An unsaturable pathway is supplemented by a saturable system. Planta **181:** 576–582

Lanfermeijer FC, van Oene MA, Borstlap AC (1992) Compartmental analysis of amino-acid release from attached and detached pea seed coats. Planta **187:** 75–82

Lejay L, Tillard P, Lepetit M, Olive F, Filleur S, Daniel-Vedele F, Gojon A (1999) Molecular and functional regulation of two NO_3^- uptake systems by N- and C-status of Arabidopsis plants. Plant J **18:** 509–519

Lhuillier-Soundélé A, Munier-Jolain N, Ney B (1999) Influence of nitrogen availability on seed nitrogen accumulation in pea. Crop Sci **39:** 1741–1748

Lohaus G, Büker M, Hußmann M, Soave C, Heldt HW (1998) Transport of amino acids with special emphasis on the synthesis and transport of asparagine in the Illinois low protein and Illinois high protein strains of maize. Planta **205:** 181–188

Lohaus G, Moellers C (2000) Phloem transport of amino acids in two *Brassica napus* L. genotypes and one *B. carinata* genotype in relation to their seed protein content. Planta **211:** 833–840

Mancini C, Carbonara AO, Heremans JF (1965) Immunochemical quantification of antigens by single radial immunodiffusion. Immunochemistry **2:** 235–254

Miflin BJ, Lea PJ (1977) Amino acid metabolism. Annu Rev Plant Physiol **28:** 299–329

Minchin PEH, Thorpe MR, Farrar JF, Koroleva OA (2002) Source-sink coupling in young barley plants and control of phloem loading. J Exp Bot **53:** 1671–1678

Miranda M, Borisjuk L, Tewes A, Heim U, Sauer N, Wobus U, Weber H (2001) Amino acid permeases in developing seeds of *Vicia faba* L.: expression precedes storage protein synthesis and is regulated by amino acid supply. Plant J **28:** 61–72

Montamat F, Maurousset L, Tegeder M, Frommer W, Delrot S (1999) Cloning and expression of amino acid transporters from broad bean. Plant Mol Biol **41:** 259–268

Müller M, Knudsen S (1993) The nitrogen response of a barley C-hordein promoter is controlled by positive and negative regulation of the GCN4 and endosperm box. Plant J **4:** 343–355

Okumoto S, Schmidt R, Tegeder M, Fischer WN, Rentsch D, Frommer WB, Koch W (2002) High affinity amino acid transporters specifically expressed in xylem parenchyma and developing seeds of Arabidopsis. J Biol Chem **277:** 45338–45346

Paul MJ, Foyer CH (2001) Sink regulation of photosynthesis. J Exp Bot **52:** 1383–1400

Peoples MB, Gifford RM (1990) Protein turnover. *In* DT Dennis, DH Turpin, eds, Plant Physiology, Biochemistry and Molecular Biology. Longman Scientific, Singapore, pp 448–456

Pickardt T, Meixner M, Schade V, Schieder O (1991) Transformation of *V. narbonensis* via Agrobacterium-mediated gene transfer. Plant Cell Rep **9:** 535–538

Rochat C, Boutin JP (1991) Metabolism of phloem-borne amino acids in maternal tissues of fruit of nodulated or nitrate-fed pea plants. J Exp Bot **42:** 207–214

Rolletschek H, Borisjuk L, Radchuk R, Miranda M, Heim U, Wobus U, Weber H (2004) Seed-specific expression of a bacterial phosphoenolpyruvate carboxylase in *Vicia narbonensis* increases protein content and improves carbon economy. Plant Biotechnol J **2:** 211–219

Rolletschek H, Hajirezaei M, Wobus U, Weber H (2002) Antisense-inhibition of ADP-glucose pyrophosphorylase in *Vicia narbonensis* seeds increases soluble sugars and lead to higher water and nitrogen uptake. Planta **214:** 954–964

Rontein D, Dieuaide-Noubhani M, Dufourc EJ, Raymond P, Rolin D (2002) The metabolic architecture of plant cells. Stability of central metabolism and flexibility of anabolic pathways during the growth cycle of tomato cells. J Biol Chem **277:** 43948–43960

Salon C, Munier-Jolain NG, Duc G, Voisin AS, Grandgirard D, Larmure A, Emery RJN, Ney B (2001) Grain legume seed filling in relation to nitrogen acquisition: a review and prospects with particular reference to pea. Agronomie **21:** 539–552

Schroeder HE, Schotz AH, Wardley-Richardson T, Spencer D, Higgins TJV (1993) Transformation and regeneration of two cultivars of pea. Plant Physiol **101:** 751–757

Schwender J, Ohlrogge J, Shachar-Hill Y (2004) Understanding flux in plant metabolic networks. Curr Opin Plant Biol **7:** 309–317

Sinclair TR, Purcell LC, Sneller CH (2004) Crop transformation and the challenge to increase yield potential. Trends Plant Sci **9:** 70–75

Smidansky ED, Clancy M, Meyer FD, Lanning SP, Blake NK, Talbert LE, Giroux MJ (2002b) Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield. Proc Natl Acad Sci USA **99:** 1724–1729

Smidansky ED, Martin JM, Hannah LC, Fischer AM, Giroux MJ (2002a) Seed yield and plant biomass increases in rice are conferred by deregulation of endosperm ADP-glucose pyrophosphorylase. Planta **216:** 656–664

Smith AJ, Rinne RW, Seif RD (1989) PEP carboxylase and pyruvate kinase involvement in protein and oil biosynthesis during soybean seed development. Crop Sci **29:** 349–353

Strand A, Hurry V, Henkes S, Huner N, Gustafsson P, Gardeström P, Stitt M (1999) Acclimation of Arabidopsis leaf developing at low temperature. Increasing cytoplasmic volume accompanies increased activities of enzymes in the Calvin cycle and sucrose-biosynthesis. Plant Physiol **119:** 1387–1397

Takahashi M, Uematsu Y, Kashiwaba K, Yagasaki K, Hajika M,

Matsunaga R, Komatsu K, Ishimoto M (2003) Accumulation of high levels of free amino acids in soybean seeds through integration of mutations conferring seed protein deficiency. Planta **217:** 577–586

Tegeder M, Offler CE, Frommer WB, Patrick JW (2000) Amino acid transporters are localized to transfer cells of developing pea seeds. Plant Physiol **122:** 319–326

Tillard P, Passama L, Gojon A (1998) Are phloem amino acids involved in the shoot to root control of NO_3^- uptake in *Ricinus communis* plants? J Exp Bot **49:** 1371–1379

Turpin DH, Weger HG (1990) Interactions between photosynthesis, respiration and nitrogen assimilation. *In* DT Dennis, DH Turpin, eds, Plant Physiology, Biochemistry and Molecular Biology. Longman Scientific, Singapore, pp 422–433

Weber H, Borisjuk L, Wobus U (1997) Sugar import and metabolism during seed development. Trends Plant Sci **22:** 169–174

Weber H, Borisjuk L, Wobus U (2005) Molecular physiology of legume seed development. Annu Rev Plant Biol **56:** (in press)

Weber H, Golombek S, Heim U, Borisjuk L, Panitz R, Manteuffel R, Wobus U (1998b) Integration of carbohydrate and nitrogen metabolism during legume seed development: implications for storage product synthesis. J Plant Physiol **152:** 641–648

Weber H, Heim U, Golombek S, Borisjuk L, Wobus U (1998a) Assimilate uptake and the regulation of seed development. Seed Sci Res **8:** 331–345

Wobus U, Bäumlein H, Bassüner R, Heim U, Jung R, Müntz K, Saalbach G, Weschke W (1986) Characteristics of two types of legumin genes in the field bean (*Vicia faba* L. var. minor) genome as revealed by cDNA analysis. FEBS Lett **201:** 74–79