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Amino acid permeases in developing seeds of *Vicia faba* L.: expression precedes storage protein synthesis and is regulated by amino acid supply

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Summary

Full length cDNAs encoding three amino acid permeases were isolated from seed-specific libraries of *Vicia faba*. The predicted proteins VfAAP1, VfAAP3 and VfAAP4 share up to 66% identity among themselves. Functional characterization of VfAAP1 and VfAAP3 in a yeast mutant showed that these permeases transport a broad range of amino acids. However, VfAAP1 had a preference for cysteine and VfAAP3 for lysine and arginine. *VfAAP1* was highly expressed in cotyledons at early developmental stages and moderately in other sink tissues. Its peak of expression in cotyledons corresponded to the appearance of storage protein transcripts, suggesting that this transporter fulfills an important role in providing amino acids for storage protein biosynthesis. *VfAAP3* was expressed most abundantly in maternal tissues, that is in roots, stems, gynoecia, pods and seed coats at different developmental stages. *VfAAP4* transcripts could not be detected by northern hybridization. *In situ* hybridization showed that *VfAAP1* mRNA is distributed throughout cotyledon storage parenchyma cells, but could not be detected in the abaxial epidermal cell layer. It also accumulate in the chlorenchyma and thin-walled parenchyma cells of seed coats. *VfAAP1* mRNA levels were lower in cotyledons cultured in the presence of glutamine, whereas expression of a vicilin storage protein gene was up-regulated under similar conditions. Cysteine repressed the expression of the *GUS* reporter gene under control of the *VfAAP1* promoter, suggesting that this transporter is modulated at the transcriptional level. Regulation of amino acid transport in relation to storage protein accumulation is discussed.

Keywords: amino acid permease, transporter, storage protein, legume seed development, gene regulation.

Introduction

In most plants, amino acids represent the major transport form of organic nitrogen, and are distributed through both xylem and phloem to all plant organs where they may be immediately metabolized, or used for storage protein synthesis in sink tissues. As nitrogen is a limiting factor to plant growth, understanding its metabolism has raised interest of physiologists, and later, efforts focused on the molecular characterization of genes involved in N distribution throughout the plant. After physiological evidence for proton-coupled amino acid uptake was found (reviewed by Bush, 1993), the first plant amino acid transporter AAP1/NAT2 was isolated from *Arabidopsis thaliana* through functional complementation in yeast (Frommer *et al.*, 1993; Hsu *et al.*, 1993). Afterwards, other full length cDNAs have been isolated (Ortiz-Lopez *et al.*, 2000). These transporters have been classified in subfamilies based on sequence similarities and uptake properties. Best characterized is the amino acid permease subfamily (AAP), from which at least six members isolated from *A. thaliana* have been functionally characterized *in vitro* (Rentsch *et al.*, 1998). It comprises general amino acid permeases, which transport a broad spectrum of amino

acids, and permeases that recognize only neutral and acidic amino acids. Moreover, most of these transporters have particular temporal and spatial gene expression patterns, denoting a refined network controlling gene regulation (Fischer *et al.*, 1995; Rentsch *et al.*, 1996).

Most reports on AAPs concentrate on the vegetative parts of plants. Seeds, on the other hand, are reproductive sink organs and thus provide a distinct physiological environment. During embryogenesis, solutes are transferred from the phloem through the seed's funiculus where they are symplasmically unloaded into the seed coat (Offler and Patrick, 1993; Patrick and Offler, 1995). The embryo, however, is symplasmically isolated from maternal tissues, hence assimilates have to cross an apoplastic space before being taken up by the cotyledons. Biochemical studies provided evidence that active transport systems for the uptake of amino acids are present in legume embryos (Bennett and Spanswick, 1983; Lanfermeijer *et al.*, 1990). The only report that focuses directly on molecular aspects of amino acid transport in developing legume seeds describes that *PsAAP1* is expressed in the transfer cell layer of pea cotyledons, and may play a role in the uptake of the full spectrum of amino acids released from the seed coat (Tegeder *et al.*, 2000). Moreover, *AtAAP1* expression in developing *Arabidopis* seeds supports a function in amino acid import into the endosperm and embryo (Hirner *et al.*, 1998).

Accumulation of storage compounds in seeds is a highly orchestrated process. Sugars and nitrogen compounds confer regulatory control on storage activities, and their levels are developmentally controlled during seed development (Weber *et al.*, 1997a; 1998a). Studies using cultured embryos of soybean showed that cotyledon N accumulation and concentration increased proportionally to the N concentration in the media. However, differences in the degree of accumulation were dependent on the genotype (Hayati *et al.*, 1996). In developing seeds, Hirner *et al.* (1998) have shown that expression of an *AAP* precedes that of storage protein genes, however, little is known about the mode of regulation of amino acid transporters in response to N supply during seed development, and whether their gene products have a rate-limiting role on amino acid uptake and, could therefore control storage protein biosynthesis.

Seeds of *V. faba* provide a well-defined system for analysing the relationship between amino acid transport and storage protein accumulation, because these seeds accumulate large amounts of proteins. A detailed description of seed development in *V. faba* can be found in Borisjuk *et al.* (1995). Briefly, the first phase of embryogenesis is marked by cell divisions, followed by a phase of differentiation and deposition of storage compounds. Several storage protein genes have been identified and characterized, which are developmentally controlled at different levels of regulation (Weber *et al.*, 1997a; Wobus *et al.*, 1995). Moreover, H^+ cotransporters for hexoses and sucrose have been characterized and are expressed mainly in the epidermal transfer cells of the cotyledons (Weber *et al.*, 1997b), which form a tissue specialized in transfer processes (McDonald *et al.*, 1995).

We have cloned three different *AAP* isoforms from seed-specific cDNA libraries of *V. faba*. One of these (*VfAAP1*) was expressed in developing seeds, mainly in the storage parenchyma cells of the cotyledon, preceding storage protein synthesis and was regulated at the transcriptional level by amino acids. The relevance and modulation of amino acid transport in relation to storage protein accumulation in legume seeds is discussed.

Results

CDNAs encoding three different amino acid permeases from V. faba *seeds*

PCR using degenerated primers yielded three different DNA fragments that had homology to known *AAPs*. These fragments were used as probes for screening two cDNA libraries from developing *V. faba* seeds. Five clones from an identical cDNA were isolated from a library of developing cotyledons. A full length clone, *VfAAP1*, was composed of 1590 bp, and encoded a predicted protein of 575 amino acid residues. It shared 91.2% identity at the amino

Table 1. Percentage of similarity between the predicted amino acid sequences from VfAAP1, VfAAP3, VfAAP4 and other amino acid permeases*

	VfAAP3	VfAAP4	VfAAP2[1]	PsAAP1[2]	PsAAP2[2]	AtAAP1[3]	AtAAP2[4]	AtAAP3[5]	AtAAP4[5]	AtAAP5[5]	AtAAP6[6]
VfAAP1	66.1	64.0	62.5	63.4	91.2	53.1	57.8	65.1	65.5	61.3	53.3
VfAAP3	–	58.6	59.5	58.3	67.9	49.1	54.7	61.1	60.1	59.6	48.6
VfAAP4	–	–	64.7	91.7	66.7	53.6	59.2	71.6	67.2	64.6	56.8

*Clustal method (MegAlign, DNAStar, London, UK); [1]*V. faba* (Montamat *et al.*, 1999); [2]Pea (Tegeder *et al.*, 2000); [3–6]*A. thaliana* ([3]Frommer *et al.*, 1993; [4]Kwart *et al.*, 1993; [5]Fischer *et al.*, 1995; [6]Rentsch *et al.*, 1996)

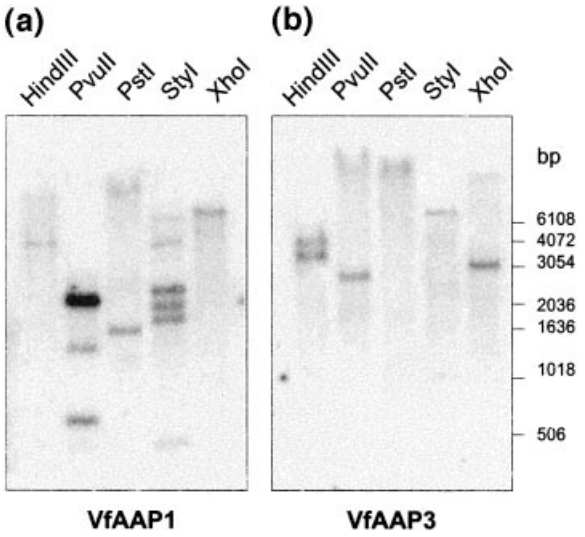

Figure 1. Southern blot analysis of *VfAAP1* and *VfAAP3*.
Ten μg total DNA were digested with *Hind*III, *Pvu*II, *Pst*I, *Sty*I or *Xho*I, separated on a 1% agarose gel, and hybridized with ^{32}P labelled cDNA probes of *VfAAP1* (a) or *VfAAP3* (b).

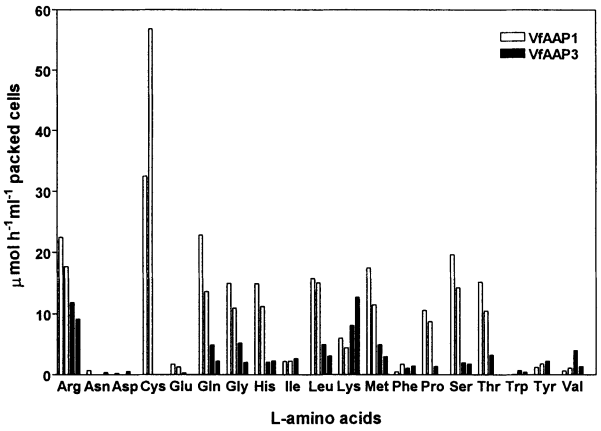

Figure 2. Amino acid uptake promoted by VfAAP1 and VfAAP3 into a yeast mutant.
Uptake rates into the *plas*23-4B yeast mutant expressing either *VfAAP1* or *VfAAP3* complete cDNAs were directly measured for each [U]-^{14}C-labelled L-amino acid at 1 mM concentration. Background uptake of the mutant transformed with an 'empty' vector was subtracted. Bars represent independent experiments.

acid level with a putative AAP (PsAAP2) isolated from pea by Tegeder *et al.* (2000) (Table 1). Twenty clones representing another cDNA were isolated from a seed coat specific library. One of the clones containing a complete open reading frame was denominated *VfAAP3*[1] (1878 bp). Its predicted protein sequence, made of 486 amino acid

[1]the designation *VfAAP2* has been used for a previously identified *AAP* of *V. faba* (Montamat *et al.*, 1999)

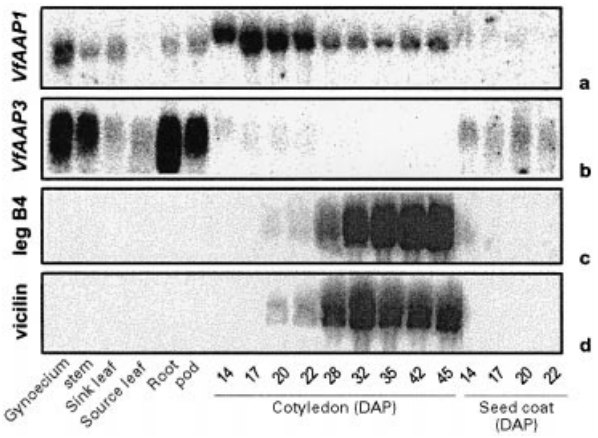

Figure 3. Analysis of transcript accumulation of *VfAAP1*, *VfAAP3*, legumin B4 and vicilin in different tissues and seed developmental stages of *V. faba*.Northern blots were hybridized with ^{32}P-labelled cDNA probes of *VfAAP1* (a), *VfAAP3* (b), legumin B4 (c) and vicilin (d). Ten μg total RNA, isolated from different tissues and seed developmental stages, were loaded per lane. DAP: days after pollination.

residues, is 67.9 and 61.1% identical to PsAAP2 and AtAAP3 (from *A. thaliana*; Fischer *et al.*, 1995), respectively (Table 1). Since the third *AAP* fragment was highly similar to the pea clone *PsAAP1* (Tegeder *et al.*, 2000), its full length cDNA (1450 bp, called *VfAAP4*) could be cloned by PCR with primers based on the *PsAAP1* sequence using the seed coat library as template. Its predicted protein sequence was 481 amino acids long and shared 91.7% identity with PsAAP1 (Table 1). The predicted VfAAP1, VfAAP3 and VfAAP4 proteins are highly hydrophobic, and their hydrophobicity profiles overlap those of the *A. thaliana* AAPs (data not shown), which suggests that they also have 11 transmembrane domains (Chang and Bush, 1997).

Southern hybridizations using total DNA showed that *VfAAP1* and *VfAAP3* do not cross-hybridize under high stringency conditions (Figure 1). *VfAAP1* revealed single bands only for *Hind*III and *Xho*I. The other three enzymes showed more bands than expected from the restriction sites in the translated region. Therefore, either additional sites are present in its introns or another gene highly homologous to *VfAAP1* may exist. *VfAAP3*, on the other hand, revealed single bands for *Pvu*II, *Pst*I, *Sty*I and *Xho*I digestions, and two *Hind*III bands, suggesting a single copy gene (Figure 1).

Amino acid uptake in a yeast mutant reveals that VfAAP1 preferably transports cysteine, and VfAAP3, lysine and arginine

For functional characterization, *VfAAP1* and *VfAAP3* cDNAs were transformed into *plas*23-4B yeast mutant. This mutant carries a mutation in the *SHR*3 gene, which

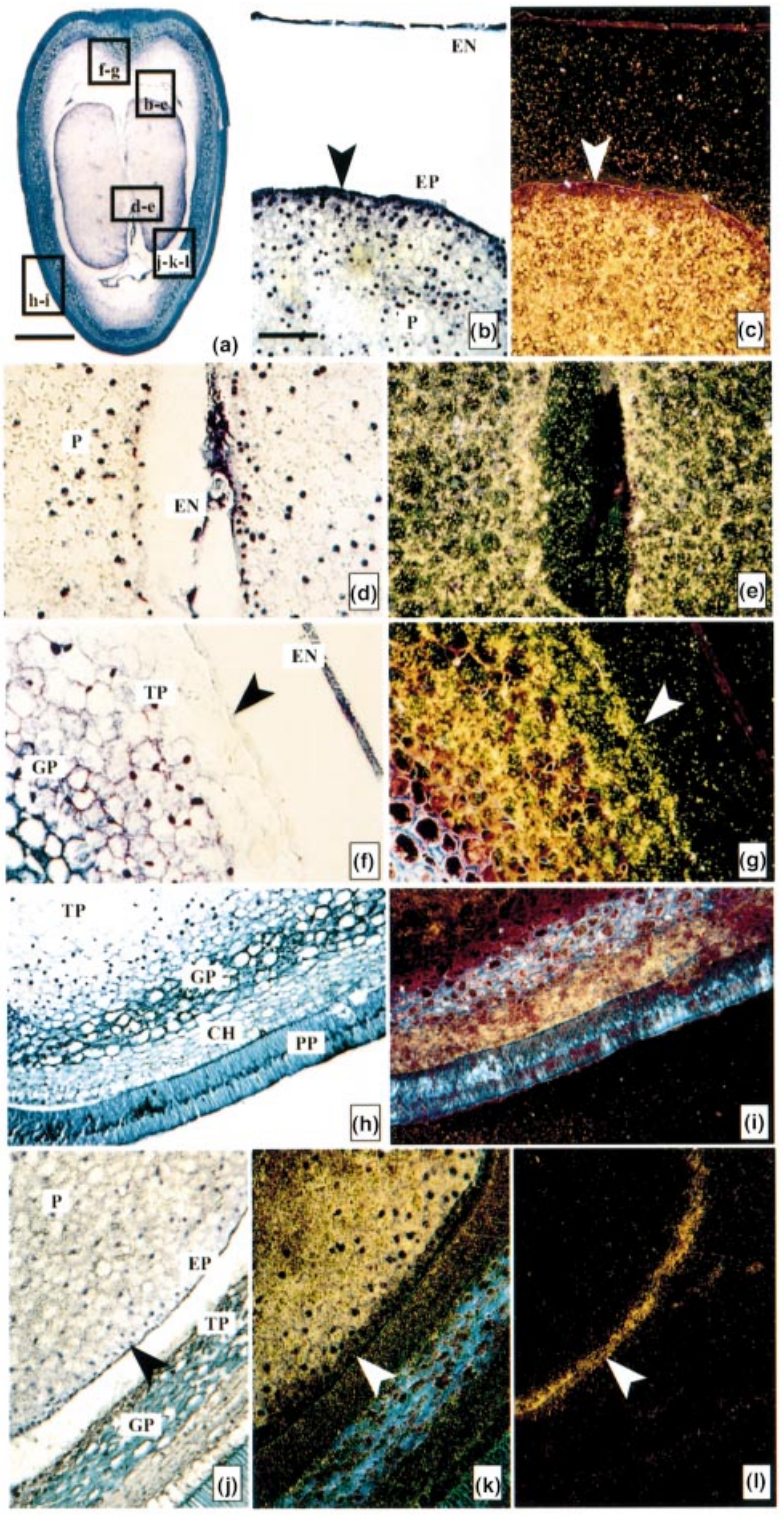

Figure 4. Transcript distribution of *VfAAP1*, *VfSUT1* and vicilin in transversal sections of a *V. faba* developing seed (stage V).
(a) overview of the seed stained with toluidine-blue. The boxed areas are enlarged in the subsequent pictures.
(b, d, f, h and j) light-field with toluidine-blue staining.
(c, e, g, i, k and l) dark-field micrographs corresponding to the light-field pictures showing *in situ* hybridization using ^{33}P labelled cDNA probes of *VfAAP1* (c–i), vicilin (k) and *VfSUT1* (l)with label seen as white grains.
(b and c) outer region of the cotyledon with labelling of the *VfAAP1* probe in the storage parenchyma (P), but not the epidermal transfer cells (EP) or endosperm (EN).
(d and e) inner portion of the cotyledon with *VfAAP1* labelling in the storage parenchyma.
(f and g) inner portion of the seed coat near the funiculus. Labelling is found in the transfer cells of the thin-walled parenchyma (TP), but not in the ground parenchyma (GP) or endosperm.
(h and i) outer region of the seed coat probed with *VfAAP1* is labelled in the chlorenchyma (CH) and thin-walled parenchyma. No label was found in the palisade epidermis (PP) or ground parenchyma where the bluish colour results from autofluorescence.
(j and k) labelling of the vicilin probe is found in the parenchyma but not in the abaxial epidermal cells of the cotyledon.
(l) labelling of the *VfSUT1* probe is found only in the transfer cells of the cotyledon.
Arrowheads show transfer cells of cotyledons and seed coat. Bars represent 0.8 and 0.1 mm in a and b–l, respectively.

prevents proper targeting of its own amino acid permeases to the plasma membrane, and inhibits growth in media where amino acids are the only source of nitrogen (Ljungdahl *et al.*, 1992). Control experiments with cells transformed with the 'empty' vector revealed no or very low background uptake for all amino acids tested (data not shown). These were subtracted from the experimental values of amino acid uptake by yeast functionally express-

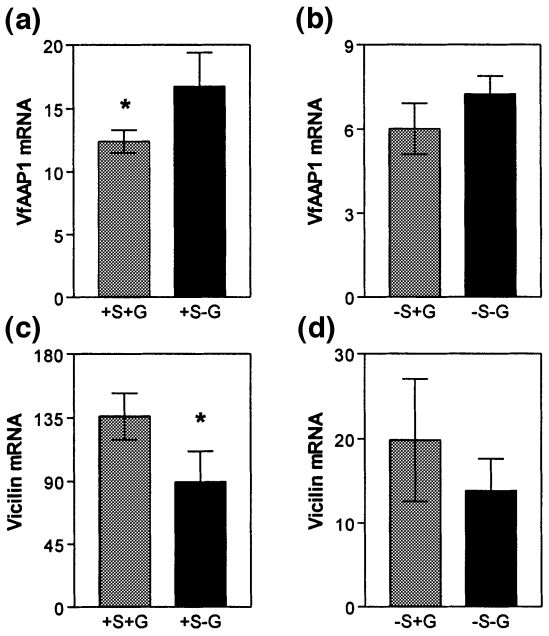

Figure 5. Effect of metabolites on the accumulation of *VfAAP1* and vicilin mRNA in cotyledons (stage V) cultured *in vitro*.
Transcript analysis of *VfAAP1* (a and b) and vicilin (c and d) after *in vitro* culture of cotyledons in the presence (+) or absence (–) of 150 mM glutamine (G) and 150 mM sucrose (S). Signals on the blots were quantified by a phospho-imager, and are shown in relative units. Each bar represents the mean of three replications ±SE. (*) statistically significant ($P < 0.05$).

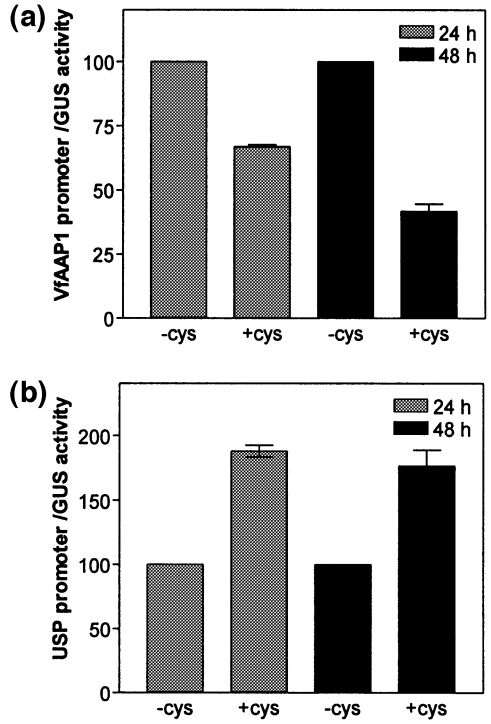

Figure 6. Effect of cysteine on transient *GUS* expression driven by the *VfAAP1* or *USP* promoter in *N. plumbaginifolia* protoplasts.
Protoplasts were transformed with *VfAAP1* promoter/*GUS* (a) or *USP* promoter/*GUS* construct (b), and cultured either in the presence of 1 mM cysteine or under amino acid starvation. GUS activities of four transformation events were measured after 24 and 48 h. Values are given in relative units (control without cysteine = 100%) ±SE ($P < 0.05$).

ing *VfAAP1* or *VfAAP3*. VfAAP1 mediated the transport of a wide range of amino acids, with high uptake rates for cysteine, arginine, glutamine, serine, leucine, methionine, histidine, glycine and threonine. A clear preference was seen for the uptake of cysteine, which was up to 2.5-fold higher than that of the second fastest taken up amino acid (Figure 2). VfAAP3 also transported several amino acids, however, at lower rates when compared with VfAAP1 in the yeast system, with a preference for the basic amino acids lysine and arginine (Figure 2). Neither acidic nor aromatic amino acids were taken up to a larger extent by these permeases. The kinetics of [U]-^{14}C labelled L-arginine uptake revealed Km values of 46.0 and 82.4 μM for VfAAP1 and VfAAP3, respectively, indicating that these AAPs have high substrate affinity.

MRNA distribution analysed by northern and in situ *hybridization*

Accumulation of *VfAAP1* transcripts, as analysed by northern hybridization, was highest in gynoecia and early developing cotyledons, that is stages IV and V (Borisjuk *et al.*, 1995). Expression in cotyledons dropped to a lower and constant level at later stages. Moderate expression levels were observed in stems, roots, pods and sink leaves, but not in source leaves, and low levels were found in seed coats (Figure 3a). The expression profile indicated that this transporter provides amino acids primarily to gynoecia and cotyledons, and to a lower extent to other sink tissues. *VfAAP3* mRNA was abundant in gynoecia, stems, roots and pod tissues, and present in moderate amounts in sink and source leaves, as well as in seed coat tissues of different developmental stages, but nearly absent in cotyledons (Figure 3b). *VfAAP4* did not reveal detectable mRNA signals in any of the tissues analysed (data not shown). For comparison, the blots were hybridized with cDNA probes encoding the storage protein genes legumin B4 (Wobus *et al.*, 1986) and vicilin (Bassüner *et al.*, 1987). Transcripts of these storage protein genes appear at the time when *VfAAP1*-mRNA levels start to decline (Figure 3c,d).

In situ hybridization of seed sections using the *VfAAP1* cDNA as a probe, revealed that this transporter is expressed throughout the whole storage parenchyma tissue of cotyledons (Figure 4c,e), with no signals in the abaxial epidermal transfer cells (Figure 4c). The endosperm was not labelled (Figure 4c,g). In seed coats, *VfAAP1* probe labelling was restricted to the chlorenchyma and thin-walled parenchyma tissues (Figure 4g,i). No signals were seen in palisade or ground parenchyma tissues (Figure 4i).

In controls with a vicilin probe, labelling was distributed in the storage parenchyma cells, but not in the abaxial epidermis of the cotyledons (Figure 4k), whereas when a sucrose transporter (*VfSUT1*; Weber *et al.*, 1997b) probe was used as a control, labelling was restricted to the cotyledon epidermal transfer cells (Figure 4l).

VfAAP1- *and storage protein-mRNA levels are regulated by metabolites*

In order to examine whether *VfAAP1*-mRNA levels can be modulated by amino acid availability, cotyledons at the mid-cotyledon stage (stage VI; Borisjuk *et al.*, 1995) were cultured *in vitro*. One cotyledon of a seed was cultured in the presence of glutamine, which is used as the major source of N by developing cotyledons (Miflin and Lea, 1977), whereas the other cotyledon was cultured under amino acid starvation. Experiments were performed both in the presence and absence of sucrose. *VfAAP1*-mRNA levels were lower in cotyledons cultured in media supplemented with glutamine. This tendency was observed both in the presence and absence of sucrose, however, significant changes were only seen when sucrose was present (Figure 5a,b). The vicilin-mRNA levels were higher when glutamine was added to the medium, with significant changes seen only in the presence of sucrose (Figure 5c,d).

The 5′-flaking region (1702 bp long) of *VfAAP1* was cloned by genome walking. Computer predictions revealed the potential regulatory TATA- and CAAT boxes and poly A signals, as well as a putative seed specific nitrogen responsive GCN4-/RY-like element (Bäumlein *et al.*, 1992; Wolfner *et al.*, 1975). The 5′-flanking region was inserted into a vector upstream of the β-glucuronidase (*GUS*) gene, and transformed into protoplasts derived from embryogenic suspension cultures of *Nicotiana plumbaginifolia*, a system previously shown to allow the analysis of heterologous promoters (Reidt *et al.*, 2000). GUS activity driven by the *VfAAP1* 5′-upstream region was up to 110-fold higher then in the controls lacking this region (data not shown), indicating that the *VfAAP1* 5′-upstream region included a functional promoter. To determine whether this promoter can be regulated by cysteine, which is the preferentially transported amino acid by VfAAP1, cysteine was added to the media after transformation of protoplasts with the *VfAAP1* promoter/*GUS* construct. GUS activities measured 24 and 48 h after transformation were approximately 40% and 55% lower, respectively, than in controls under amino acid starvation (Figure 6a). For comparison, protoplasts were transformed with a *USP* promoter/*GUS* construct. The *USP* promoter (from *U*nknown *S*eed *P*rotein) is seed specific and is expressed and regulated similarly to storage protein genes (Bäumlein *et al.*, 1991). After 24 and 48 h, GUS activities driven by the *USP* promoter were approximately 80% higher in protoplasts cultured with cysteine than in controls cultured under amino acid starvation (Figure 6b).

Discussion

Three amino acid transporters from seed-specific cDNA libraries of V. faba *belong to the AAP subfamily*

Three full length cDNAs (*VfAAP1*, *VfAAP3* and *VfAAP4*) sharing homology to the AAP subfamily of amino acid transporters (Fischer *et al.*, 1995) have been isolated after screening two seed-specific libraries from *V. faba*. Comparisons between their predicted protein sequences showed that VfAAP1 and VfAAP4 share high degrees of identity with PsAAP2 (a putative AAP; 91.2%) and PsAAP1 (91.7%), respectively, isolated from pea (Tegeder *et al.*, 2000). Because *V. faba* and pea are phylogenetically closely related, these clones may represent the orthologue forms of the same transporters in these two species. The pea counterpart of VfAAP3 remains to be identified. Our newly isolated AAPs differ from those (one full length and three partial cDNAs) previously isolated from *V. faba* (Montamat *et al.*, 1999), indicating that at least seven different *AAP* genes are present in the genome of this species. In *A. thaliana*, six AAP isoforms have been functionally characterized *in vitro* (Fischer *et al.*, 1995; Kwart *et al.*, 1993; Rentsch *et al.*, 1996). This apparent redundancy of such transporters in a genome has frequently been suggested to reflect a strict regulatory control, substantiated by individual expression profiles in the plant (Fischer *et al.*, 1998).

V. faba AAPs showed hydrophobic profiles that superimposed those of the *A. thaliana* AAPs, and therefore the 11 transmembrane model proposed by Chang and Bush (1997) is applicable to these proteins as well.

VfAAP1 and VfAAP3 are transporters for neutral and basic amino acids with different substrate specificity

The yeast mutant *plas*23-4B, which has a reduced amino acid uptake capacity due to improper targeting of its endogenous amino acid permeases (Ljungdahl *et al.*, 1992), was used for characterizing the uptake of VfAAP1 and VfAAP3. This mutant was suitable for measuring the direct uptake of amino acids since it did not exhibit efficient transport of any of the amino acids tested. Direct measurements are more likely to give reliable results compared with competition experiments since it is not known whether the competitor is actually taken up, and because these transporters may have multiple amino acid binding sites which could lead to ambiguous results (Boorer *et al.*, 1996). VfAAP1 and VfAAP3 exhibited high substrate affinities and transported a broad spectrum of amino acids (Figure 2). Both high and low affinity AAPs are

known, and broad substrate specificity is a feature that is characteristic of most of them (Rentsch *et al.*, 1998). VfAAP1 transported most of the neutral and basic amino acids at high uptake rates, and did not transport acidic or aromatic amino acids efficiently. Among the tested amino acids, cysteine was clearly preferred by VfAAP1 (Figure 2). Kwart *et al.* (1993) showed that a 4-fold excess of cysteine was able to inhibit approximately 90% of proline uptake by AtAAP2, but a comparison of the direct uptake of these substrates was not provided. VfAAP3 showed a broad substrate specificity, a lower transport activity in the yeast mutant compared with VfAAP1 and a preference for the basic amino acids arginine and lysine (Figure 2). Therefore, with respect to substrate specificity, it functions similarly to AtAAP3 and AtAAP5 (Fischer *et al.*, 1995). VfAAP1 and VfAAP3 are peculiar in the sense that they did not take up acidic amino acids. In support of our observations, the uptake of glutamate by vesicles of pea cotyledons occurred in a passive-like manner (de Jong and Borstlap, 2000a). All AAPs of *A. thaliana* so far analysed were able to transport glutamic acid, which is in many plants one of the preferred transport forms of reduced nitrogen. In *V. faba* and pea, however, glutamine is used as the major source of N by embryos throughout their development (Miflin and Lee, 1977; Murray and Cordova-Edwards, 1984), which may explain to some extent the differences between *V. faba*'s and *A. thaliana*'s AAPs regarding the uptake of glutamate. Complementation of the yeast mutant by VfAAP4 was not carried out since the probable orthologue of this permease (PsAAP1) has already been functionally characterized by Tegeder *et al.* (2000), and similar results could be anticipated.

VfAAP1 *expression during seed development precedes storage protein synthesis*

VfAAP1 transcripts accumulate primarily in cotyledons at early stages of development (stages IV and V, Borisjuk *et al.*, 1995), and to a lower extent in other sink tissues. Its mRNA level peaked at the time when storage protein transcripts appeared, and then declined to a lower and relatively constant level. The concentration of free amino acids in the cotyledons increases rapidly during the period of seed filling, and decreases during storage protein synthesis (Weber *et al.*, 1996). Moreover, timing and location of storage protein gene expression are directly coupled to storage protein deposition (Weber *et al.*, 1998b). Thus, increases in *VfAAP1* mRNA accumulation, amino acid concentration and storage protein gene transcripts happen in a sequential manner, and suggest a potential primary role of VfAAP1 in supplying amino acids for storage protein synthesis. A similar function has been proposed for the *AtAAP1* gene from *A. thaliana*, which is expressed in the endosperm and cotyledons of developing seeds (Hirner *et al.*, 1998). In contrast to our observations, Bennett and Spanswick (1983) and Lanfermeijer *et al.* (1990) provided evidence that in young pea cotyledons (corresponding to the stage V in *V. faba*) passive uptake of amino acids would be of primary importance, and that an active uptake system would only become apparent in late cotyledon development. In addition, *VfAAP1* may have a secondary function in supplying amino acids to other sink organs as well, since it is also expressed, albeit to a lower extent, in gynoecia, stems, sink leaves, roots and pods, but not in source leaves. *VfAAP3* mRNA accumulated in all maternal tissues analysed, mostly in gynoecia, stems, roots, pods and moderately in sink and source leaves, as well as in seed coat tissues of different developmental stages. This suggests a general 'house keeping' function in the plant's transport of amino acids. It was not possible to detect any level of expression from *VfAAP4* by Northern analysis. Interestingly, despite a high sequence similarity (over 90% of identical amino acids) between the predicted VfAAP1 and VfAAP4 proteins and the pea proteins PsAAP2 and PsAAP1 (Tegeder *et al.* 2000), respectively, their gene expression profiles are strikingly different suggesting that these two species have developed a refined and distinct mode of regulation.

VfAAP1 *is expressed in the cotyledon storage parenchyma similar to storage protein genes, and not in the transfer cells like other transporters*

Epidermal transfer cells play a key role in the active uptake of assimilates by the embryos (Weber *et al.*, 1998a), and are the exclusive site of expression of hexose and sucrose carriers within *V. faba* cotyledons (Weber *et al.*, 1997b). In pea cotyledons, an amino acid permease was reported to be expressed in this cell layer also (Tegeder *et al.*, 2000). However, our *in situ* hybridization experiments localized *VfAAP1* mRNA only in the storage parenchyma of *V. faba* cotyledons. The labelling pattern was rather similar to that found for storage protein genes like vicilin (Figure 4k) and legumin B4 (Weber *et al.*, 1998b). The presence of an amino acid transporter in the storage parenchyma cells has been hypothesized by de Jong and Borstlap (2000a) to explain discrepant uptake rates of L-valine and sucrose by pea cotyledon vesicles. Speculations on a possible need of an active amino acid transport system in storage parenchyma cells would include relocation of amino acids through the cotyledons and/or retrieval from intercellular spaces of 'leaked' amino acids (Ruiter *et al.*, 1984). Further studies are necessary to solve these questions.

VfAAP1 mRNA was also found in the chlorenchyma and thin-walled parenchyma cells of the seed coat. The thin-walled parenchyma exhibit numerous wall in-growth projections and is responsible for the exchange of assimilates between the coat cells and the apoplast (Offler and

Patrick, 1993). The mode of assimilate unloading from the seed coat cells is still controversial. A putative sucrose H^+-antiporter localized in the thin-walled parenchyma of *V. faba* has been proposed (Patrick and Offler, 1995), whereas in pea unloading has been shown to be entirely passive (de Jong *et al.*, 1996). Although efflux of amino acids mediated by a H^+-coupled transporter is thermodynamically feasible, so far it could not be experimentally attributed to the AAPs (Fischer *et al.*, 1995). On the other hand, H^+-symporters seem to be responsible for the retrieval of amino acids from the apoplast back to the seed coats cells in pea (de Jong and Borstlap, 2000b). Since there is no evidence that VfAAP1 has a role in seed coat unloading, it is more likely that it takes part in retrieving amino acids from the apoplast. The function of an amino acid permease in the chlorenchyma is unclear, but it underlines once more that *VfAAP1* is expressed in seed tissues that are symplasmically connected.

Amino acids modulate VfAAP1 *gene transcription and storage protein gene expression in an antagonistic manner*

The expression of *VfAAP1* was repressed in cotyledons cultured in the presence of glutamine, whereas the vicilin gene expression increased (Figure 5). Weber *et al.* (1998b) have shown that another storage protein gene, the legumin B4 gene, is also up-regulated by glutamine. During seed development, *VfAAP1* and storage protein genes are expressed in a sequential manner (Figure 3), and the decline in the steady-state level of *VfAAP1* mRNA is temporarily coupled to the highest concentration of free amino acids in cotyledons *(*Weber *et al.*, 1996*),* thus we presume that this transporter may also respond to assimilate availability *in vivo*. A decrease in C or N assimilates induces genes involved in their acquisition, while abundance of these resources induces genes associated with their use and storage (Koch, 1996; Weber *et al.*, 1998b). This seems to apply to *VfAAP1* as well. Changes in *VfAAP1* and vicilin mRNA levels were more pronounced in the presence of sucrose. Indeed, observations made by Barneix *et al.* (1992) suggest that the C: N ratio exported from vegetative tissues is correlated to the level of storage protein accumulation, however, whether C levels may affect amino acid import by the embryo remains to be shown. Moreover, in yeast, *AAP* genes are transcriptionally regulated by an extracellular amino acid sensor (Didion *et al.*, 1998; Iraqui *et al.*, 1999). Although extracellular sensing of amino acids may also exist in plants, in the present experiments sensing could also have taken place intracellularly since Weber *et al.* (1998b) found that the content of free amino acids in cotyledons cultured in the presence of glutamine was approximately 2.5 times higher than that in cotyledons cultured under amino acid starvation.

Expression of the *GUS* reporter gene driven by the *VfAAP1* promoter was gradually and drastically reduced in the presence of cysteine, pointing to a transcriptional regulation of the *VfAAP1* promoter. In yeast, *GAP1*, a general amino acid permease, is induced under poor nitrogen supply, by regulatory mechanisms acting at transcriptional and post-transcriptional levels (Grenson, 1992). Among the regulatory elements found in the *VfAAP1* promoter, a GCN4/RY-like motif (TGCATG-ATTTGCAT; pos. –372 bp from the translation start) is a likely candidate for conferring amino acid responsiveness to this transporter. In plants, the GCN4 elements act as enhancers in several storage protein genes (Bäumlein *et al.*, 1992; Müller and Knudsen, 1993), or as silencers in amino acid biosynthetic and non-storage protein genes (Cock *et al.*, 1992; Fiedler *et al.*, 1993). The GCN4-like RY-motifs play a key role in seed-specific gene regulation in coordination with other *cis*-acting elements (Bäumlein *et al.*, 1992; Reidt *et al.*, 2000). The putative RY-motif of the *VfAAP1* promoter is located 99 bp upstream of a potential TATA-box. Such a distance is an usual requirement for functional RY-motifs (Reidt *et al.*, 2001). In a transient assay with a C-hordein promoter, the GCN4 motif induced a reporter gene in the presence of amino acids (Müller and Knudsen, 1993), whereas in yeast, it induced amino acid biosynthetic genes under amino acid starvation (Wolfner *et al.*, 1975). The present results are in general agreement with the mode of regulation of genes involved in N metabolism.

In conclusion, we have shown that at least three amino acid permeases are expressed in *Vicia faba* seeds. VfAAP1 transports a broad range of amino acids with a preference for cysteine, and is strongly expressed in developing seeds. Its mRNA was found in the storage parenchyma tissue, but not in the abaxial epidermal cell layer of the cotyledons. The maximum expression of *VfAAP1* in cotyledons during seed development corresponds to the appearance of storage protein gene transcripts and to the highest concentration of free amino acids. During storage protein synthesis, levels of *VfAAP1* mRNA and of free amino acids decline (Weber *et al.*, 1996), consistent with the presumed role of this permease in providing amino acids that will be used for the synthesis of storage proteins. Amino acid supply modulates *VfAAP1* and storage protein genes in an antagonistic manner at the transcription level, and may, to some extent, influence the switch between amino acid import and storage phase.

Experimental procedures

Plant material

Plants from *V. faba* L. var. *minor* cv. 'Fribo' (Genebank, IPK, Gatersleben, Germany) were grown in soil in growth chambers supplied with artificial light (16 h light/8 h dark regime), at 20°C.

Seeds were collected 4 h after beginning of the light period, and sorted on basis of the number of days after pollination (DAP).

Molecular cloning

In an attempt to isolate different amino acid transporter genes expressed in seeds of *V. faba*, two cDNA libraries, one prepared from developing cotyledons (Heim *et al*., 1993) and the other from seed coats, were screened as described in Buchner *et al*. (1996). Fragments obtained by PCR using degenerated primers based on database *AAP* sequences (5′-CAYATHACIGCIGTIATHGG-3′/5′-ATYTCIACIGGRAARTAIACIGT-3′ primer combination) were sequenced, labelled with ^{32}P-dCTP (Random Primer Labelling Kit, Amersham Pharmacia Biotech, Buckinghamshire, UK) and used as probes.

Southern, Northern and in situ *hybridizations*

For Southern hybridizations, 10 μg total DNA were digested with *Hind*III, *Pvu*II, *Pst*I, *Sty*I and *Xho*I, blotted and hybridized as described in Heim *et al*. (1996). Complete cDNAs were used as probes after labelling with ^{32}P-dCTP. For Northern blotting, total RNA was isolated from different tissues, separated on a gel according to Heim *et al*. (1993). Northern hybridizations were performed at 65°C and washed at high stringency. Fragments obtained by enzymatic digestions, which included both coding and uncoding regions of the cDNAs, were used as probes after labelling with ^{32}P-dCTP. Signals on filters were quantified using a phospho-imager. *In situ* hybridizations followed the protocol described in Weber *et al*. (1995). Slides were hybridized overnight at 42°C with ^{33}P-dCTP labelled probes.

Heterologous expression in a yeast mutant

Full length cDNAs were cloned in sense orientation into NEV-E yeast shuttle vector (Sauer and Stolz, 1994), and transformed into the *plas*23-4B yeast mutant (Ljungdahl *et al*., 1992). Negative controls consisted in the NEV-E vector without insert. Uptake experiments were carried out as described by Sauer *et al*. (1983). Cells were grown in SC-D medium supplemented with 0.6 mM histidine, harvested in the logarithmic growth phase, and 20 μl packed cells were resuspended in 1 ml 30 mM phosphate buffer (pH 6.0). For all experiments, 100 μl cell samples (containing 200 μCi U-^{14}C labelled of a L-amino acid and 1 mM of the same unlabeled amino acid) were harvested every 2 min, during 10 min, filtered and washed. Amino acids taken up were measured by scintillation spectrometry. Representative values are given after subtracting background uptake measured with the negative control.

In vitro *culture of cotyledons*

Cotyledons were cultured as described in Weber *et al*. (1996). One cotyledon from a seed was incubated in Petri dishes containing 6 ml medium for cotyledon culture (Millerd *et al*., 1975) supplemented with 150 mM glutamine, with or without 150 mM sucrose, while the other cotyledon from the same seed was incubated without amino acids. Osmotic conditions were maintained by addition of sorbitol. Sealed Petri dishes were incubated in growth chambers for 3 d at 20°C under slight shaking. Seeds were harvested, washed and frozen for future RNA isolation.

Promoter isolation and activity in cell cultures

The *VfAAP1* 5′-upstream region was cloned by using the Genome Walker Kit (Gibco-BRL, Berlin, Germany). Gene specific primers were GS1: 5′-AACCCCCGAGAATGGTGTGAACAGCTTCC-3′, and nested GS2: 5′-CGCCCATGCCAACGACAACACTCCTG-3′. PCR conditions followed manual's instructions, except that the first PCR reaction was performed at 65°C. Primers based on the sequence of the PCR product obtained by genome walking were designed and used for further PCR with undigested leaf DNA as template to confirm its sequence. Constructs for transient expression were made by cloning the *VfAAP1* promoter or the *USP* promoter (Bäumlein *et al*., 1991) upstream of the β-glucuronidase (*GUS*) reporter gene. Protoplasts were isolated from embryogenic suspension cultures of *N. pumbaginifolia* grown to *log*-phase as described in Reidt *et al*. (2000). The purified protoplasts were resuspended in 330 μl $MgCl_2$-mannitol at a density of approximately 10^6 protoplasts ml^{-1}, and transformed with 5 μg of plasmid DNA by heat-shock and PEG 6000 treatment. For assessing the effect of amino acids in the media, each transformed protoplast batch was divided into two plates. One of the plates was supplied with 1 mM cysteine, while the other remained under amino acid starvation. Plates were incubated for 24 or 48 h in the dark. After harvesting the protoplasts, GUS activity was measured by using the GUS-Light™ Kit (Tropix, Bedford, USA). For statistical analyses, the effect of amino acids was calculated as a percentage in relation to the control under amino acid starvation in each transformation.

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