# MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF AMINO ACID AND PEPTIDE TRANSPORTERS IN DEVELOPING SEEDS OF *VICIA FABA* L.

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To Laura, *im memorium*

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| *5'-UR* | 5'-upstream region |
| AAP | amino acid permease |
| ATF | amino acid transporter family |
| DAP | days after pollination |
| DW | dry weight |
| NTR | nitrate transporter |
| OPT | oligopeptide transporter |
| ORF | open reading frame |
| POT | proton-coupled oligopeptide transporter |
| PTR | peptide transporter |
| SUT | sucrose transporter |
| USP | unknown seed protein |

# CHAPTER 1

# INTRODUCTION

Nitrogen is a limiting factor to plant growth, and understanding its metabolism has soon raised interest by early physiologists. Later, with the advent of molecular techniques, efforts focused on the characterization of genes involved in its metabolism and distribution. Nitrogen uptake and assimilation have been relatively well characterized, however, less is known regarding the molecular aspects of transport of organic N compounds in higher plants. Inorganic nitrogen is taken up by the roots and its incorporation into amino acids takes place mainly in roots and mature leaves. In most plants, amino acids represent the major transport form of organic nitrogen, and account for the main component of xylem saps, and the second of phloem saps, behind sucrose only (Pate *et al.*, 1977). From the primary sites of assimilation, amino acids are distributed through the vascular system to sink organs, such as developing leaves, meristem and seeds, where they may be immediately metabolized or used for storage protein synthesis. Storage proteins accumulate transiently in vegetative tissues, as in leaves for instance, or for a long term in sink organs, such as seeds and root tubers, and are degraded and remobilized under certain circumstances during the life cycle of a plant, e.g. leaf senescence and seed germination. Part of the nutrient supply that reaches developing seeds emanates from the proteolysis of transiently accumulated storage proteins, when the transport

of peptides assumes also an important role for the rapid relocation of organic nitrogen (Sopanen *et al*., 1977; Higgins and Payne, 1978; and review by Frommer *et al.*, 1994a).

Legume seeds store high amounts of protein in their cotyledons, which confers them great economical importance. Protein synthesis in these seeds relies on the availability of nitrogenous compounds that are delivered by the phloem conduits and made accessible to the embryo, and changes of available nutrients can fundamentally alter amount and composition of storage proteins (Barneix *et al*., 1992; and review by Thorne, 1985). In addition to the genetical background, the mechanisms controlling protein accumulation respond to a series of environmental and physiological aspects (see below).

## 1.1. SEED DEVELOPMENT IN RELATION TO STORAGE PRODUCT ACCUMULATION IN *VICIA FABA*

Developing legume seeds are typical sink organs in which the anatomical and physiological aspects of metabolite assimilation have been extensively studied (Offler and Patrick, 1993; Patrick and Offler, 1995; Wang *et al*., 1995). *V. faba* seeds accumulate storage proteins as the main energy reserve and provide, therefore, a suitable system for analyzing the relationship between transport of nitrogen compounds and storage protein accumulation. Moreover, its large seeds enable combined efforts at physiological and molecular levels. 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 an embryo undergoing intensive cell divisions (stage I-IV), followed by a phase of cell differentiation and deposition of storage compounds in the cotyledons (stages V-VII). Accumulation of starch and proteins starts in the inner adaxial region of the cotyledon at mid developmental stage (stage V) and gradually expands, reaching its whole extension at late embryogenesis (stage VII; Figure 1). Storage proteins are deposited in vacuoles frequently referred as protein bodies and serve as source of carbon, nitrogen and sulfur for the growing seedling upon germination. Globulins, which form the bulk of storage proteins, are proportionally rich in nitrogen, but poor in sulfur-containing amino acids. The second most abundant class of proteins found in the seeds are the albumins, which frequently contain elevated amounts of sulfur compared to globulins. In many legumes, including *V. faba*, vicilins and legumins, which are the two major classes of globulins, consist of oligomers formed by non-identical subunits (reviewed by Nielsen *et al*., 1997a).

Availability and partitioning of sugars and nitrogen compounds confer regulatory control on storage activities, and their levels are developmentally controlled during seed development (reviewed by Weber *et al.*, 1997a; 1998a; Wobus and Weber, 1999). Evidence shows that sucrose works as a signaling molecule for the beginning of the storage (Wang and Hedley, 1993; Weber *et al.*, 1998a, b), and that a high sucrose to hexoses ratio in the embryo is responsible for triggering differentiation and storage processes (Weber *et al.*, 1995). Expression of vicilin and legumin genes is tightly coupled to the cessation of mitosis, cell expansion and endopolyploidization (Borisjuk *et al.*, 1995; Wobus *et al.*, 1995).

The amount of protein accumulated is regulated at different levels, including the availability of assimilates and genetic background (reviewed by Weber *et al.*, 1997a; 1998a; Wobus and Weber, 1999). Studies using soybean cultured embryos showed that cotyledon N accumulation and concentration increased in a direct proportion to the N concentration in the media, however, differences in the degree of accumulation were dependent on the genotype used (Hayati *et al.*, 1996). Similarly, significant correlation has been found between seed protein and free amino acid concentration in the cotyledons of *V. faba* (Barratt, 1982). Using two genotypes that differed in their protein contents, Golombek *et al.* (2001) have shown that despite no major differences in the amount of amino acids delivered by the seed coats, the genotype with higher protein had higher capability to take up the amino acids. Growing evidence suggests that the synthesis of storage proteins is regulated by the embryo itself rather than by the maternal plant (Golombek *et al.*, 2001).

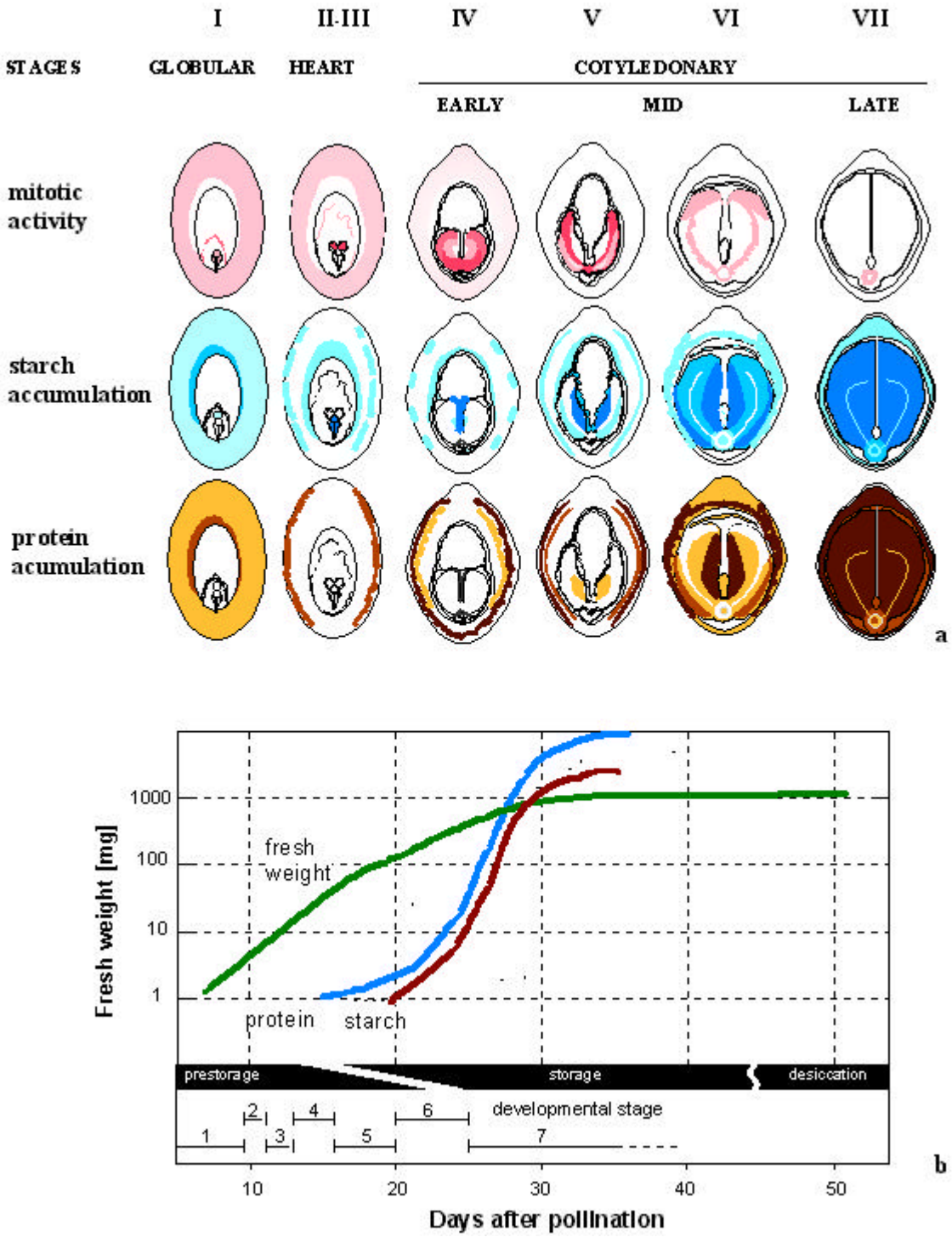



**Figure 1. *V. faba* seed development.**

*According to morphological and physiological changes seed development in* V. faba *can be divided in 7 stages (**a**). Mitotic activity is followed by differentiation creating a developmental gradient within the seed, to which the accumulation of starch and proteins is directly coupled. The storage phase begins in stage VI, with cessation of fresh weight gain (**b**). Reproduced from Borisjuk et al. (1995) and Weber et al. (1997a).*

## 1.2. PLANT AMINO ACID AND PEPTIDE TRANSPORTERS

Shortly after a proton-coupled uptake system for amino acids had been proposed by Bush (1993), the first plant amino acid transporter AtAAP1/NAT2 was isolated from *Arabidopsis thaliana* through functional complementation in yeast by two independent groups (Frommer *et al.*, 1993; Hsu *et al.*, 1993). Afterwards, several other members of the amino acid transporter super-family (ATF1) have been identified. Based on sequence similarities and uptake properties these members have been classified into subfamilies (for review see Rentsch *et al.*, 1998). Certainly, the amino acid permease (AAP) subfamily is the best represented, from which at least six isoforms are present in *Arabidopsis*, including both high and low affinity transporters (Fischer *et al.*, 1995; Kwart *et al.*, 1993; Rentsch *et al.*, 1996). Several members of this subfamily were shown to function as proton symporters (Boorer *et al.*, 1996; Borrer and Fischer, 1997), and they can be further sub-divided in regard to the transported substrate into general or neutral/acidic transporter systems (Fischer *et al.*, 1995). The *Arabidopsis* AAPs show overlapping uptake properties, however, most of them are characterized by particular temporal and spatial mRNA expression patterns, denoting a refined network controlling gene regulation (see Fischer *et al.*, 1998).

In addition to amino acid transporters, proton-coupled oligopeptide (POT) transporters have also been identified in bacteria, yeast, animals and plants (reviewed by Fei *et al.*, 1998). In plants, biochemical analyses showed that active transport of peptides is frequently related to periods of rapid proteolysis, such as seed germination and leaf senescence, when efficiency of transport may be increased by direct uptake of peptides (Sopanen *et al.*, 1977; Higgins and Payne, 1978). Expression in a yeast mutant of two peptide transporters, one isolated from *A. thaliana* (Frommer *et al.*, 1994b; Rentsch *et al.*, 1995; Song *et al.*, 1996) and the other from barley (West *et al.*, 1998), showed that these transporters have low selectivity, mediating the transport of several di- and tripeptides, and as the amino acid permeases, they include both high and low affinity systems.

Further reading on different molecular and physiological aspects of plant amino acid and peptide transport can be found in one of the several reviews that have been published during the last few years (Fischer *et al.*, 1998; Rentsch *et al.*, 1998; Ortiz-Lopez *et al.*, 2000; Delrot *et al.*, 2000).

## 1.3. ACTIVE POSTPHLOEM TRANSPORT OF ASSIMILATES INTO THE SEEDS

Most reports on AAPs concentrate on the vegetative parts of the plants and source organs. Seeds, on the other hand, are sink organs and thus provide a distinct physiological environment. Assimilates are transported through the phloem sieve elements and reach the seeds through the funiculus. Phloem unloading and postphloem transport through the seed coat occur symplasmically (Offler and Patrick, 1993; Patrick and Offler, 1995). The embryo, however, is symplasmically isolated from maternal tissues, and assimilates need to cross an apoplastic space before reaching it. Unloading from the seed coat into the apoplast happens at the thin-walled parenchyma (Wang *et al.*, 1995), a tissue which covers the whole inner surface of the coat and is morphologically specialized in metabolite transfer (Offler and Patrick, 1993). The mechanisms involved in unloading processes are still controversial; a putative sucrose $H^+$-antiporter localized in the thin-walled parenchyma of *V. faba* seed coats has been proposed (Patrick and Offler, 1995), whereas in pea unloading has been shown to be entirely passive (De Jong *et al.*, 1996). Assimilates are then taken up by the embryo from the surrounding apoplastic space. Saturable transport systems have been characterized for sugar (McDonald *et al.*, 1996) and amino acids uptake (Lanfermeijer *et al.*, 1990) by the embryos, indicating the presence of energy dependent carriers. During early seed development in pea, a nonsaturable amino acid transport system is of primary importance, and later, when ca. 2/3 of the storage proteins have been deposited, a saturable system emerges (Lanfermeijer *et al.*, 1990). In soybeans, about half of the glutamine uptake into cotyledons was shown to be energy dependent (VerNooy *et al.*, 1986).

*AtAAP1* and *AtAAP2* from *Arabidopsis* were the first *AAP*s to be shown to be expressed in seeds, indicating a potential role in supplying the seeds with organic nitrogen during development and germination (Kwart *et al.*, 1993; Hirner *et al.*, 1998) (Figure 2). Legume cotyledons are covered by a layer of cells that is differentiated into an epidermis, which plays a primary role in the active uptake of nutrients by the embryo (Gunning and Pate, 1974; McDonald *et al.*, 1996; Offler *et al.*, 1989); this is the exclusive site of expression of hexose and sucrose (*VfSUT1*) $H^+$ co-transporters within *V. faba* cotyledons (Weber *et al.*, 1997b). 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).

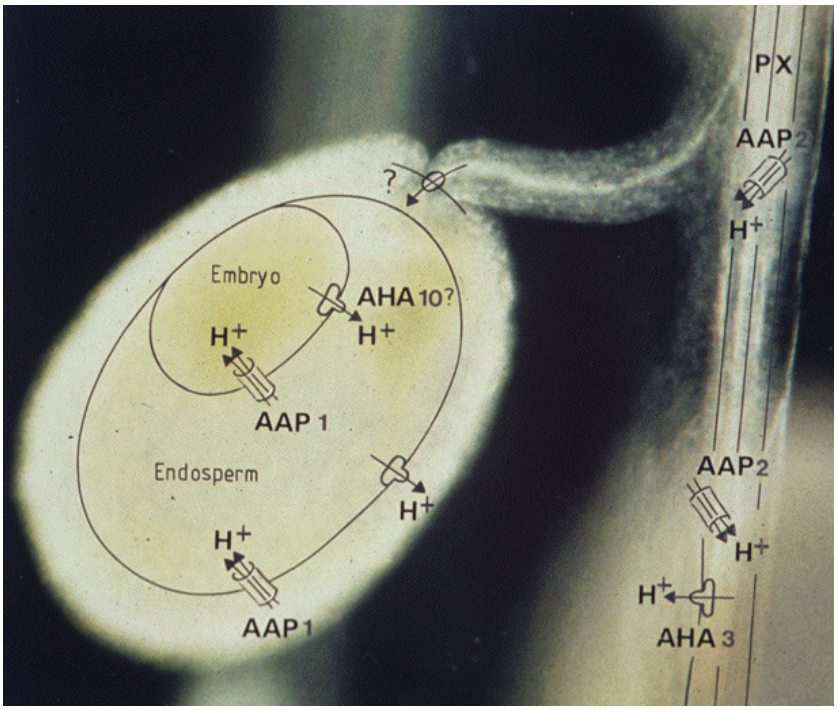



**Figure 2. Model for amino acid import into seeds.**
AtAAP2 *is expressed in the vascular system of stems and siliques where it might function in (i) phloem loading, (ii) retrieval of amino acids and (iii) in xylem-to-phloem transfer.* AtAAP1 *might be responsible for active uptake of amino acids into the endosperm and the embryo (taken from Hirner* et al*., 1998).*

Peptides also seem to be actively transported in seeds. Biochemical studies have shown that a peptide transport system is present during germination of barley grains (Higgins and Payne, 1978). Among the peptide transporters isolated from plants, *AtOPT1* from arabidopsis was found to be expressed in embryos, implicating a role in the nourishment of developing seeds (Rentsch *et al*., 1995). Moreover, transgenic *Arabidopsis* plants with reduced levels of *AtOPT1* mRNA had fewer and larger seeds per silique (Song *et al*., 1997). In barley, a low affinity peptide transporter was expressed in the scutellum of germinating seeds, and seems to be responsible for rapid absorption of peptides originated from the hydrolysis of endosperm storage proteins (West *et al*., 1998). No peptide transporter has so far been identified in legume seeds.

In addition to the transport of photoassimilates into the embryo, the thin-walled parenchyma of *V. faba* seed coats exhibits numerous wall ingrowth projections and is responsible for the exchange of assimilates between the coat cells and the apoplast (Grusak and Minchin, 1988; Offler and Patrick, 1993; Offler *et al.*, 1989). Since $H^+$ co-transporters do not seem to mediate efflux, expression of the *VfSUT1* in thin-wall parenchyma cells offers evidence for active retrieval of assimilates from the apoplast back to the seed coat (Weber *et al.*, 1997b). Possible reasons why the seed coat cells would rely on active transport to take up sugars from the apoplast are of speculative nature, but it could be to supply the coat cells with nutrients for their own metabolism and/or to keep an osmotic gradient (De Jong *et al.*, 1996). Moreover, using detached seed coats from pea, De Jong and Wolswinkel (1995) and De Jong *et al.* (1997) have proposed that the seed coat parenchyma cells do not retrieve amino acids from the apoplast, however, in a recent work, these authors provided evidence for the presence of $H^+$ symporters for amino acids in seed coats (De Jong and Borstlap, 2000a).

## 1.4. OBJECTIVE OF THE PRESENT WORK

Little is known about the function and mode of regulation of amino acid and peptide transporters during seed development in relation to the deposition of storage proteins. Similarly, there is no or little evidence on whether their gene products have a rate-limiting role on the uptake of assimilates delivered by the phloem and could, therefore, influence assimilate partitioning and storage protein accumulation. As an attempt to address these questions, this work aimed to identify and isolate cDNAs clones of genes involved in the active transport of amino acids and peptides in developing *V. faba* seeds.

Characterization of these transporters was carried out at several different levels, including uptake properties, expression analyses, regulation by metabolites and plant transformation, and provided new insights on the function and regulation of these genes. When inserted into a bigger context, these data allowed a comparison with what had been known for the transport of sugars and for the importance of assimilate uptake on the deposition of storage compounds.

Briefly, three different *AAP* and two *OPT* isoforms were isolated from *V. faba* seeds. In developing cotyledons, *VfAAP1* is expressed in the storage parenchyma and *VfOPT1*, in the transfer cells. *VfAAP1* and a storage protein gene are regulated by assimilates in an antagonistic manner, while *VfAAP1* expression was shown to be regulated at the transcriptional level. Moreover, transgenic plants carrying the *VfAAP1* cDNA have altered contents of nitrogen, sulfur and starch. Together these data substantiate the relevance of amino acid and peptide transport for storage protein accumulation during development of legume seeds.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. PLANT MATERIAL

*Vicia faba* L. var. *minor* cv. 'Fribo' and *Vicia narbonensis* plants, originated from the IPK Genebank (Gatersleben, Germany), were grown in soil in growth chambers supplied with artificial light (16 h light/8 h dark regime) at 20-25°C. Samples were collected 4 h after beginning of the light period, and seeds were sorted on basis of the number of days after pollination (DAP) .

## 2.2. MOLECULAR CLONING

Degenerated primers were designed based on conserved regions of known *AAP*s (forward: 5'-CAY ATH ACI GCI GTI ATH GG-3'; reverse: 5'-ATY TCI ACI GGR AAR TAI ACI GT-3') and *OPT*s (forward: 5'-TTY GGW GCY GAY CAR TTT G-3' and 5'-CYM TGY ACD GTK ACD CAR GTN G; reverse: 5'-CTG GAG ATT GRT CRT AGA ARA AC-3'). PCR with the degenerated primers was carried out using one cDNA library of developing cotyledons (Heim *et al*., 1993) and another of developing seed coats as templates. The

fragments obtained were cloned into pUC18 and sequenced. Positively identified clones were labeled with [α-$^{32}$P]dCTP (Random Primer Labeling Kit, Amersham Pharmacia Biotech, Buckinghamshire, UK), and used as probes for screening the cDNA libraries in order to isolate the respective full length cDNAs. Libraries were screened as described in Buchner *et al.* (1996) at a temperature of 60°C for hybridization and washing.

The 5'-upstream region (*5'-UR*) of *VfAAP1* was cloned by using the Genome Walker Kit (GIBCO BRL, Life Technologies, Paisley, UK). Gene specific primers were GS1: 5'-AAC CCC CGA GAA TGG TGT GAA CAG CTT CC-3', and nested GS2: 5'-CGC CCA TGC CAA CGA CAA CAC TCC TG-3'. PCR conditions followed manual's instructions, except that the first PCR was performed at 65°C with 10 extra cycles. A PCR fragment was cloned into pUC18, and confirmed by sequencing to be the *VFAAP1 5'-UR*. Three combinations of additional forward and reverse primers were designed based on the sequence of the *VfAAP1 5'-UR* and further PCRs using total leaf DNA were done for confirmation.

## 2.3. AMINO ACID UPTAKE AND KINETIC CHARACTERIZATION OF TRANSPORTERS IN YEAST MUTANTS

Constructs were made by cloning *VfAAP1* and *VfAAP3* in sense orientation into the NEV-E vector (Sauer and Stolz, 1994) for transformation of the yeast mutant *plas*23-4B (*MAT*α, *shr3-23*, *ura3-52*, *his4*Δ*29*; Ljungdahl *et al.*, 1992), and by cloning *VfOPT1* into pDR195 for transformation of *LR2* (*MAT*α, *hip1-614*, *his4-401*, *can1*, *ino1*, *ura3-52*, *ptr2*Δ::hisG; Rentsch *et al*., 1995). Negative controls consisted of vectors without inserts. Uptake by *plas*23-4B transformed with either *VfAAP1* or *VfAAP3* was performed as described by Sauer *et al*. (1983). Briefly, cells were grown in SC medium + 0.6 mM histidine, and harvested in the logarithmic growth phase. Twenty 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 labeled of a L-amino acid and variable concentrations 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 were given after subtracting background uptake measured with the negative control. All transport experiments were

performed at least twice. Complementation of the *LR2* mutant transformed with *VfOPT1* was performed by plating cells in SC medium supplemented with 10 mM His-Ala. Positive and negative controls consisted, respectively, of cells carrying the *AtOPT1* (Rentsch *et al*., 1995) and *AtAAP2* (Kwart *et al*., 1998) cDNAs of *A. thaliana*.

## 2.4. NORTHERN AND SOUTHERN HYBRIDIZATIONS

Total RNA isolation was performed as described by Heim *et al*. (1993), and separated in 1.2% agarose gels containing 15% formaldehyde, and blotted overnight onto Hybond-$N^+$ nylon membranes (Amersham Pharmacia Biotech). Various fragments obtained by enzymatic digestions, which excluded the polyA tail from the cDNAs, were used as probes after labeling with [$\alpha$-$^{32}$P]dCTP. Hybridizations were performed at 65°C, and washing at high stringency according to Church and Gilbert (1984). Signals on filters were quantified using a Fuji-BAS phosphoimager (Fuji Photo Film C., Tokyo, Japan).

For Southern hybridizations, ten µg DNA were digested with *Hind*III, *Pvu*II, *Pst*I, *Sty*I or *Xho*I, and separated in a 1% agarose gel, which was blotted overnight onto a Hybond-$N^+$ nylon membrane. Complete cDNAs were used as probes after labeling with [$\alpha$-$^{32}$P]dCTP. Hybridizations were performed at 65°C, and membranes were washed twice with 2xSSPE/0.1% (w/v) SDS, twice with 1xSSPE/0.1% (w/v) SDS, and once with 0.5xSSPE/0.1% (w/v) SDS at 65°C for 15 min each.

## 2.5. SEMI-QUANTITATIVE RT-PCR

For quantification of *VfOPT2* transcripts, RT-PCR was performed with Oligo $(dT)_{23}$ primer (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) using 1 µg total RNA extracted as in Heim *et al*. (1993) as template. RT-PCR was preceded by a 'hot start' (5 min 70°C) and followed by a standard PCR with a combination of primers specific for the *VfOPT2* cDNA (forward: 5'-AGT GGG TTG GGC TGT AGG GTA TGG-3' and reverse: 5'-ATG CTG GAG GGA TAT CAA AAT GTG -3').

## 2.6. *IN SITU* HYBRIDIZATION

Sample fixation and slide preparation followed the protocol described in Weber *et al.* (1995) with the only exception that polyA coated slides (Sigma-Aldrich Chemie GmbH) were used. Fragments obtained by enzymatic digestions for excluding the polyA tails of the *VfAAP1*, *VfOPT1*, *VfSUT1* and *vicilin* cDNAs were randomly labeled with [$\alpha$-$^{33}$P]dCTP and used as probes. Slides were hybridized overnight at 42°C, and washed with 50% formamide in 0.1x SSC. After drying, slides were coated with photoemulsion (LM-1, Amersham Pharmacia), exposed for 3-10 days at 4°C, and developed with D-19 developer (Eastman Kodak, Rochester, USA). If necessary, slides were counterstained with 0.05% toluidine blue.

## 2.7. *IN VITRO* CULTURE OF DEVELOPING COTYLEDONS

Cotyledons from freshly harvested seeds (stage V, see Figure 1) were cultured as described in Weber *et al*. (1996). One cotyledon from a seed was incubated in a Petri dish containing 6 ml medium for cotyledon culture (Millerd *et al.*, 1975) supplemented with 150 mM glutamine, either with or without 150 mM sucrose, while the other cotyledon from the same seed was incubated without amino acids (Figure 3). 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 and northern analyses.

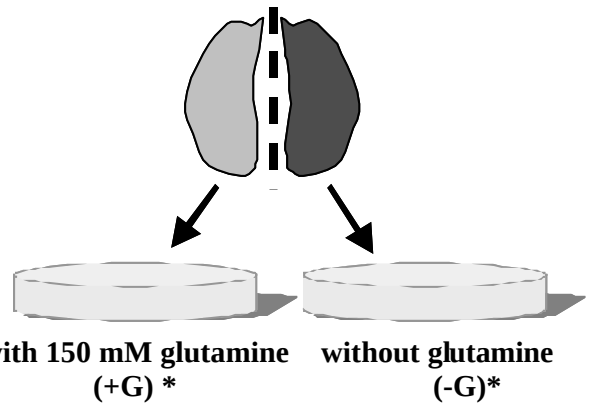



**Figure 3. *In vitro* culture of cotyledons.**

*One cotyledon of a seed was cultured in the presence of 150 mM glutamine (+G) and the other was cultured under amino acid starvation (-G). Experiments were performed either in the presence (+S) or absence (-S) of 150 mM sucrose. Total RNA was extracted from cotyledons after 3 d incubation.*

## 2.8. TRANSIENT EXPRESSION IN *NICOTIANA PLUMBAGINIFOLIA* PROTOPLASTS

To amplify the two *VfAAP1 5'-UR*s immediately before the predicted translation start, i.e. the first ATG of the *VfAAP1* open reading frame (ORF), PCR was performed with forward (5'-GGA TTG TTA AG*A* AG*C* TTT ATG GAG-3') and reverse (5'-CAT CAA ATT CTT CT*G* CAG GGT GT-3') primers that introduced, respectively, *Hind*III and *Pst*I restriction sites in the original sequences of the *VfAAP1 5'-UR*s (point mutations are indicated in italic, and the translation start is underlined). Resultant PCR products (1.5 Kb long) were re-cloned into pUC18, and fragments resulting from *Hind*III and *Pst*I double digestion were ligated in sense orientation upstream of the β-glucuronidase (*GUS*) reporter gene. Controls consisted of the vector carrying the *GUS* gene lacking a promoter and a vector carrying the *USP* promoter (Bäumlein *et al.*, 1991) upstream of the *GUS* gene. Protoplasts were isolated from embryogenic suspension cultures of *N. pumbaginifolia* grown to *log*-phase as described in Reidt *et al.* (2000). Purified protoplasts were resuspended in 330 µl $MgCl_2$-mannitol at a density of ca. $10^6$ protoplasts/ml, and 10 µg of plasmid DNA were used to transform the protoplasts by heat-shock and PEG 6000 treatment. 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 percentage in relation to the control under amino acid starvation in each transformation.

## 2.9. STABLE PLANT TRANSFORMATION

The full length cDNA from *VfAAP1* was cloned under the control of the *legumin B4* (*LegB4*) promoter (Bäumlein *et al.*, 1992) into the binary vector pGA482 (An *et al.*, 1987) for transformation of *V. narbonensis* plants. Transformation was done by *Agrobacterium*-mediated gene transfer according to Pickardt *et al.* (1991). The presence of the transgene was confirmed by PCR using leaf DNA as template with a forward primer specific to the *LegB4* promoter in combination with a reverse primer specific to the *VfAAP1* cDNA.

## 2.10. EXTRACTION AND DETERMINATION OF PROTEINS, STARCH, SUGARS, TOTAL NITROGEN, SULFUR AND CARBON

Seeds from transgenic plants and wild types were freeze-dried until a constant weight and the cotyledons were pulverized in a ball-mill. For protein determination, 50 mg of the powder were extracted in 0.5 M sodium phosphate buffer pH 7, 0.5 M NaCl, for 0.5 h following the Bradford protein assay with bovine serum albumine as a standard. Soluble carbohydrates were extracted with 80% ethanol at 80°C for 1 h. After centrifugation, the supernatant was evaporated and dissolved in sterile water for enzymatic carbohydrate determination (Boeringer, Mannheim, Germany). The remaining insoluble material was used for starch determination after solubilizing in 1 N KOH and hydrolysing with amyloglucosidase (Heim *et al*., 1993). The concentration of total nitrogen, sulfur and carbon was measured by a CHN analyzer (CHN-O-Rapid, Foss-Heraeus, Germany).

# CHAPTER 3

# RESULTS

## 3.1. CLONING THREE AMINO ACID PERMEASES (*AAPS*) AND TWO PEPTIDE TRANSPORTERS (*OPTS*) FROM *VICIA FABA* SEEDS

### 3.1.1. *Primary and secondary structure of the AAPs*

PCR with degenerated primers yielded three distinct DNA fragments belonging to homologues of the AAP subfamily of amino acid transporters (BLASTX search; Altschul *et al*., 1997), which were used as probes for screening two cDNA libraries of developing *V. faba* seeds. Five clones from an identical cDNA were isolated from the library of developing cotyledons. A full length clone, named *VfAAP1*, is composed of 1577 bp and encodes a predicted protein of 53,0 kD and 475 amino acid residues (Figure 4a). No positive clone was obtained by screening this library with the other *AAP* fragments as probes. Twenty clones representing another cDNA were isolated from a seed coat specific library. One of the clones, denominated *VfAAP3* [(1)] (1878 bp), contains a complete open reading frame and encodes a

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

predicted protein sequence made of 486 amino acid residues (53,1 kD; Figure 4b). Since the third *AAP* fragment is 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. *VfAAP4* encodes a predicted protein sequence of 53,4 kD and 481 amino acid residues (Figure 4c).

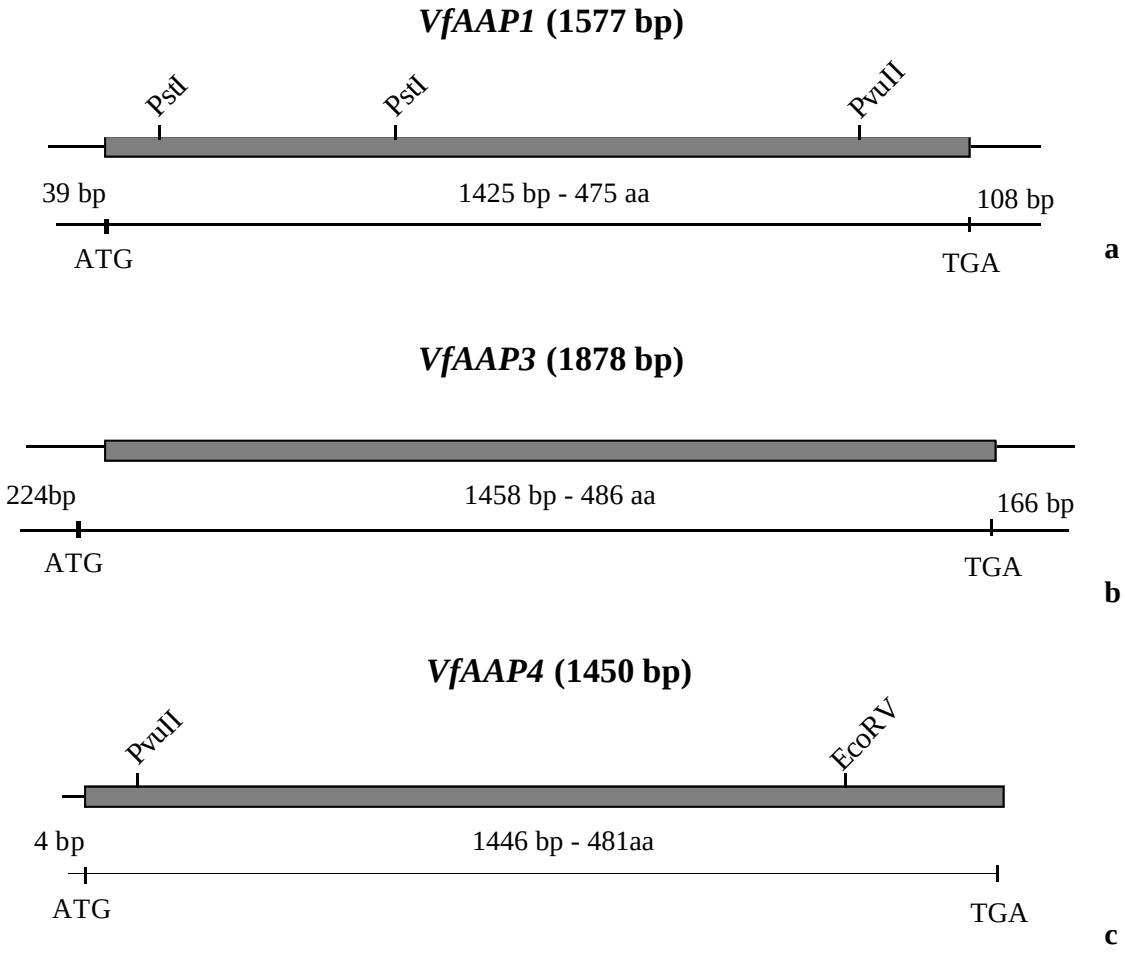



**Figure 4. Schematic representation of the *AAP* clones isolated from cDNA libraries of developing *Vicia faba* seeds.**

VfAAP1 ***(a)***, VfAAP3 ***(b)*** *and* VfAAP4 ***(c)*** *are full length cDNAs, in which the beginning and the end of the predicted open reading frame (ORF) is indicated by 'ATG' and 'TGA', respectively. The length of the ORFs and untranslated regions are indicated in base pairs (bp) and of the predicted protein sequences in amino acid residues (aa). Restriction sites of most common enzymes are shown.*

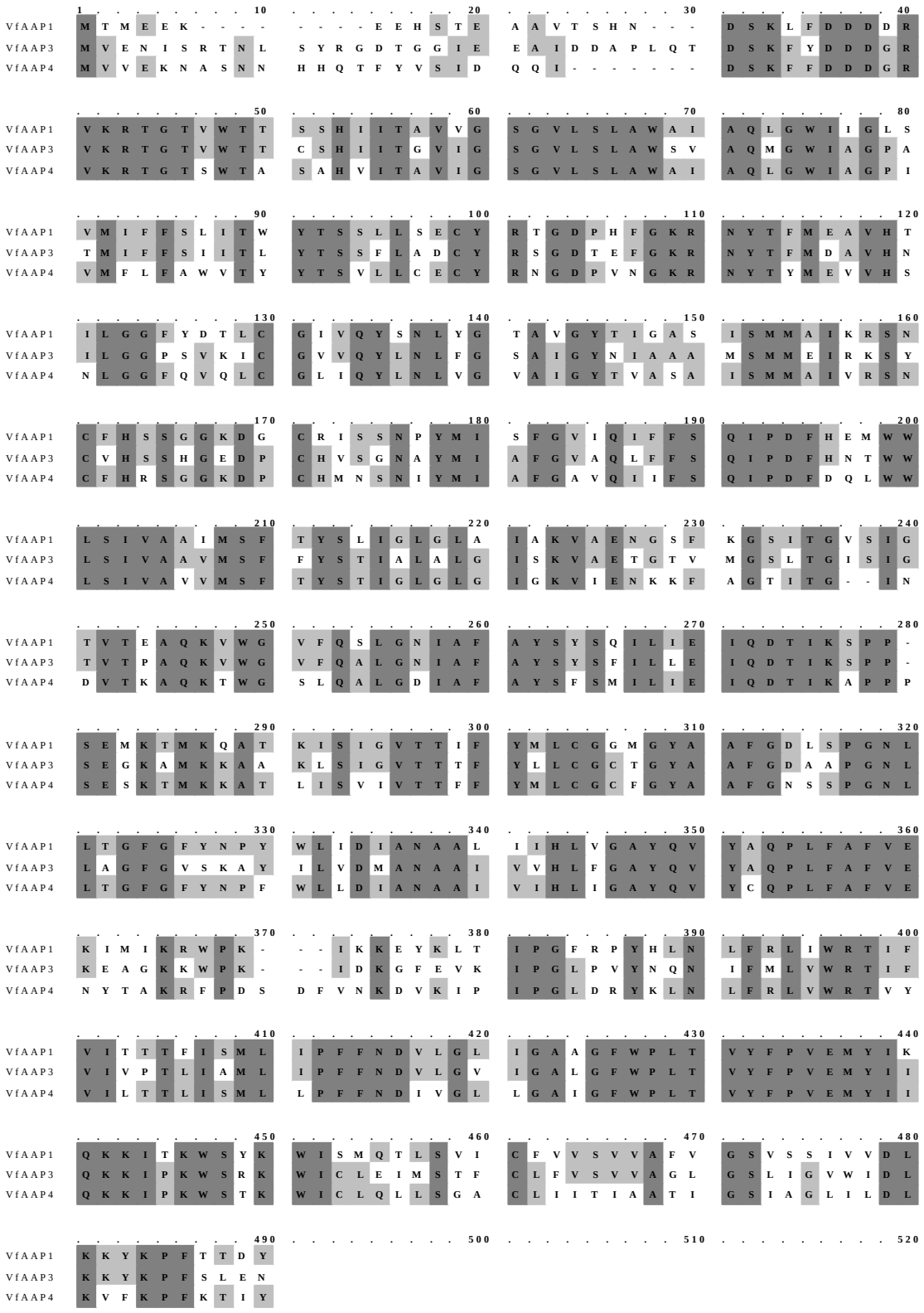



**Figure 5 . Alignment of VfAAP1, VfAAP3 and VfAAP4 predicted protein sequences.**

*Conserved regions are spread over the whole extension of these proteins, except for the first 30 amino acid residues and the region at pos. 370-372, in which VfAAP4 has three additional amino acid residues (Clustal method; Omiga 2.0, Oxford Molecular, England).*

VfAAP1, VfAAP3 and VfAAP4 predicted proteins share up to 66% of identity among themselves. An alignment showed that their conserved regions are spread through their whole extension, with exception of the first 30 amino acid residues at the N-terminus and the region corresponding to pos. 370-372 of the alignment, in which VfAAP4 has three additional residues in comparison to VfAAP1 and VfAAP3 (Figure 5). VfAAP1 is 91.2 % identical to a putative amino acid permease (PsAAP2) isolated from pea. VfAAP3 is 67.7% and 61.1% similar to PsAAP2 and to AtAAP3 from *A. thaliana*, respectively, while VfAAP4 shares 91.7% identity with PsAAP1 from pea (Table 1). Similarly, in a phylogenic tree VfAAP1 and VfAAP4 cluster together with PsAAP2 and PsAAP1, respectively. Moreover, the legume AAPs are more closely related to the *A. thaliana* isoforms AtAAP2-5 then to AtAAP1 and AtAAP6 (Figure 6).

VfAAP1, VfAAP3 and VfAAP4 are predicted to be highly hydrophobic, which is a common feature of membrane integrated proteins. The hydrophobic profiles of these proteins overlap those of the *A. thaliana* AAPs (Figure 7a), therefore the 11 transmembrane domain (TMD) model proposed by Chang and Bush (1997) could also be applied to the *V. faba* AAPs. A topology model was generated for VfAAP1 as shown in Figure 7b. The accuracy of this method was checked by running a topology prediction for the AtAAP1 protein as well, which generated a model similar to that based on experimental evidence provided by Chang and Bush (1997). Three potential N-glycosylation sites are present in each of the VfAAPs (data not shown). However, according to the 11-TMD model, only the potential N-glycosylation site at pos. 216 of VfAAP1 is located in a extracellular loop (Figure 7b), and would therefore be accessible at the ER lumen for glycosylation. N-terminal signal peptides were predicted in VfAAP1 and VfAAP4, with most probable cleavage sites occurring between the amino acid residues at positions 13-14 (TEA-AV) and 10-11 (SNN-HH), respectively, suggesting that these are secretion proteins. No signal peptide was predicted for VfAAP3 (60 bp truncated N-terminus; SignalP 2.0; Nielsen *et al*., 1997b).

**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[5] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 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]Fisher *et al*., 1995; [6]Rentsch *et al*., 1996).

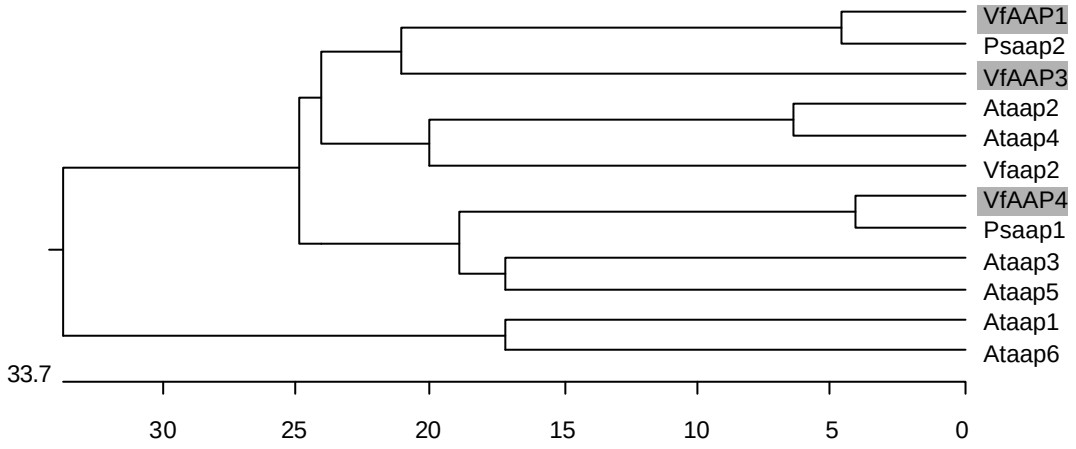



**Figure 6. AAP phylogenic tree**.

*Based on the alignment between the predicted protein sequences of VfAAP1, VfAAP3, VfAAP4 and other amino acid permeases by Clustal method (MegAlign, DNAStar).*

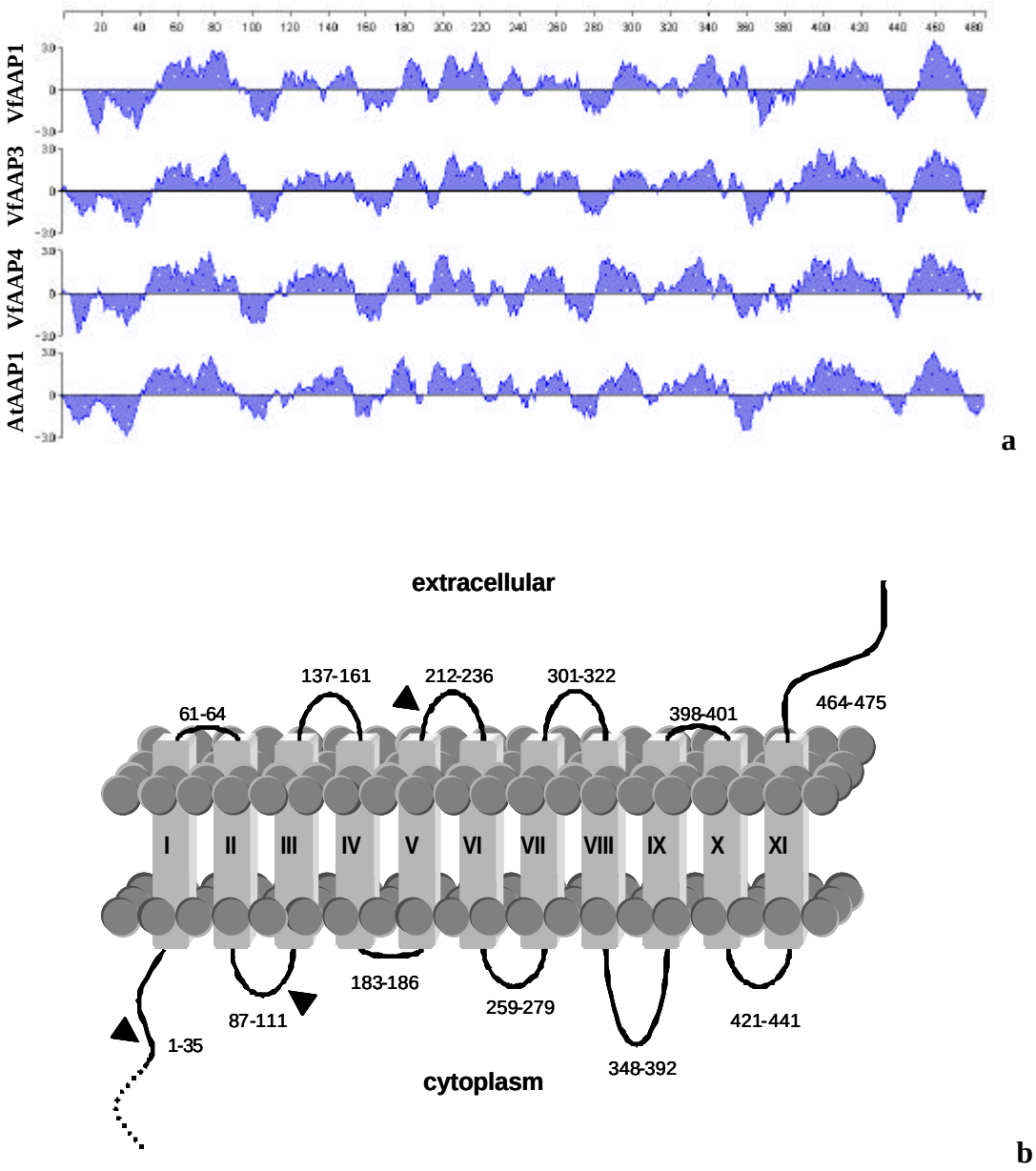



**Figure 7. AAP secondary structure predictions.**

***a.*** *Prediction of hydrophobic regions of VfAAP1, VfAAP3, VfAAP4 and AtAAP1. Positive values indicate hydrophobic regions (window size of 9; Kyte and Doolittle, 1982); and* ***b.*** *topology model for VfAAP1 based on the method of Tusnády and Simon (1998). Relative positions of first and last amino acid residue of each loop are indicated. A predicted N-terminal signal peptide (pos. 1-13) is shown by a dotted line. Arrowheads indicate putative N-glycosylation sites.*

### 3.1.2. *Primary and secondary structure of the OPTs*

PCR with degenerated primers using the cotyledon cDNA library as template yielded two distinct DNA fragments homologous to peptide transporters of the POT superfamily (BLASTX Search), which were used as probes for screening the *V. faba* seed-specific cDNA libraries. From the cotyledon library, ten clones representing the same cDNA were isolated. A full length cDNA was called *VfOPT1* (2028 bp), which encodes a predicted protein of 64,5 kD and with 584 amino acid residues (Figure 8a). Neither library screening nor further PCR succeeded on isolating the full length cDNA of the second peptide transporter (*VfOPT2*, 1041 bp; Figure 8b).

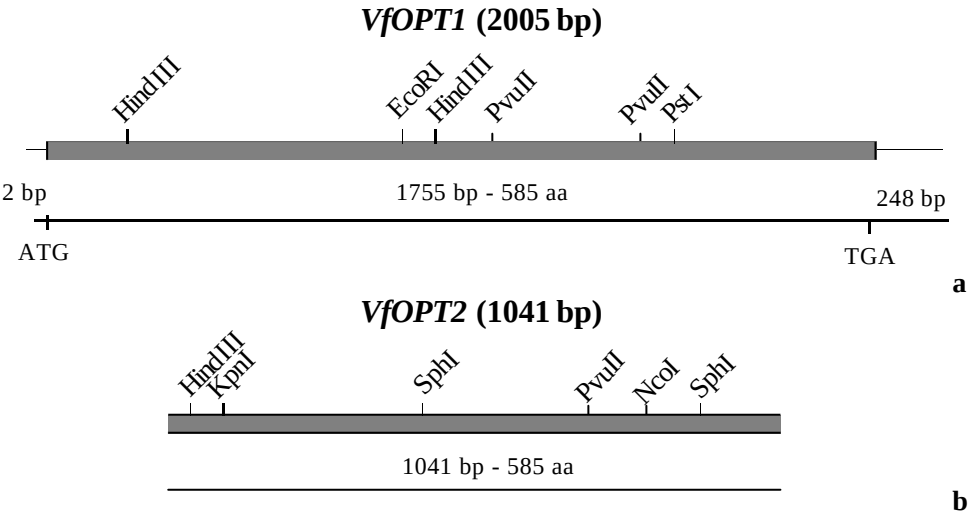



**Figure 8. Schematic representation of the *OPT*s cloned from developing seeds of *V. faba*.**

VfOPT1 ***(a)*** *is a full length cDNA, in which the beginning and the end of the predicted ORF is indicated by 'ATG' and 'TGA', respectively. The length of the ORF and untranslated regions are indicated in base pairs (bp) and of the predicted protein sequence in amino acid residues (aa).* VfOPT2 ***(b)*** *consists of a PCR fragment, whose ORF is incomplete. Restriction sites of most common enzymes are shown.*

Database searches revealed that VfOPT1 is closely related to putative peptide transporters of almond (80.3% identity; accession AF213936) and tomato (79.8%; accession AF016713). On

the other hand, VfOPT2 shares only 54.7% identity with a putative soybean nitrate/peptide transporter (GmNTR1-3; Yokoyama *et al.*, 2001) and *ca*. 40% with other peptide transporters (Table 2). Similarly, in a phylogenic tree VfOPT1 clusters together with the peptide transporters from almond and tomato, whereas VfOPT2 is more distantly related to other OPTs, and forms another cluster along with GmNTR1-3 (Figure 9). An alignment between the predicted OPT proteins revealed a region of hyper-variability including apparent insertions and/or deletions, in which VfOPT2 has an additional stretch of 26 amino acid residues (Figure 10). The predicted proteins of VfOPT1 and VfOPT2 are highly hydrophobic, suggesting that they are membrane integral proteins. Computer predictions failed to establish a reliable topology model since a range of 9-12 transmembrane domains has been predicted for VfOPT1 depending on the software used (data not shown). No signal peptide was predicted in the first 60 bp of the VfOPT1 amino acid sequence (SignalP 2.0).

**Table 2.** Percentage of similarity between the predicted protein sequences from VfOPT1, VfOPT2 and other peptide transporters*

| | VfOPT2 | AtOPT1[1] | HvOPT1[2] | LeOPT1[3] | PdOPT[4] | GmNTR1-3[5] |
|---|---|---|---|---|---|---|
| VfOPT1 | 42.2 | 60.2 | 56.3 | 79.8 | 80.3 | 40.9 |
| VfOPT2 | - | 43.5 | 42.8 | 40.3 | 41.6 | 54.7 |

*Clustal method (DNAStar); [1]*A. thaliana* (Rentsch *et al*., 1995) ; [2]barley (West *et al*., 1998); [3]tomato (putative OPT, AF016713); [4]almond (putative OPT, AF213936); [5]soybean (putative nitrate/peptide transporter; Yokoyama *et al*., 2001).

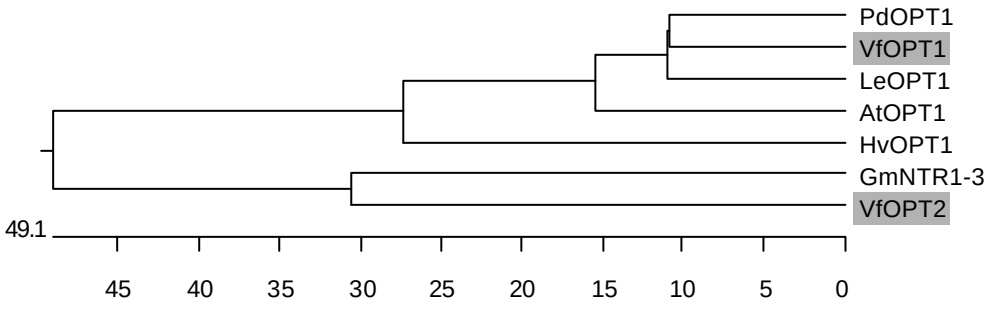
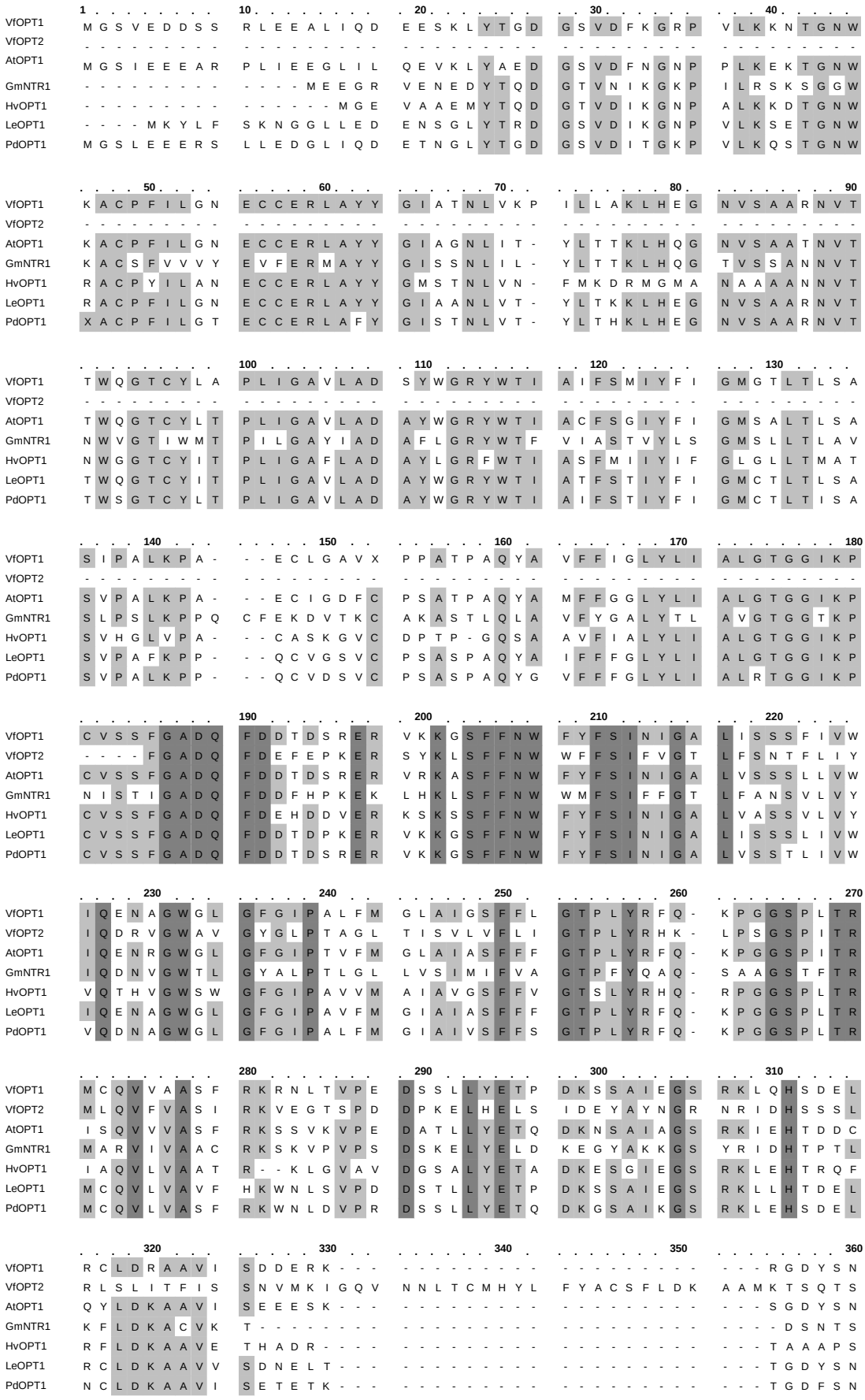



**Figure 9. OPT phylogenic tree.**

*Based on the alignment between the predicted proteins sequences of VfOPT1, VfOPT2 and other peptide transporters by Clustal method (MegAlign, DNAStar).*


VfOPT1
VfOPT2
AtOPT1
GmNTR1
HvOPT1
LeOPT1
PdOPT1

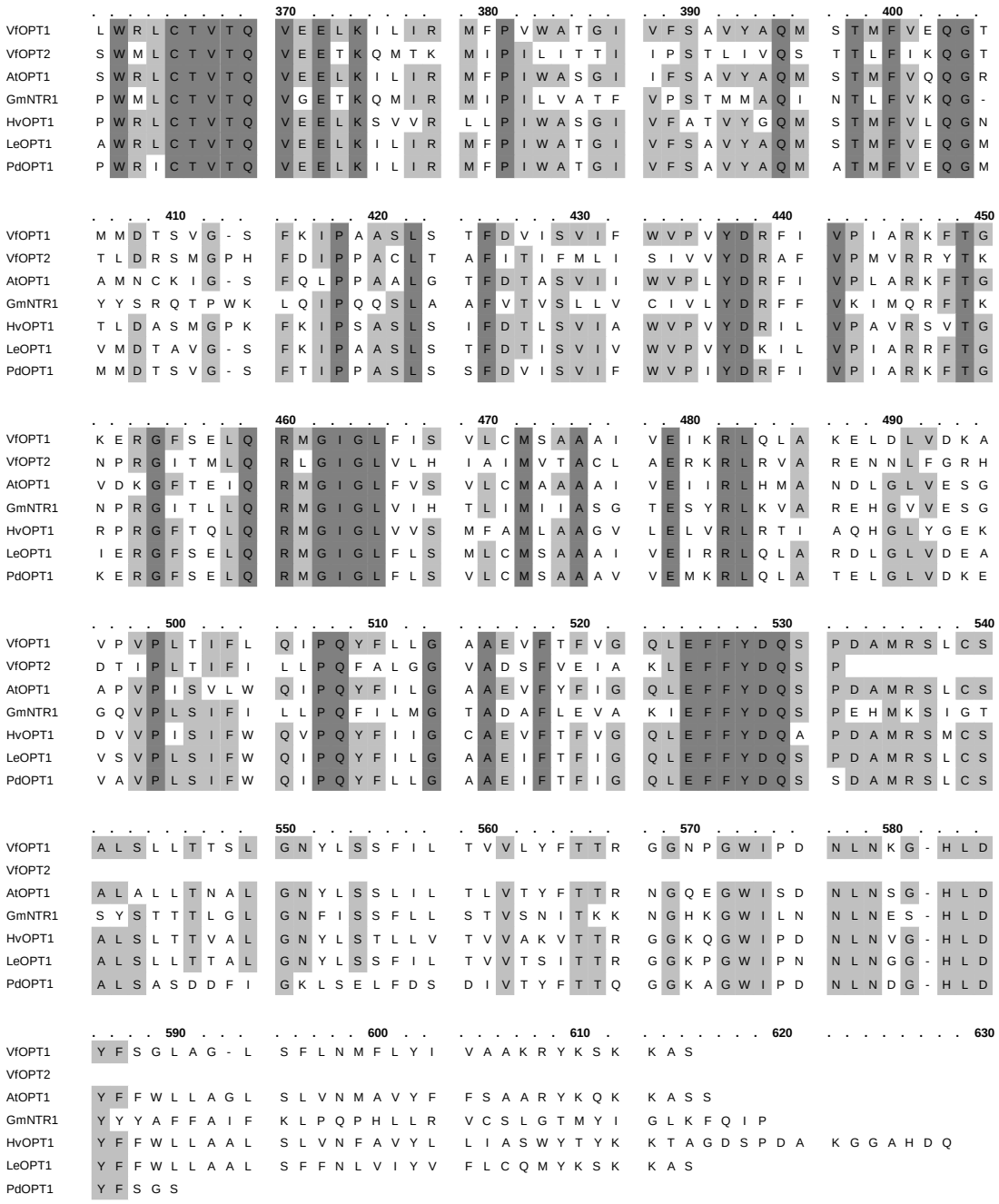



**Figure 10. Alignment between the predicted protein sequences of VfOPT1, VfOPT2 and other plant OPT proteins.**

*Note that VfOPT2 has 26 additional amino acid residues at pos. 331-354, which are located within a region of low homology among all the aligned sequences (Clustal method; Omiga 2.0, Oxford Molecular, England). AtOPT1 (*A. thaliana*; Rentsch* et al.*, 1995), GmNTR1 (soybean; Yokoyama* et al.*, 2001), HvOPT1 (barley; West* et al.*, 1998), LeOPT1 (tomato; AF016713), PdOPT1 (almond; AF213936).*

## 3.2. GENOMIC ORGANIZATION

### 3.2.1. *Southern analyses of* VfAAP1 *and* VfAAP3

Southern analyses were performed to determine the copy number of *VfAAP1* and *VfAAP3* genes. Distinct patterns were seen after hybridizing the blots at high stringency with the complete cDNAs of *VfAAP1* and *VfAAP3* as probes. *VfAAP1* showed single bands only for *Xho*I digestion, and several bands for the other four enzymes (Figure 11a). *Pvu*II and *Sty*I digestions yielded a number of strongly hybridizing bands larger than expected from their restriction sites in the *VfAAP1* cDNA (see Figure 4a), suggesting either that the *VfAAP1* gene has additional sites for these enzymes in its introns or that two or more copies of this gene are present. *VfAAP3*, on the other hand, revealed a simpler hybridization pattern, with single bands for *Pvu*II, *Pst*I, *Sty*I and *Xho*I digestions, and two *Hind*III bands (Figure 11b). Therefore it is likely that *VfAAP3* is a single copy gene. These results indicate, furthermore, that although the predicted protein sequences of VfAAP1 and VfAAP3 share 66.1% identity, their genes do not cross-hybridize under high stringency conditions.

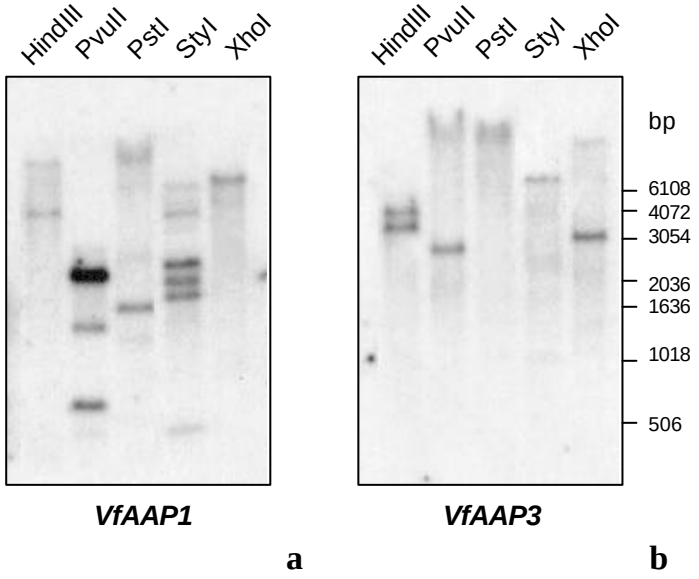



**Figure 11. Southern blot analysis of *VfAAP1* and *VfAAP3*.**

*Ten mg 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 labeled cDNA probes of* VfAAP1 ***(a)*** *or* VfAAP3 ***(b)***.

### 3.2.2. *Isolation of the* VfAAP1 *5'-upstream region*

To better examine the structure of the *VfAAP1* gene, its 5'-upstream region (*5'-UR*; 1.5 Kb long) was cloned by genome walking. In addition to the *5'-UR*, the first 340 bp of the *VfAAP1* coding region, interrupted at pos. +100 [(2)] by a 70 bp intron, was cloned. Sequence analyses revealed that not all clones were identical, but that they could be divided into two groups of *5'-UR*s (so called *VfAAP1 5'-URa* and *5'-URb*) sharing ~95% identity to each other. These findings were confirmed by further PCR using undigested leaf DNA as template.

An alignment of the *5'-UR*s is shown in Figure 12. Apart from single base exchanges, the two *5'-URs* differ in three insertions and/or deletions: (i) the sequence TAAAATATTTTTT and AAAGTTTTA found in *5'-URb* at pos. – 482/– 470 and – 409/– 400, respectively, are absent in *5'-URa*. By inserting the latter 9 bp, a string similar to an endosperm- (E-) element (TGTAAAG; Kreis *et al*., 1985) is created in *5'-URb*; and (ii) *5'-URa* has a tandem repeat (GATTTGCATGATTTGCAT; pos. – 376/– 359) that appears only once in *5'-URb* (GATTTGCAT). By duplicating these 9 bp, a putative RY-element (CATGCA), which is known essential for the proper regulation of several seed specific genes (Bäumlein *et al*., 1992), is created in the lower DNA strand of *5'-URa*. Another RY-motif (pos. – 976 to – 970) is found in of both *5'-UR*s. Moreover, computer predictions revealed in both *5'-UR*s the presence of potential regulatory motifs commonly found in promoter sequences, e.g. TATA- and CAAT-boxes, besides a nitrogen responsive GCN4-like element (TGAGTCA; Wolfner *et al*., 1975) (Promoter 2.0 Prediction Server, http://www.cbs.dtu.dk; Figure 13). Three base substitutions are observed in the 70 bp intron, whereas the first 340 bp of the *VfAAP1* coding region were identical between the two types of clones carrying either *5'-URa* or *5'-URb* (data not shown). These results confirm the existence of at least two copies of the *VfAAP1* gene, which slightly differ from each other in the 5'-upstream and untranslated regions.

[(2)] positions are given in relation to the predicted translation start, i.e. the first in frame ATG, of the aligment consensus of the two *5'-UR*s.

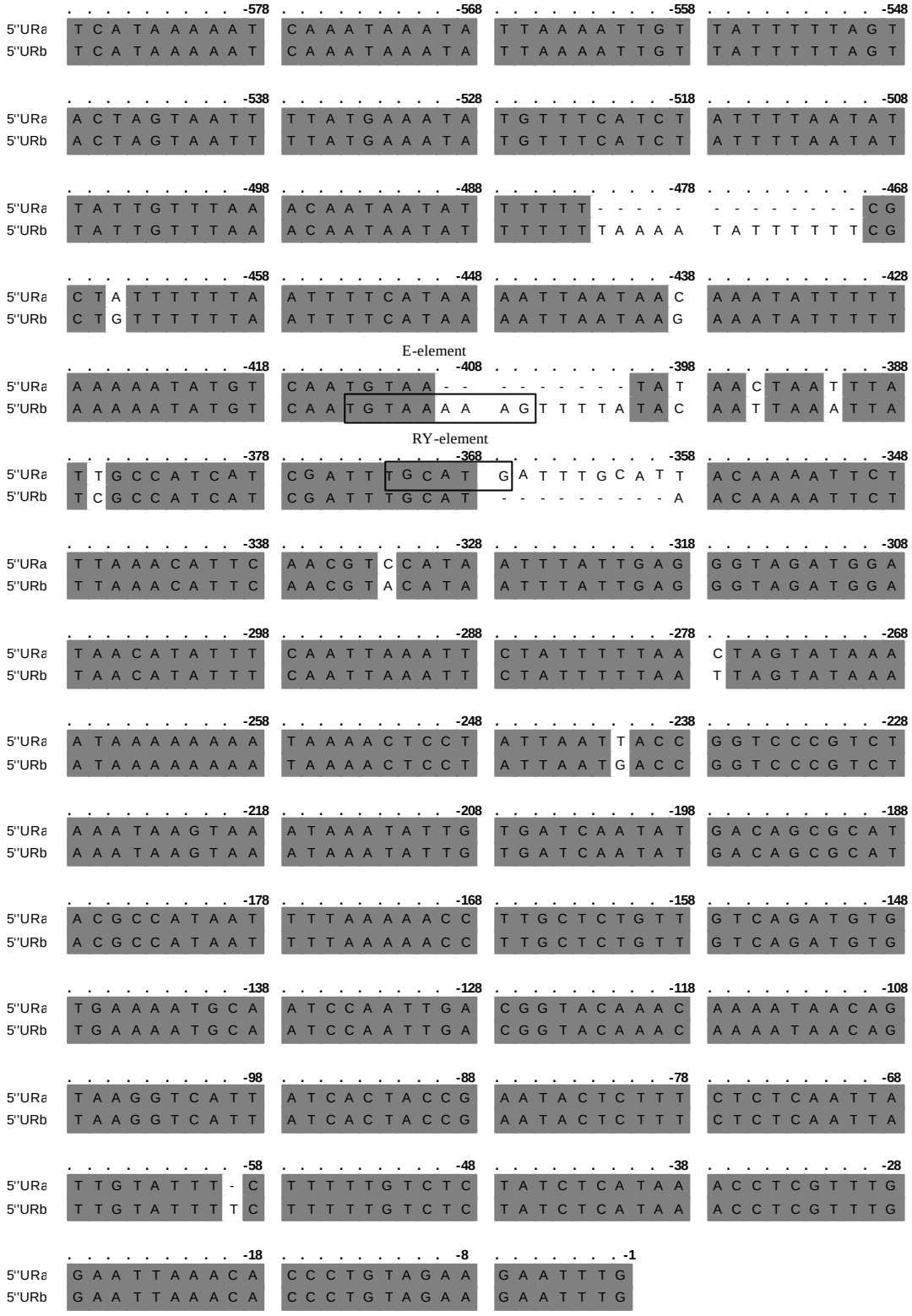



**Figure 12: Partial alignment between *VfAAP1 5'-URa* and *5'-URb***

*Note the presence of three short insertion/deletions between pos. –482 and –359 bp. The putative regulatory motifs created by these insertions/deletions are indicated. Positions are given in relation to the predicted translation start.*

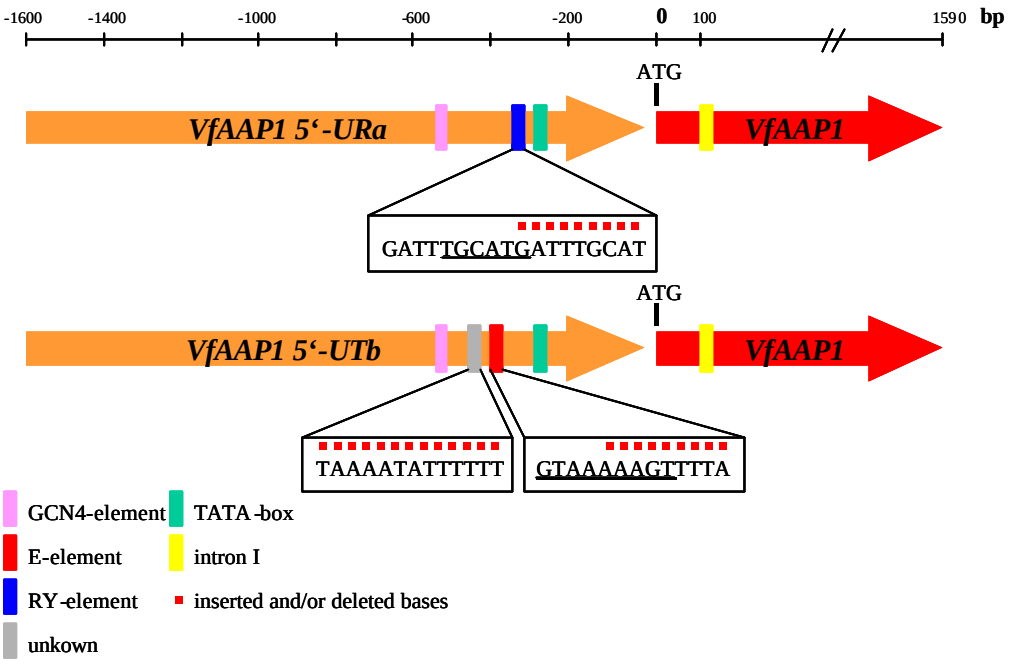



**Figure 13. Schematic comparison between the two types of *VfAAP1* 5'-upstream regions.**

*The two* VfAAP1 *5'-upstream regions (*5'-URa *and* 5'-URb*) differ from each other mainly by three insertions and/or deletions (shown in the boxed areas). These differences created a putative RY-element and an endosperm (E-)-element (underlined bases) which are present either in* 5'-URa *or in 5'-URb, respectively. The first potential TATA-box and GCN4-element upstream of the translation start (ATG) are indicated, which are common to both 5'-URs. A 70 bp intron is located at pos. +100. Positions are given in relation to the ATG.*

## 3.3. YEAST FUNCTIONAL COMPLEMENTATION AND TRANSPORTER UPTAKE PROPERTIES

### 3.3.1. *Uptake kinetics of VfAAP1 and VfAAP3*

For functional characterization, *VfAAP1* and *VfAAP3* full length cDNAs were cloned into the NEV-E vector (Stolz and Sauer, 1984) and transformed into the *plas*23-4B yeast mutant. This mutant carries a mutation in the *SHR3* gene that prevents proper targeting of its own amino

acid transporters 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' NEV-E vector revealed no or very low uptake for all amino acids tested (data not shown). Background uptake was subtracted from the experimental values. Uptake rate was directly measured for each [U]-$^{14}$C-labeled L-amino acid at 1 mM concentration. Under these conditions, it was observed that VfAAP1 mediates the transport of a wide range of amino acids, with high uptake rates for cysteine, arginine, glutamine, serine, leucine, methionine, histidine, glycine and threonine. VfAAP1 shows a clear preference for cysteine, whose uptake rate is more than 2-fold higher than that of any other amino acid (Figure 14). VfAAP3 also transports several amino acids, however at lower rates when compared to VfAAP1 in the yeast system, with a preference for the basic amino acids lysine and arginine (Figure 14). Neither acidic nor aromatic amino acids were taken up to a larger extent by these permeases. The kinetics of [U]-$^{14}$C labeled L-arginine uptake revealed $Km_{ARG}$ values of 46.0 and 82.4 µM for VfAAP1 and VfAAP3, respectively, indicating that these AAPs have high substrate affinity.

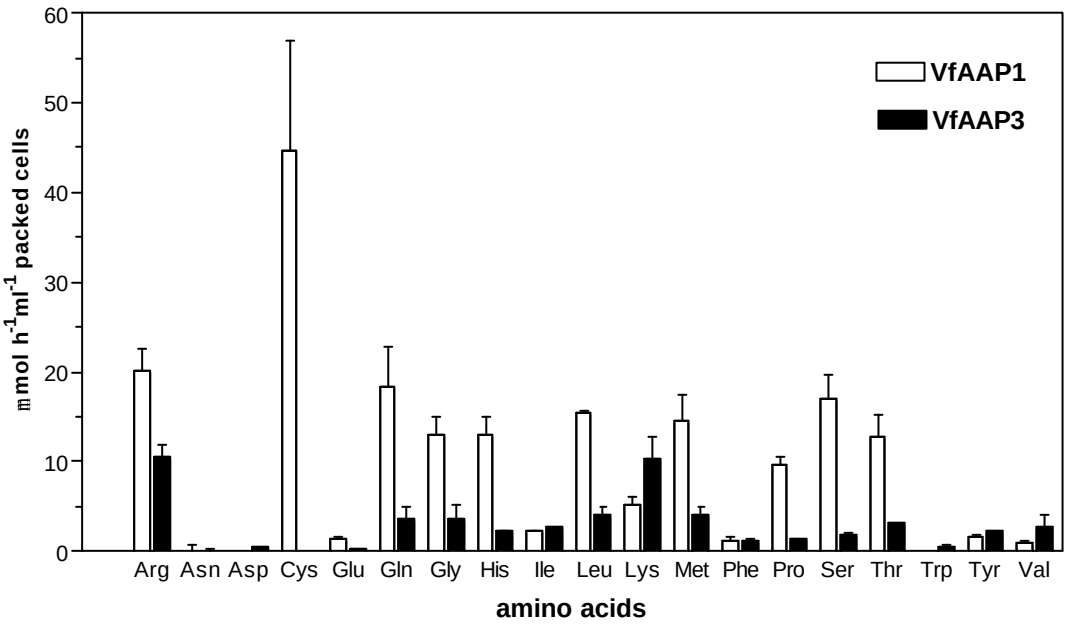



**Figure 14. Amino acid uptake promoted by VfAAP1 and VfAAP3 into a yeast mutant.**

*Direct uptake activity of 1 mM [U]-$^{14}$C-labeled L-amino acids into the* plas*23-4B yeast mutant expressing either* VfAAP1 *or* VfAAP3 *complete cDNAs. Background uptake of the mutant transformed with an 'empty' vector was subtracted.*

### 3.3.2. *Complementation of mutant growth by VfOPT1*

The *VfOPT1* cDNA was inserted into the pDR195 vector and transformed into the *LR2* yeast mutant, which carries a mutation in the *PTR2* gene and is unable to grow in media containing di-peptides as the only source of nitrogen (Rentsch *et al*., 1995). *LR2* cells transformed with *VfOPT1* grow efficiently on a medium containing 10 mM His-Ala, similar to cells carrying the *AtOPT1* cDNA from *A. thaliana*, whereas *LR2* cells transformed with an amino acid permease (*AtAAP2*) or with an 'empty' pDR195 vector, as negative controls, are not able to grow under similar conditions (Figure 15a). *LR2* cells grow efficiently on a non-selective medium containing 20 mM histidine regardless of the construct they are carrying (Figure 15b).

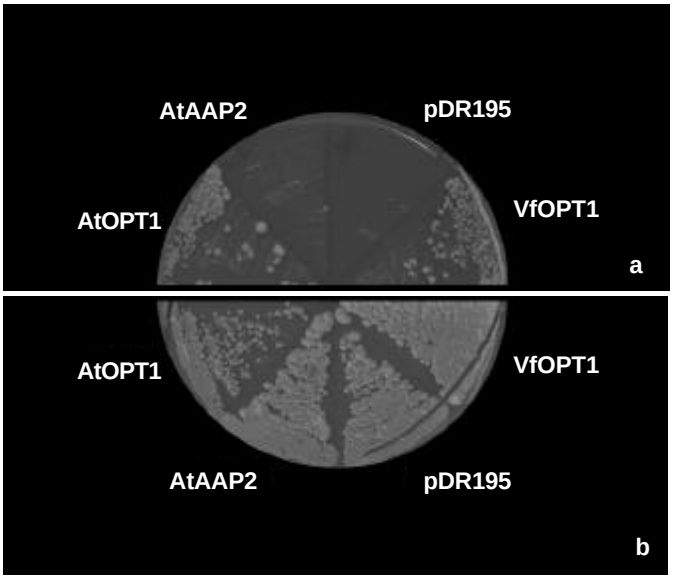



**Figure 15. Functional complementation of the *LR2* yeast mutant by VfOPT1.**
*Selective growth of mutant strains carrying the cDNAs of* VfOPT1, AtOPT1 *(as positive control),* AtAAP2 *(negative control) or pDR195 'empty' vector (negative control) in SC medium supplemented with 10 mM His-Ala (**a**) and non-selective growth in SC medium supplemented with 20 mM His (**b**).*

## 3.4. *AAP*S AND *OPT*S EXPRESSION THROUGHOUT THE PLANT AND DURING SEED DEVELOPMENT

Northern analyses revealed that the accumulation of *VfAAP1* transcripts is highest in gynoecia and early developing cotyledons, i.e. stages IV and V (see Figure 1; Borisjuk *et al.* 1995). Expression levels in cotyledons at later stages are lower and constant. Moderate expression levels were found in stems, roots, pods and sink leaves, but not in source leaves, and low levels in seed coats (Figure 16a). The expression profile indicates that this transporter provides amino acids primarily to cotyledons, and to a lower extent to other sink tissues. *VfAAP3* mRNA is 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 16b). *VfAAP4* did not reveal detectable signals in any of the tissues analyzed following northern hybridizations (data not shown). For comparison, cDNAs encoding the sucrose transporter (*VfSUT1*; Weber *et al.*, 1997b) and the storage proteins legumin B4 (*LegB4*; Heim *et al.*, 1989) and vicilin (Bassüner *et al.*, 1987) were used as probes to hybridize the same blots. *VfSUT1*-mRNA level is high in gynoecia and cotyledons of stages IV and V, and lower in older cotyledons. *VfSUT1* is moderately expressed in stems, roots, pods, seed coats and sink leaves, but not in source leaves (Figure 16c). Its mRNA distribution pattern resembles, therefore, that of *VfAAP1*, excepting that *VfSUT1* seems to have an overall higher expression level and that during cotyledon development its expression declines earlier than that of *VfAAP1*. *LegB4* and *vicilin* genes are expressed exclusively in cotyledons. The first detectable transcripts appear at mid-cotyledon stage (around the 20 DAP), when *VfAAP1*-mRNA level decline starts (Figures 16d and e).

*VfOPT1* is highly expressed in roots, shoots and developing cotyledons, and moderately expressed in gynoecia. Expression levels in source and sink leaves are low. During development of cotyledons, *VfOPT1* mRNA accumulate moderately during stage VI, reaches a peak around the 25-28 DAP (early stage VII), and decreases towards seed maturation (Figure 17a). Detection of *VfOPT2* transcripts by northern analyses has failed, but succeeded by RT-PCR using *VfOPT2* specific primers. During cotyledon development, a faint right sized band is first seen at 32 DAP, increases in intensity reaching a peak at 42 DAP, and quenched at 45 DAP. No bands are seen in the seed coats (Figure 17b). Weak bands were also seen when RNA from shoots and roots were used as templates (data not shown). These results

showed that both *VfOPT1* and *VfOPT2* reach their maximum transcript accumulation later than *VfAAP1*, when storage protein genes are already highly expressed. Taken together, these observations suggest that *VfAAP1* plays a more important role during early cotyledon development, and that the two peptide transporters should play a more important role during later cotyledon development.

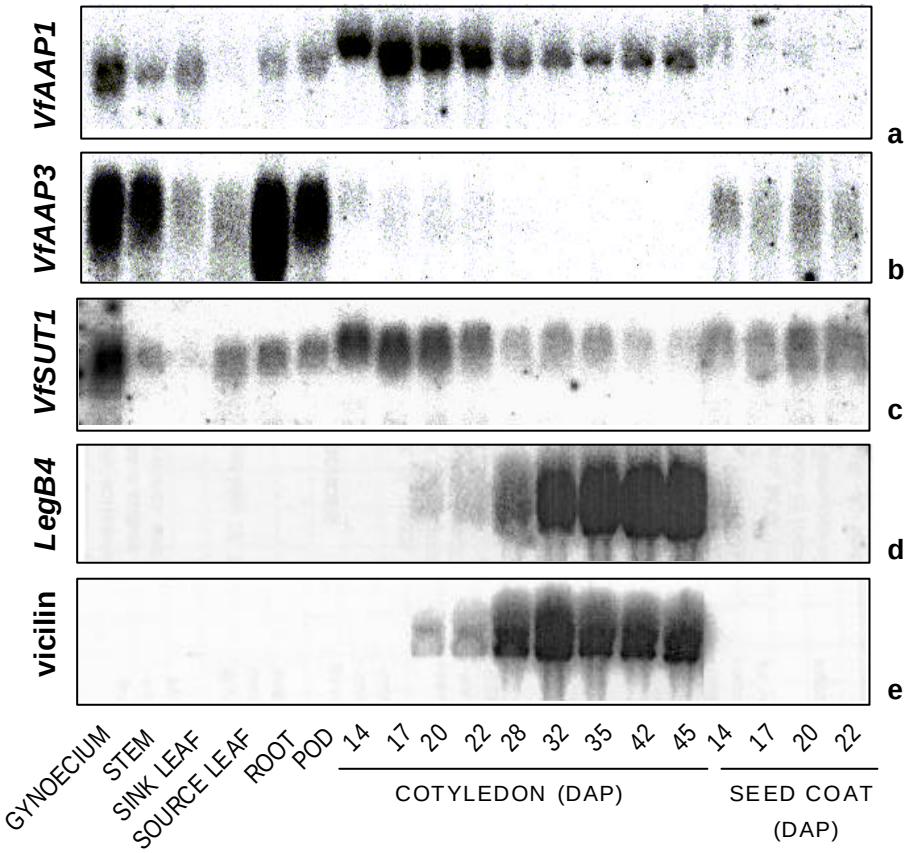



**Figure 16. Transcript accumulation of *VfAAP1, VfAAP3, VfSUT1, LegB4* and *vicilin* in different tissues and seed developmental stages of *V. faba*.**

*Northern hybridizations of using* VfAAP1 ***(a)***, VfAAP3 ***(b)***, VfSUT1 ***(c)***, LegB4 ***(d)*** *and* vicilin ***(e)*** *as probes. Ten micrograms of total RNA extracted from different tissues were loaded per lane. DAP: days after pollination.*

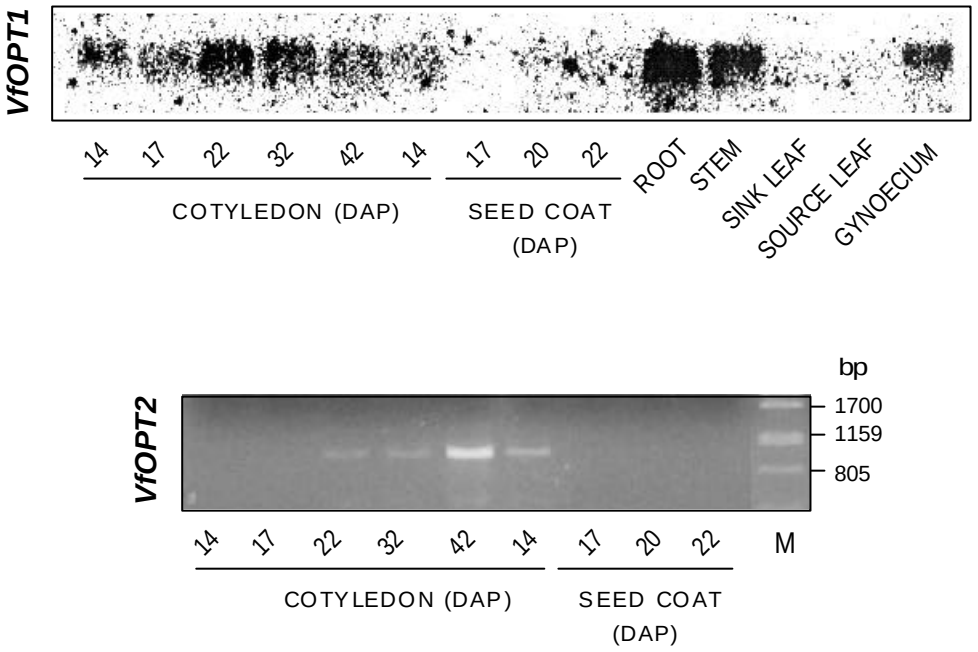



**Figure 17. Transcript accumulation of *VfOPT1* and *VfOPT2*.**

***a:*** *Northern hybridization of using* VfOPT1 *as a probe. Ten micrograms of total RNA extracted from different tissues were loaded per lane.* ***b:*** *Semi-quantitative RT-PCR using* VfOPT2 *specific primers and 1 mg total RNA from as template. M: marker. DAP: days after pollination.*

## 3.5. CELL TYPE-SPECIFIC MRNA DISTRIBUTION IN THE SEEDS

*In situ* hybridizations were performed in order to compare the expression patterns of *VfAAP1*, *VfOPT1*, *VfSUT1* and the *vicilin* gene within a developing seed. Cross sections of seeds at stage V were used (Figure 18a). Hybridizations using the *VfAAP1* cDNA as a probe, revealed that this transporter is expressed throughout the whole storage parenchyma of the cotyledons, but not in the epidermal transfer cells (Figures 18b and d). No label is found in the endosperm (Figures 16b and f). In seed coats, *VfAAP1* probe labeling is restricted to the chlorenchyma and thin-walled parenchyma tissues (Figures 18f and h). No signals are seen in palisade or ground parenchyma tissues (the bluish color seen in the ground parenchyma is derived from autofluorescence caused by phenolic compounds present in these cells; Figure 18h). The labeling of the *VfOPT1* probe is seen only in the epidermal transfer cell layer of the cotyledons, and not over the storage parenchyma (Figure 19b). In cotyledons, labeling of the *vicilin* probe shows that this storage protein gene is co-expressed with *VfAAP1* in the storage parenchyma, and not in the epidermal cell layer (Figure 20b). On the other hand, *VfSUT1* transcripts are localized only in the cotyledon transfer cells of the cotyledons, resembling the hybridization pattern of the *VfOPT1* probe (Figure 20c). The mRNA-distribution patterns of the *vicilin* gene and *VfSUT1* are consistent with previous reports (Borisjuk *et al*., 1995; Weber *et al*., 1997b).

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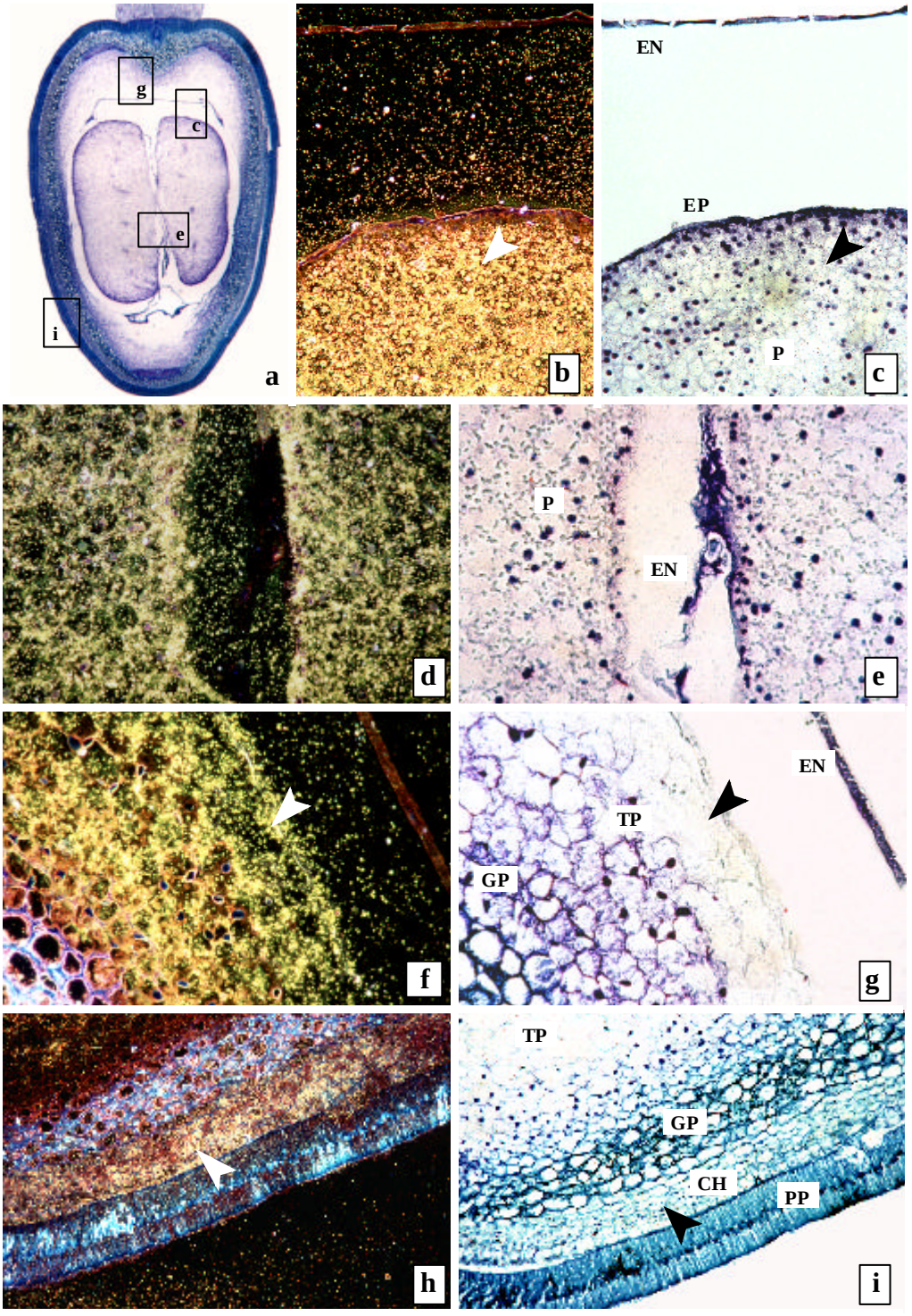
**Figure 18 (following page). Transcript distribution of *VfAAP1* in transversal sections of a *V. faba* seed.**

***a**: Overview of the seed stained with toluidine-blue. The boxed areas are enlarged in the following pictures. **b, d, f** and **h**: dark-field micrographs showing* in situ *hybridization using $^{33}P$ labeled cDNA probes of* VfAAP1, *with label seen as white grains. **c, e, g** and **i**: light-field with toluidine-blue staining showing the corresponding areas of the dark-field pictures. **b**: Outer adaxial region of the cotyledon with labeling in the storage parenchyma (P), but not the epidermal transfer cells (EP) or endosperm (EN). **d**: Inner portion of the cotyledon with labeling in the storage parenchyma. **f**: Inner portion of the seed coat near the funiculus. Labeling is found in the transfer cells of the thin-walled parenchyma (TP), but not in the ground parenchyma (GP). **h**: Outer region of the seed coat is labeled in the chlorenchyma (CH) and thin-walled parenchyma. No label was found in the palisade epidermis (PP) or ground parenchyma where the bluish color results from autofluorescence.*


g
c
e
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a
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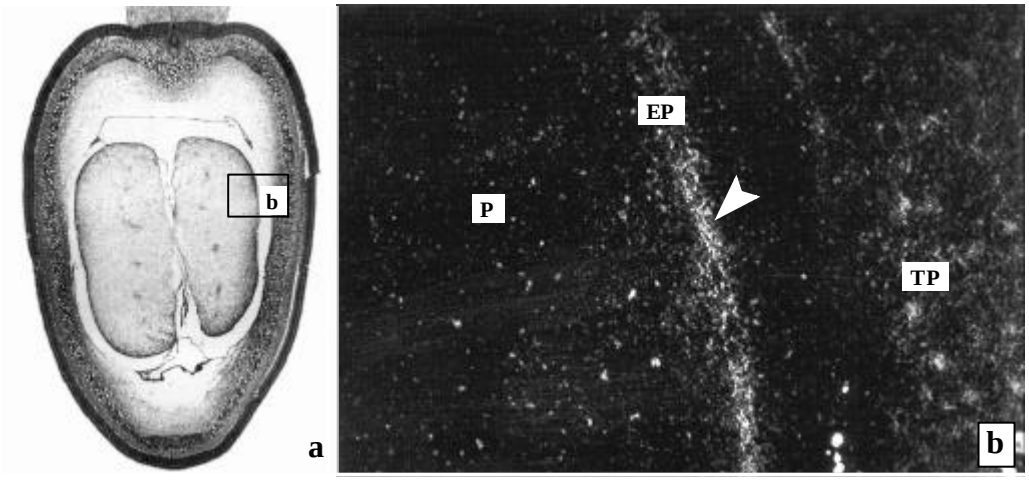


**Figure 19. *VfOPT1* transcript distribution of in a transversal section of a *V. faba* seed.**
***a****: Overview of the seed stained with toluidine-blue. The boxed area is enlarged in the following picture.* ***b****: dark-field micrograph showing* in situ *hybridization using* $^{33}P$ *labeled cDNA probe of* VfOPT1, *with label seen as white grains in the epidermal transfer cell layer (EP) of the cotyledon (marked by an arrowhead). P: cotyledon storage parenchyma; TP: thin-walled parenchyma of the seed coat.*

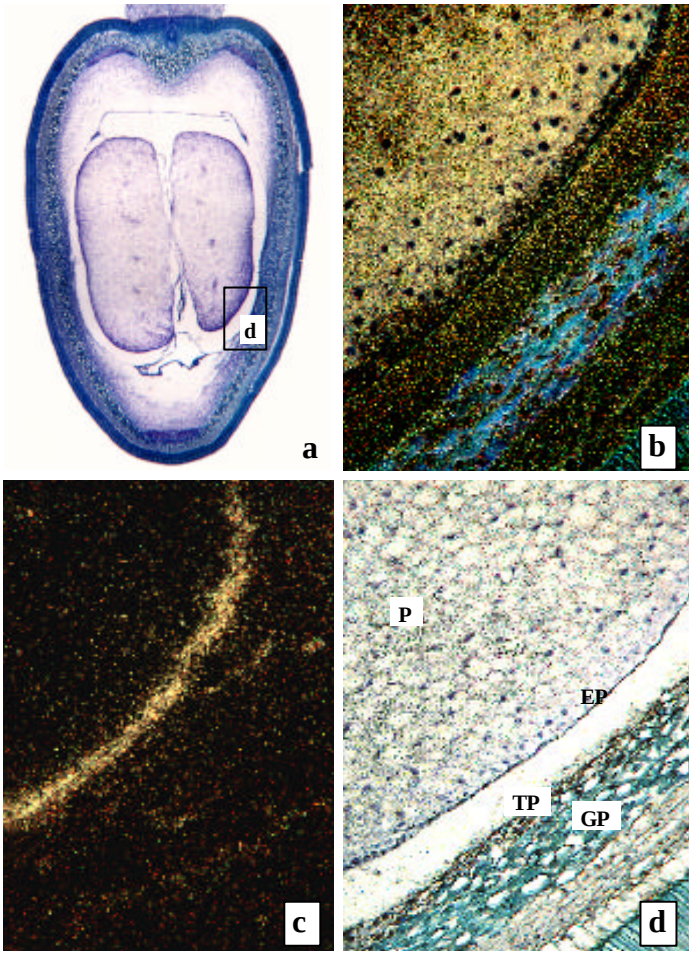


**Figure 20. Transcript distribution of the *vicilin* gene and *VfSUT1* in transversal sections of a *V. faba* seed.**

***a**: Overview of the seed stained with toluidine-blue. The boxed area is enlarged in the following pictures. **b** and **c**: dark-field micrographs showing* in situ *hybridization using $^{33}P$ labeled cDNA probes of* vicilin *(**b**) and* VfSUT1 *(**c**), with label seen as white grains. **d**: light-field with toluidine-blue staining showing the corresponding areas of the dark-field pictures. **b**: Labeling of the* vicilin *probe is found in the parenchyma (P) but not the epidermal cells of the cotyledon (EP). **c.** Labeling of the* VfSUT1 *probe is found only in the epidermal cell layer of the cotyledon. Arrowheads show transfer cells of cotyledons and seed coat. TP: thin-walled parenchyma of the seed coat; GP: ground parenchyma.*

## 3.6. REGULATION OF THE *VFAAP1* GENE

### 3.6.1. *Modulation of* VfAAP1 *expression in cotyledons cultured* in vitro

In order to examine whether *VfAAP1*-mRNA levels can be modulated by assimilate availability, cotyledons of developmental stage V were cultured *in vitro* under different metabolite supply, i.e. one cotyledon of a seed was cultured in the presence of glutamine, and the other cotyledon was cultured under amino acid starvation. Experiments were performed both in the presence or absence of sucrose. Northern analyses with RNA isolated from these cotyledons showed that *VfAAP1*-mRNA levels are lower in cotyledons that have been cultured in media supplemented with glutamine. This tendency is observed both in the presence or absence of sucrose, however, significant changes occur only when sucrose is present (Figures 21a and b). For comparison, the *vicilin* cDNA was used as a probe. The mRNA levels of this storage protein gene are higher when glutamine is added to the media, with significant changes seen only in the presence of sucrose (Figures 21c and d). Transcript levels of both *VfAAP1* and *vicilin* gene are ca. 2- and 6-fold higher, respectively, in cotyledons incubated in the presence of sucrose alone (seen by the relative mRNA levels on the Y axis of the graphs in Figure 21).

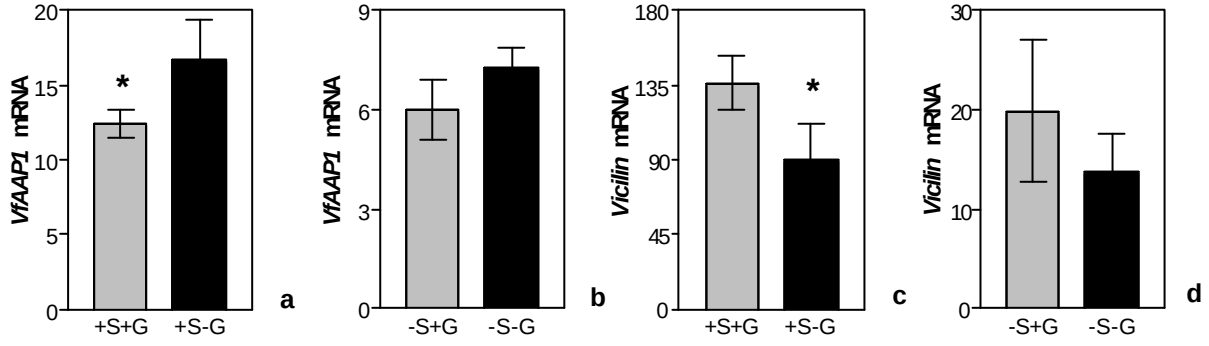


**Figure 21. Effect of metabolites on the accumulation of *VfAAP1* and *vicilin* mRNA in cotyledons cultured *in vitro*.**

*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). mRNA levels (in relative units) represent the mean of three replications ± SE. (*) statistically significant (P < 0.05).*

### 3.6.2. *GUS expression driven by the* VfAAP1 *promoters and regulatory effects of cysteine*

To assess the functionality of the *VfAAP1* 5'-uptstream regions, *5'-URa* and *5'-URb* were inserted in sense orientation upstream to the β-glucuronidase (*GUS*) reporter gene, and transformed transiently into protoplasts isolated from embryogenic cell suspensions of *N. plumbaginifolia*. GUS activity driven by *5'-URa* and *5'-URb* were up to 150- and 190-fold higher, respectively, then in controls without a promoter, indicating that these regions included functional promoter sequences (Figure 22).

Cysteine, which is the preferentially transported amino acid by VfAAP1, was added to the media to determine whether this system is suitable for studying possible effects of amino acids on transcriptional regulation. After 24 and 48 h of transformation, GUS activities driven by the *VfAAP1 5'-URa* in protoplasts cultured in the presence of cysteine are ~ 40% and 55% lower, respectively, than in controls under amino acid starvation (Figure 23a). For comparison, protoplasts were transformed with a *USP* promoter/*GUS* construct. The *USP* promoter (for *U*nknown *S*eed *P*rotein) is seed specific and it is expressed and regulated similarly to storage protein genes (Bäumlein *et al.*, 1991a). After 24 and 48 h, GUS activities driven by the *USP* promoter are ~ 80 % higher in protoplasts cultured with cysteine than in controls cultured under amino acid starvation (Figure 23b). A similar approach was carried out to compare the effects of cysteine between the two *5'-UR*s. GUS activities driven by *5'-URa* and *5'-URb* are ~ 55% and 70% lower, respectively, than in controls under amino acid starvation (Figures 24a and b).

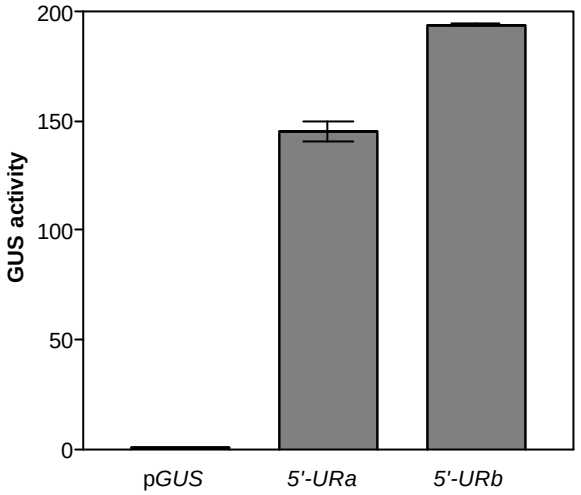


**Figure 22. *GUS* transient assay of *VfAAP1 N. plumbaginifolia* protoplasts.**

*GUS activities (relative units) in protoplasts transformed with* VfAAP1 5'URa *or* 5'-URb *upstream the* GUS *gene are given in comparison to the GUS activity in protoplasts transformed with a construct lacking a promoter (p*GUS*).*

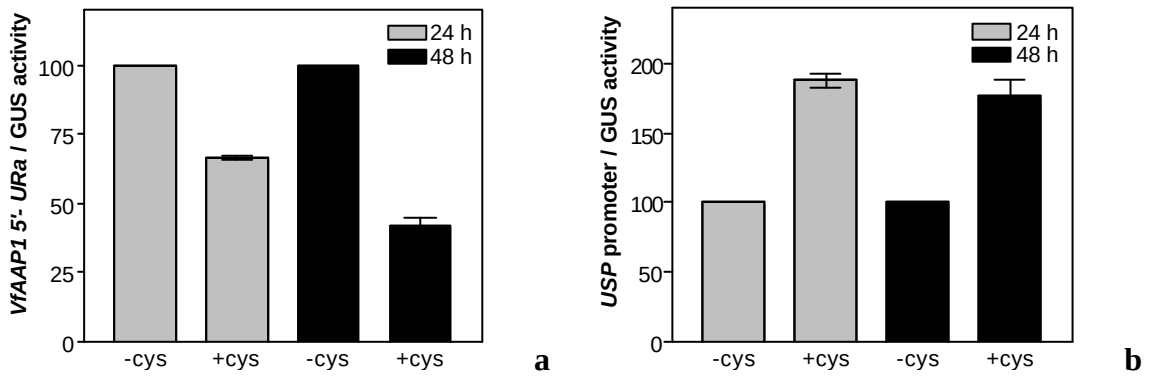


**Figure 23. Effect of cysteine on transient GUS expression driven by the *VfAAP1 5'-URa* or *USP* promoter in *N. plumbaginifolia* protoplasts.**

*Protoplasts were transformed with a* VfAAP1 5'-URa*/*GUS ***(a)*** *or an* USP *promoter/*GUS *construct* ***(b)****, and cultured either in the presence of 1mM cysteine (+cys) or without cysteine, i.e. under amino acid starvation (-cys). GUS activities were measured after 24 and 48 h, and average values of two experiments ± SE are given in relative units (control without cysteine = 100%).*

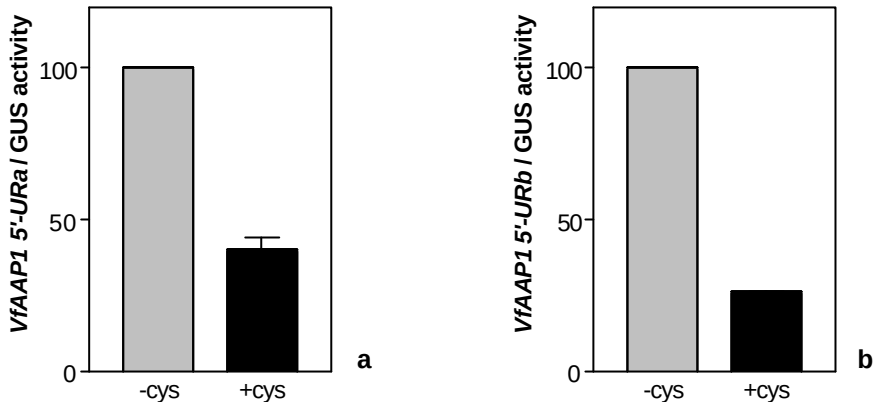


**Figure 24. Effect of cysteine on transient *GUS* expression driven by the *VfAAP1* 5'-upstream regions (*5'-URs*) in *N. plumbaginifolia* protoplasts.**

*Protoplasts were transformed with* VfAAP1 5'-URa ***(a)*** *or* VfAAP1 5'-URb ***(b)*** *upstream the* GUS *gene, and cultured either in the presence of 1mM cysteine (+cys) or without cysteine, i.e. under amino acid starvation (-cys). GUS activities are given in percent values of two experiments ± SE (control without cysteine = 100%).*

## 3.7. *VICIA NARBONENSIS* PLANTS CARRYING THE *VFAAP1* CDNA UNDER CONTROL OF THE *LEGB4* PROMOTER

A transgenic approach aiming the overexpression of *VfAAP1* was carried out by transforming *V. narbonensis* plants (a species closely related to *V. faba* that is routinely used for this purpose; Pickardt *et al*., 1991; Weber *et al*., 1998b, 2000) with constructs carrying the *VfAAP1* cDNA in sense orientation downstream of the *LegB4* promoter. This promoter confers strong expression only in the parenchyma cells of *V. narbonensis* cotyledons during mid- to late stages of development (Weber *et al*., 1998b). The transgenic genotype was confirmed by PCR in several independently regenerated $F_0$ plants (Figure 25). These plants were allowed to self-pollinate and PCR-positive plants were propagated up to the $F_3$ generation. Because more life cycles are usually necessary to produce homozygous transgenic lines (see Weber *et al*., 2000), the present results should be considered of preliminary nature. Northern analysis of total RNA isolated from embryos at late development (stage VII) showed that in three lines (Vn10, Vn20 and Vn26) *VfAAP1* transcript levels are higher than in wild

type plants (Figure 26a). When the coding region of the *LegB4* cDNA was used as a probe, it was observed that mRNA levels of the endogenous *LegB4* gene are also increased in these lines (Figure 26b). Seeds produced by these three lines and by line Vn21 (in which *VfAAP1* overexpression remains to be shown) are not totally uniform, i.e. the majority of seeds are smaller and exhibit a wrinkled phenotype, however some have a normal appearance (Figure 27). It was impossible, however, to determine the percentage of wrinkled *versus* normal seeds once it is frequently difficult to determine a border between these two types. Dry weight of Vn10, Vn20 and Vn21 seeds is significantly lower than that of the wild types, whereas Vn26 seed weight is not altered (Figure 28a). Further biochemical analyses carried out with these same seeds showed that total nitrogen and sulfur contents are significantly increased on a percentage basis in all four lines (Figures 28b and c), whereas starch contents are reduced (Figure 28d). Total carbon is slightly reduced only in line Vn20 and total sugars are kept unchanged (Figures 28e and f). The total globulin fractions of lines Vn20 and Vn21 are increased in *ca*. 35%, whereas albumin contents in lines Vn20 and Vn21 are unaltered and reduced, respectively (Figures 28g and h).

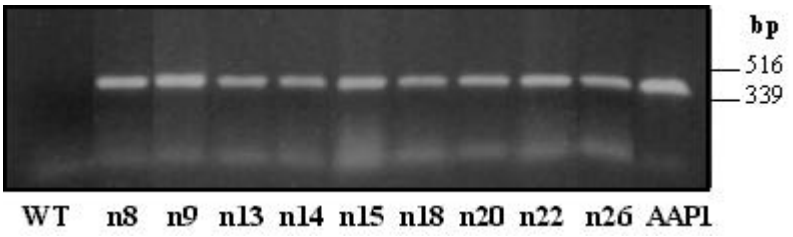


**Figure 25. PCR on total leaf DNA of *V. narbonensis* plants transformed with a *LegB4* promoter/*VfAAP1* cDNA construct.**

*Wild type (WT; as negative control); transformed lines (n8-n26);* VfAAP1 *miniprep DNA (AAP1, as positive control).*

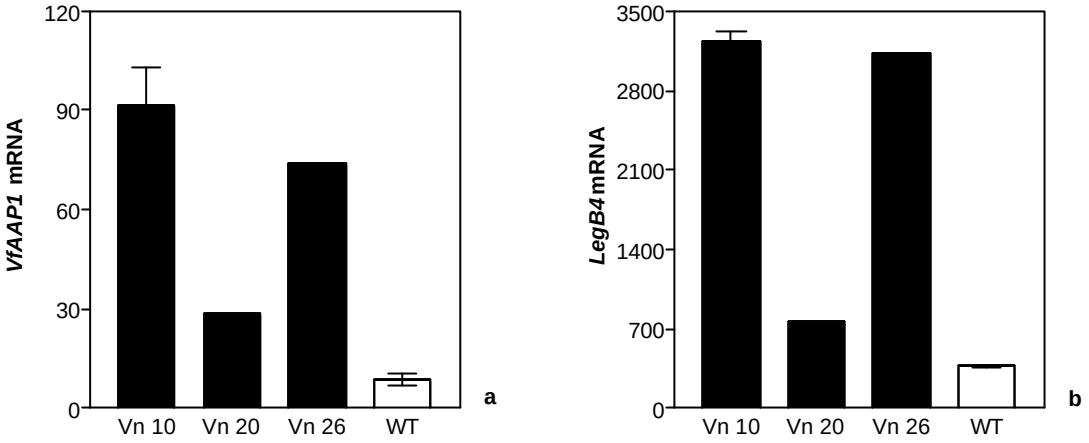


**Figure 26. Transcript accumulation of *VfAAP1* and *LegB4* gene in different *V. narbonensis* lines carrying the *VfAAP1* cDNA under control of the *LegB4* promoter.**

*Northern blots were hybridized with the coding region of VfAAP1 (**a**) or LegB4 (**b**). Ten micrograms of total RNA extracted from embryos at late development (stage VII) were loaded per lane. Transcript levels were quantified by scanning. Vn10, Vn20 and Vn26: transgenic lines; WT: wild type.*


**Figure 27. Seed morphology in transgenic *V. narbonensis* plants.**

V. narbonensis *plants carrying the* VfAAP1 *cDNA under control of the* LegB4 *promoter (Vn10, Vn20 and Vn21) produced seeds ranging from normal to smaller and wrinkled phenotypes in comparison to wild type seeds (WT).*

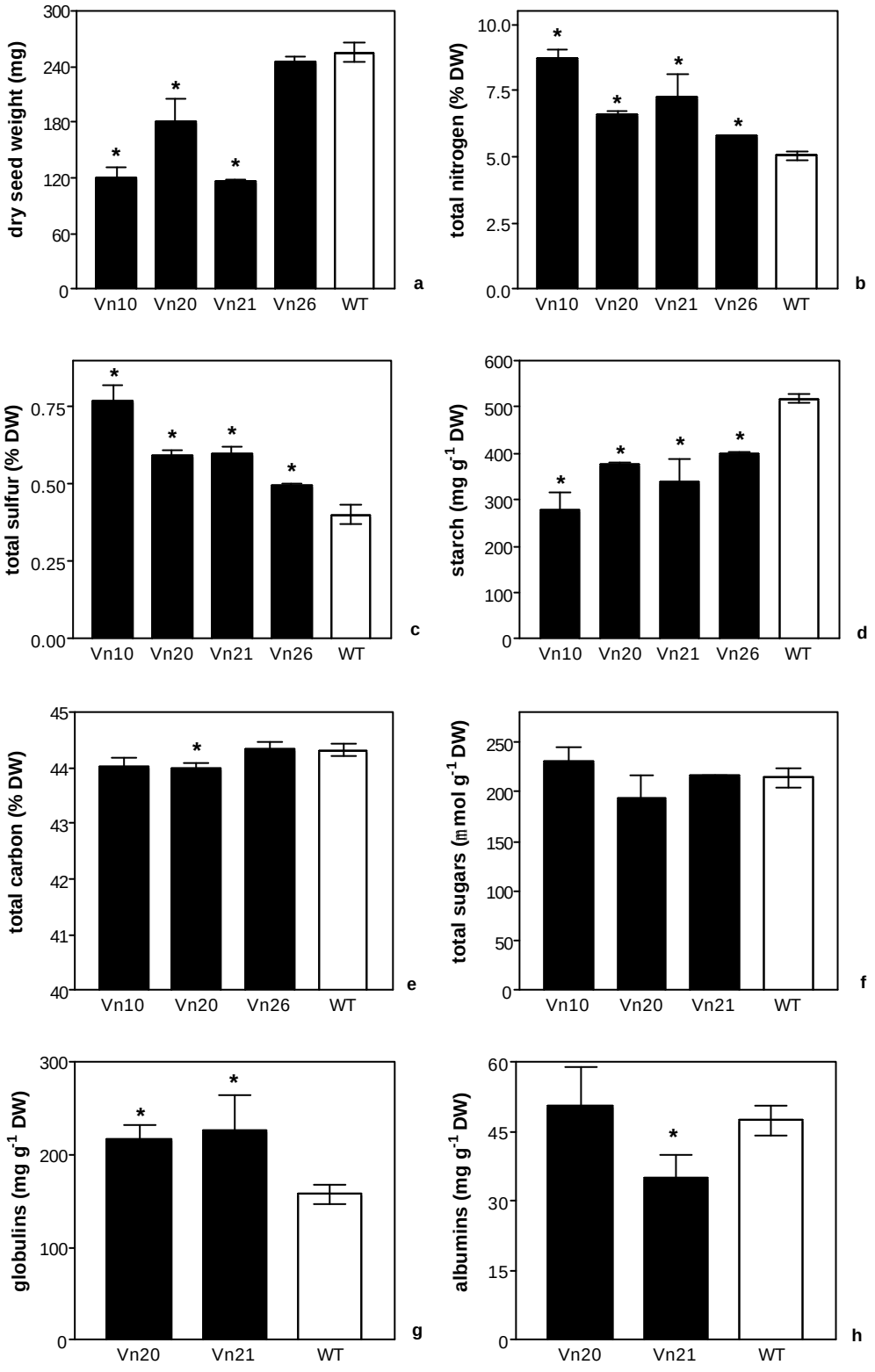
dry seed weight (mg)
300
240
180
120
60
0
Vn10 Vn20 Vn21 Vn26 WT
a
total nitrogen (% DW)
10.0
7.5
5.0
2.5
0.0
Vn10 Vn20 Vn21 Vn26 WT
b
total sulfur (% DW)
0.75
0.50
0.25
0.00
Vn10 Vn20 Vn21 Vn26 WT
c
starch (mg g-1 DW)
600
500
400
300
200
100
0
Vn10 Vn20 Vn21 Vn26 WT
d
total carbon (% DW)
45
44
43
42
41
40
Vn10 Vn20 Vn26 WT
e
total sugars (mmol g-1 DW)
250
200
150
100
50
0
Vn10 Vn20 Vn21 WT
f
globulins (mg g-1 DW)
300
200
100
0
Vn20 Vn21 WT
g
albumins (mg g-1 DW)
60
45
30
15
0
Vn20 Vn21 WT
h

**Figure 28 (previous page). Biochemical analyses of mature cotyledons from *V. narbonensis* lines carrying the *VfAAP1* cDNA under control of the *LegB4* promoter.**

*Dry seed weight (**a**) and contents of contents of total nitrogen (**b**), total sulfur (**c**), starch (**d**), total carbon (**e**), total sugars (**f**), globulins (**g**) and albumins (**h**) were measured in transgenic lines (Vn10, Vn20, Vn21 and Vn26) and wild type plants (WT). DW: dry weight. $P<0.05$, $n=4$ (except for t21, where $n=2$).*

# CHAPTER 4

# DISCUSSION

## 4.1. THE *AAP* AND *OPT* GENE FAMILIES IN *V. FABA*

Three amino acid permeases (*VfAAP1*, *VfAAP3* and *VfAAP4*) and two peptide transporters (*VfOPT1* and *VfOPT2*) were isolated from two cDNA libraries of *V. faba* developing seeds. The three newly identified *AAP*s differ from those (one full length cDNA, and three partial cDNAs) previously isolated from *V. faba* (Montamat *et al.*, 1999), whereas so far no peptide transporter has been identified in this species. Hence, at least seven different *AAP* and two *OPT* genes are expressed in *V. faba*. In other plants, these two types of transporters also belong to gene families. In *A. thaliana*, six AAP isoforms have promoted uptake of amino acids *in vitro* (Fischer *et al.*, 1995; Kwart *et al.*, 1993; Rentsch *et al.*, 1996) and at least 30 others remain to be functionally characterized (Entrez Protein Database; www.ncbi.nlm.nih.gov:80/entrez). The family of peptide transporters in *A. thaliana* seems to be equally composed of numerous members (Entrez Protein Database), but so far only one isoform from this species has been functionally characterized *in vitro*[3] (Frommer *et al.*,

[3] the first peptide transporter to be cloned from *A. thaliana* by Steiner *et al*. (1994) was later shown to be a fungal PTR (Steiner *et al*., 2000).

1994b; Rentsch *et al.*, 1995; Song *et al.*, 1996). Moreover, a large number of putative peptide transporters are expressed in soybeans seedlings (Public Soybean EST Project, Shoemaker *et al.*, unpublished results). The apparent redundancy of such transporters in a genome has frequently been justified by a strict regulatory control, substantiated by individual expression profiles throughout the plant (Fischer *et al.*, 1995, 1998; Rentsch *et al.*, 1998). The proportion of genes involved on amino acid and peptide transport found in *A. thaliana* is remarkably higher than in unicellular organisms, which evidences the importance of these genes for long distance transport of assimilates in higher plants (The Arabidopsis Genome Initiative, 2000).

The predicted protein sequences of VfAAP1 and VfAAP4 share a high degree of identity with PsAAP2 (a putative AAP) and PsAAP1 from pea (Tegeder *et al.*, 2000), respectively (Table 1). Because pea and *V. faba* are closely related, these clones may represent the orthologue forms of the same transporters in these two species. Likewise, VfAAP3's counterpart in pea would remain to be identified. Concerning sequence similarity, the legume AAPs are more closely related to the *A. thaliana* isoforms AtAAP2-5, than to AtAAP1 and 6 (Figure 6), but whether this has a practical relevance on the function of these permeases remains to be shown. VfOPT1 is closely related to putative peptide transporters isolated from almond and tomato, whereas VfOPT2 seems to belong, along with GmNTR1-3 (Yokoyama *et al.*, 2001), to another OPT sub-family.

The *V. faba* amino acid and peptide transporter proteins are highly hydrophobic, which is characteristic for membrane integral proteins. Experimental evidence showed that AtAAP1 has 11 transmembrane domains (TMD), leading to a model in which its N-terminus is located in the cytoplasm and the C-terminus faces the outside of the cell (Chang and Bush, 1997). Because the hydrophobic profiles of VfAAP1, VfAAP3 and VfAAP4 overlap that of AtAAP1 it is likely that these proteins share a similar membrane topology (Figure 7). The *A. thaliana* AAPs show great sequence variability in the loop between TMD5 and TMD6 of this model. These sequences have been proposed as a possible site responsible for substrate recognition (Chang and Bush, 1997). Similarly, a region with low similarity between the VfAAPs, in which VfAAP4 has three additional amino acid residues (see alignment in Figure 5), is located in the loop between TM9 and TM10 of the VfAAP topology model, and may also be involved in substrate recognition. The N-termini of VfAAP1 and VfAAP4 were predicted as signal peptides (SignalP 2.0; Nielsen *et al.*, 1997b), and may be involved in the targeting of the proteins to the membrane. In sucrose transporters this region plays a significant role in

transport efficiency (Schulze *et al.*, 2000). Moreover, according to the topology model, one N-glycosylation site in VfAAP1 is located in an extracellular loop (at pos. 216) and would therefore be accessible for glycosylation in the ER lumen. Glycosylation has been experimentally demonstrated for cationic amino acid transporters (Kim *et al.*, 1991), but not for an AAP (Chang and Bush, 1997). VfOPTs are also hydrophobic, but attempts to make a topology model have failed. VfOPT2 has an additional stretch of 30 amino acid residues within a highly variable hydrophilic region (see Figure 10), which may be an indication that this region is involved in substrate recognition.

## 4.2. VFAAP1 AND VFAAP3 ARE HIGH AFFINITY TRANSPORTERS FOR SMALL, NEUTRAL AND BASIC AMINO ACIDS EXHIBITING 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 measuring uptake properties of VfAAP1 and VfAAP3. This mutant was suitable for measuring the direct uptake of amino acids once it did not exhibit efficient transport of any of the amino acids tested. Direct measurements are more likely to give reliable results as compared to 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 had high substrate affinity and were able to transport a broad spectrum of amino acids (Figure 14). 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 amino acids tested, cysteine was clearly preferred by VfAAP1, since the uptake rate for this amino acid was at least 2-fold higher than for the others. Kwart *et al.* (1993) showed that a 4-fold excess of cysteine was able to inhibit *ca.* 90% of proline uptake by AtAAP2, but the direct uptake of cysteine has not been shown. VfAAP3 showed a broad substrate specificity and, at least in the yeast mutant, lower transport activity compared to VfAAP1 (Figure 14). VfAAP3 showed a preference for the basic amino acids arginine and lysine, therefore, with regard to substrate specificity, it is related to AtAAP3 and AtAAP5. VfAAP1 and VfAAP3 are peculiar in the sense that they did not take up acidic amino acids, whereas all *A. thaliana* AAPs so far analyzed 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, glutamine is used as the major source of N (Miflin *et al.*, 1977; Murray and Cordova-Edwards, 1984), which may explain to some extent the differences between *V. faba* and *A. thaliana* AAPs with regard to the uptake of glutamate. In support of our observations, a permease from castor bean was shown also to transport neutral and basic, but not acidic amino acids (Neelam *et al.*, 1999), and the uptake of glutamate by vesicles from pea cotyledons occurred in a passive-like manner (De Jong and Borstlap, 2000b).

Functional characterization of VfOPT1 was done by complementation of the yeast strain *LR2*, which carries a mutation in the *PTR2* gene and requires high concentrations of histidine for efficient growth (Rentsch *et al.*, 1995). Upon supplementation with His-Ala, VfOPT1 restored growth of the mutant, implying that cells had taken up this di-peptide (Figure 15). One peptide transporter from *A. thaliana*, isolated independently by two groups (Frommer *et al.*, 1994b; Rentsch *et al.*, 1995; Song *et al.*, 1996), and one from barley (West *et al.*, 1998) are so far the only plant OPTs that have been functionally characterized. They are rather non-specific and mediate the uptake of a wide range of di- and tri-peptides (Rentsch *et al.*, 1995). Kinetic studies were not carried out with VfOPT1, therefore it is still unclear whether or not this OPT also takes up a broad range of peptides.

## 4.3. *AAPs* AND *OPTs* HAVE SPECIFIC AND DEVELOPMENTALLY CONTROLLED EXPRESSION PATTERNS

### 4.3.1 *Expression of VfAAP1 and VfOPT1 in cotyledons and synthesis of storage proteins – are these events connected?*

*VfAAP1* is expressed primarily in cotyledons, and to a lower extent in other sink tissues. During cotyledon development, *VfAAP1* m-RNA level peaks simultaneously with the appearance of storage protein transcripts, and then declines to a lower and constant level (Figure 16). The concentration of free amino acids in cotyledons increases rapidly during the period of seed filling, and decreases during storage protein synthesis (Weber *et al.*, 1996). Expression of *VfAAP1* and storage protein genes along with the variation of amino acid concentration happen in a concerted manner (see Figure 30) and correlate well with a

potential primary role of VfAAP1 in supplying amino acids that will be used 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). VfAAP1 may have a secondary function in supplying amino acids to other sink organs as well, since it is also expressed in gynoecia, stems, sink leaves, roots and pods, but not in source leaves. *VfAAP3* mRNA accumulates in all maternal tissues analyzed, 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 transport of amino acids through the plant. In contrast to *VfAAP1* and *VfAAP3*, it was not possible to detect any expression of *VfAAP4* by northern analysis. Interestingly, despite high sequence similarity (over 90 % of identical amino acids residues) between VfAAP1 and VfAAP4 and the pea PsAAP2 and PsAAP1 predicted protein sequences (Tegeder *et al.*, 2000), respectively, their expression profiles were strikingly different. *VfAAP1* was highly expressed in *V. faba* seeds but *PsAAP2* expression in pea could not be detected at all, and *VfAAP4*/*PsAAP1*, vice-versa. These observations suggest that these two species have developed a refined and distinct mode of regulation.

*VfOPT1* was mainly expressed in developing cotyledons, but also to a high extent in roots, source and sink leaves, shoots, flowers and seed coats, suggesting that this transporter may have a 'house keeping' role, similarly to *VfAAP3*. During cotyledon development, *VfOPT1* mRNA accumulates slightly later than *VfAAP1* (Figure 16). The second peptide transporter, *VfOPT2*, is expressed mainly in embryos at late-cotyledon stage (Figure 17). As compared to *VfOPT1* expression in cotyledons, *VfOPT2* transcript level is lower (it only be detected by RT-PCR and not by northern analyses) and peaks slightly later during embryo development. An *OPT* of *A. thaliana* is constitutively expressed in all plant organs, including embryos at the heart stage of development (Rentsch *et al.*, 1995; Song *et al.*, 1996), and a reduction on its expression level seems to be responsible for delayed flowering and arrestment of seed development in anti-sense transgenic plants (Song *et al.*, 1997). High expression levels of an *OPT* was found in germinating barley grains, when storage proteins are degraded and used to nourish the seedling (West *et al.*, 1998). Activities of peptide transporters seem to be related to moments of intensive proteolysis, such as wounding, senescence and degradation of storage proteins, when the transport of peptides assumes a great importance for the rapid export of organic nitrogen (Higgins and Payne, 1978; reviewed by Frommer *et al.*, 1994a). Assuming that expression of *OPT*s in cotyledons may also be related to high levels of protein hydrolysis,

it is worth mentioning that these *OPT*s are highly regulated during late seed maturation when leaf senescence in legumes is initiated (e.g. Crafts-Brandner *et al.*, 1996). In soybeans an increase in amino acid uptake was temporarily related to leaf senescence and appears to permit yellow cotyledons to accumulate amino acids, C and N beyond the cessation of sucrose import (VerNooy *et al.*, 1986). A possible correlation between expression of *VfOPT1* and *VfOPT2* in cotyledons and proteolysis of transiently accumulated proteins remains to be confirmed. On the other hand, there is apparently no correlation between *OPT* expression profiles in developing cotyledons and protease genes active in the seeds, once several protease genes are already expressed in young cotyledons (Fischer *et al.*, 2000) and seed coats (Batchelor *et al.*, 2000).

Taken together, the present results show that during seed development of *V. faba*, amino acid and peptide transporters are differently expressed and under developmental control. Active uptake of amino acids seems to play a more important role at seed filling phase and beginning of storage protein synthesis, whereas peptides transporters would play a role later, when high amounts of storage proteins have already been deposited in the cotyledons.

### 4.3.2. VfAAP1 *is co-expressed with storage protein genes in the cotyledon storage parenchyma, and not as other transporters, including VfOPT1, in the transfer cells*

*In situ* hybridization revealed that *VfAAP1* is expressed throughout the storage parenchyma tissue of the cotyledons, but not in the epidermal cell layer. A similar pattern was seen for a *vicilin* storage protein gene. In contrast, *VfOPT1* expression was restricted to the cotyledon epidermal cells, similar to the expression of *VfSUT1*. Epidermal transfer cells play a key role in the active uptake of assimilates by the cotyledons (Gunning and Pate, 1974; Offler *et al.*, 1989; Weber *et al.*, 1998a). In pea cotyledons, an amino acid transporter was reported to be expressed in this cell layer (Tegeder *et al.*, 2000). *VfAAP1* mRNA distribution in cotyledons is peculiar in this regard because it was not related to transfer cells but to the storage parenchyma. The presence of an amino acid transporter in this latter tissue has been hypothesized by De Jong and Borstlap (2000b) to explain discrepant uptake rates of L-valine and sucrose by pea cotyledon vesicles. Speculations about a possible need of an active transport system in these symplasmically connected cells would include relocation of amino

acids within the cotyledons and/or retrieval of 'leaked' amino acids from intercellular spaces (Ruiter *et al.*, 1984). Symplasmic active transport of sucrose in phloem conduits has been proposed so as to drive the mass flow movement by increasing the hydrostatic pressure difference (Lalonde *et al.*, 1999). Because other osmolytes, such as amino acids, also contribute to the driving force of mass flow, VfAAP1 may be involved in a similar mechanism within *V. faba* cotyledons. This possible role would differ from that proposed for hexoses and sucrose carriers of *V. faba*, whose expression is restricted to the transfer cells (Weber *et al.*, 1997b). Although several parallels can be drawn for the uptake of sugars and nitrogenous compounds by the cotyledons, it is worth mentioning that there are also notable differences between these mechanisms, and that the transfer cell layer may play different roles on the active transport of these assimilates. Firstly, the plasma membranes are more permeable to amino acids than to peptides and sugars (De Jong and Wolswinkel, 1995), and secondly, sucrose transporters are already expressed during the early development of a legume embryo (Weber *et al.*, 1997b), meanwhile the main uptake of amino acids happens passively (Lanfermeijer *et al.*, 1990). Together, these observations suggest that the transfer cells are involved in a crucial step for the active uptake of sugars and peptides by cotyledons, whereas the storage parenchyma cells, where storage protein genes are expressed, seem to be the site for active mobilization of amino acids (see scheme in Figure 29).

*VfAAP1* mRNA was also found in the chlorenchyma and thin-walled parenchyma cells of the seed coats. The thin-walled parenchyma cells exhibit numerous wall ingrowth projections and are responsible for the exchange of assimilates between the coat and the apoplast (Grusak and Minchin, 1988; Offler and Patrick, 1993; Offler *et al.*, 1989). The mode of assimilate unloading from the 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 it has been shown to be entirely passive (De Jong *et al.*, 1996). Although thermodynamically feasible, no efflux of amino acids could so far 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 coat cells in pea (De Jongand Borstlap, 2000a). 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. A role in retrieving sucrose was attributed to VfSUT1 in this tissue (Weber *et al.*, 1998b). The function of an amino acid permease in the chlorenchyma is unclear, but it evidences once more that *VfAAP1* is expressed in seed tissues that are symplasmically connected.

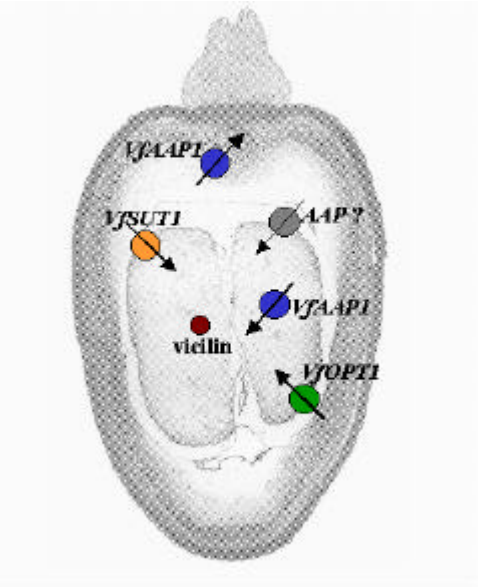


**Figure 29. Scheme showing localization of several transcripts in a transversal section of a *V. faba* seed.**

VfAAP1 *and* vicilin *transcripts are localized in the storage parenchyma of the cotyledons, whereas transcripts of* VfOPT1 *and* VfSUT1 *are found exclusively in the transfer cell layer of the cotyledons.* VfAAP1 *and* VfSUT1 *are also expressed in the seed coats. Whether another AAP is expressed in the cotyledon epidermis is unknown.*

## 4.4. REGULATION OF *VFAAP1* IS MEDIATED BY AMINO ACIDS

### 4.4.1. *Assimilates modulate* VfAAP1 *and storage protein gene expression in an antagonistic manner*

In cotyledons, *VfAAP1* is repressed and the *vicilin* gene is induced in the presence of glutamine, whereas both genes are up-regulated by sucrose (Figure 20). Another storage protein gene, the *LegB4*, is also up-regulated by glutamine (Weber *et al.*, 1998a). It has been shown that a decrease in C or N assimilates induces several genes involved in their acquisition, while abundance of these resources induces genes associated with their use and storage (reviewed by Koch, 1996; Weber *et al.*, 1998b). This seems to be valid for *VfAAP1* as

well. Changes in the *VfAAP1* expression level are more pronounced in the presence of sucrose. Similarly, Nielsen *et al.* (1998) found that the rate of protein accumulation under amino acid incubation was higher when sucrose was present, which suggests a correlation between C and N contents. The C/N ratio of assimilates exported from the vegetative tissues is correlated to the level of storage protein accumulation (Barneix *et al.*, 1993). The exact mechanism of this interaction is unknown, but it may be related to the fact that $H^+$-ATPases are induced by sugars and energize several processes in the cells (Mito *et al.*, 1996). In yeast, genes of the general amino acid transport system 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, the regulatory processes seen in the present experiments could also have taken place intracellularly since Weber *et al.* (1998b) found that cotyledons cultured in the presence of glutamine had higher contents of free amino acids, suggesting a higher uptake rate under these conditions.

During *V. faba* seed development, the contents of free amino acids and sucrose increase rapidly during the filling phase. Amino acids concentration drops drastically during protein synthesis, whereas sucrose concentration remains high for a longer time before dropping as well (Weber *et al.*, 1996). Sucrose confers regulatory control for several processes in the plant (e.g. Nielsen *et al.*, 1998), and it functions as a signaling molecule the beginning of the storage activity (Weber *et al.*, 1998a, b; and review by Wobus and Weber, 1999). Differentiation and storage processes in the embryo are triggered by a high sucrose to hexoses ratio (Weber *et al.*, 1995). On the other hand, little is known concerning possible roles of amino acids as signals for seed development. The present results suggest that the switch between amino acid import and storage protein synthesis may be controlled, at least to some extent, by amino acid concentration acting on the regulation of genes involved in these processes (Figure 30).

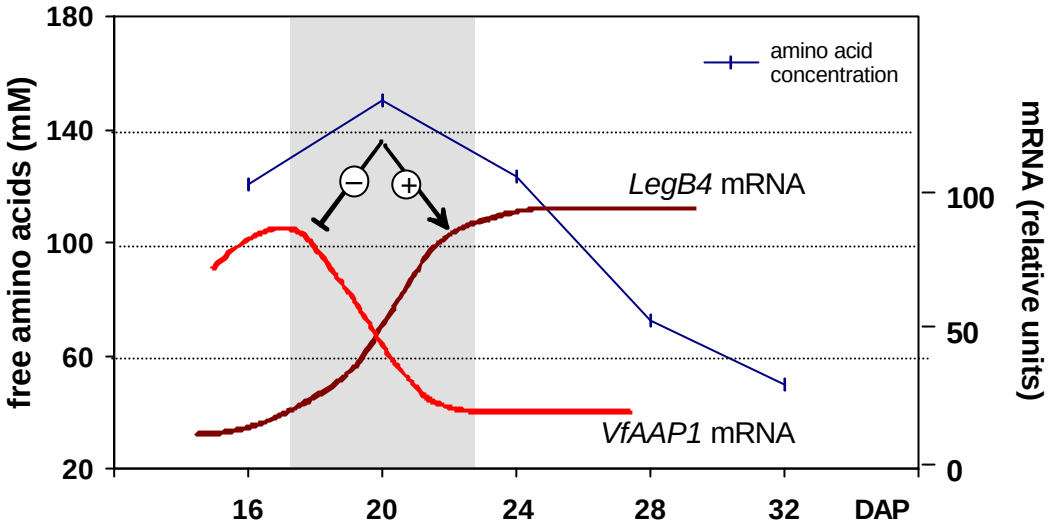


**Figure 30: Concentration of free amino acids and m-RNA levels of *VfAAP1* and *LegB4* during cotyledon development.**

*Amino acid concentration in cotyledons increases during seed filling and decreases rapidly during protein synthesis. Its peak coincides with a decline in* VfAAP1 *expression and an increase in* LegB4 *expression, which may regulate the switch between amino acid import and storage protein synthesis. The shaded area corresponds to the switch between intensive amino acid import and synthesis of storage proteins.*

### 4.4.2. *Transcriptional down-regulation of the* VfAAP1 *promoters in the presence of cysteine*

Genomic analyses revealed the existence of at least two *VfAAP1* copies (or highly similar genes), whose differences are presently seen only in their 5'-upstream region and first intron, but not in the first 340 bp of their coding region. These findings are in agreement with Southern analyses, which suggested that *VfAAP1* may be present in a low copy number (Figure 11). For characterization of the two *VfAAP1* upstream regions (*5'-URa* and *5'-URb*), transient GUS expression assays were performed in embryogenic *N. plumbaginifolia* protoplasts, a system that has been previously used for the analyses of heterologous promoters

(Reidt *et al.*, 2000). GUS activities were induced by both *5'-UR*s, indicating that these regions contain functional promoter elements (Figure 22). To analyze whether this system is suitable also for studying a possible transcriptional regulation of the *VfAAP1* promoters upon amino acid supply, cysteine was added to the culture media after transformation of protoplasts. Cysteine was chosen because this amino is preferably taken up by VfAAP1. As a control, GUS activity driven by the *USP* promoter was initially measured, and shown to be induced in the presence of cysteine (Figure 23), as it is expected since this gene is regulated similarly to storage protein genes which are normally induced by amino acids (reviewed by Motto *et al.*, 1997). It is important to note that the conditions applied under amino acid starvation are considered standard for this protoplast culture system and non-limiting. In protoplasts transformed with the *GUS* reporter gene driven by either *VfAAP1 5'-URa* or *5'-URb*, GUS activities are drastically reduced in the presence of cysteine (Figure 24), pointing to a regulatory mechanism of the *VfAAP1* genes acting at the transcriptional level. In support of this, transcription of several amino acid permease genes in yeast, such as *GAP1*, is induced under poor nitrogen supply (Grenson, 1992). Additionally, amino acid transport in yeast is regulated by targeting and turnover of the proteins. Similarly, genes of the neutral amino acid transport system found in animals are down-regulated by amino acids at the transcriptional and post-transcriptional levels. A model proposed for the transcriptional regulation of these genes includes an extracellular sensor for amino acids and an amino acid inducible transporter repressor protein. Despite experimental evidence, such a protein that represses amino acid transporter genes remains to be isolated (reviewed by Laine *et al.*, 1996). Whereas an external sensor for amino acids has been isolated in yeast, which is structurally similar to the amino acid transporter proteins themselves (Iraqui *et al.*, 1999). A comparable model via an external sensor structurally related to the transporters has been proposed for the regulation of plant sucrose transporters, implying that these proteins exercise a dual function on sugar transport and sensing (Lalonde *et al.*, 1999). It is possible that transcription of plant *AAP*s responds to similar sensing and regulatory mechanisms, as in the model shown in Figure 31. Any possible post-transcription regulation of these permeases remains to be shown.

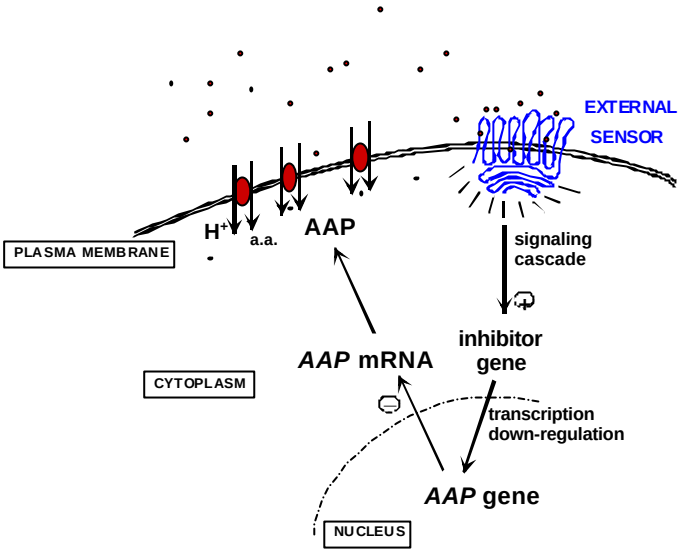


**Figure 31: A model for amino acid-dependent transcriptional repression of an AAP gene.**

*By analogy with amino acid transporters from mammal cells and plant sucrose transporters, a possible mechanism for* AAP *transcriptional regulation may involve amino acid sensing by external sensors that trigger a signaling cascade (see Iraqui* et al.*, 1999), and repress transcription of the* AAP *genes. An intermediary step may involve the induction of a repressor gene. Adapted from Laine* et al. *(1996) and Lalonde* et al. *(1999).*

Among potential regulatory elements, a GCN4-like motif found in both *VfAAP1 5'-UR*s at position – 593 to – 587 (Figure 13) is a likely candidate for conferring amino acid responsiveness to this permease (Wolfner *et al.*, 1975; Müller and Knudsen, 1993). In agreement to the present observations, it has been shown that, upon amino acid supply, the GCN4 elements act as enhancers in several storage proteins genes (Müller and Knudsen, 1993) or as silencers in amino acid biosynthetic and non-storage protein genes (Cock *et al.*, 1992; Fiedler *et al.*, 1993). Moreover, it is curious that *VfAAP1 5'-URa* and *5'-URb* differ from each other mainly by three insertions/deletions within < 130 bp region that generate potential regulatory elements in one of the *5'-UR*s and not in the other, i.e. *5'-URb* carries an endosperm-like (E-) motif (Kreis *et al.*, 1985), which is absent in *5'-URa*, whereas *5'-URa* carries a putative RY-element (Bäumlein *et al.*, 1992) that was not present in *5'-URb* (see Figure 13). Moreover, the RY-element of *5'-URa* is located 99 bp upstream a potential

TATA-box, which is an usual requirement for functional RY-motifs (Reidt *et al.* in press). The down-regulatory effect of cysteine is proportionally more pronounced in *VfAAP1 5'-URb* than in *5'-URa*, as result of a higher activity of the *5'-URb* promoter under standard conditions, i.e. under amino acid starvation, rather than its weaker activity in the presence of cysteine (see Figures 22 and 24). The RY-motif plays a key role in seed-specific gene regulation, and mutations in its sequence abolish promoter seed specificity and causes expression in leaves (Bäumlein *et al.*, 1992; Fujiwara and Beachy, 1994; Reidt *et al.*, 2000). So far, it is unclear whether the RY-motif present in VfAAP1 *5'-URa* but not in *5'-URb* is involved on the regulation of these genes, however, preliminary results show that the *5'-UR*s react differently to the presence of transcription factors known to bind the RY-motif, and may indicate a regulatory function of this motif (unpublished results). For understanding the physiological relevance of the two types of *VfAAP1* upstream regions, it is imperative to examine whether these promoters shown any difference on tissue specificity in the plant.

## 4.5. CHANGES IN N CONTENT AND PARTITIONING IN SEEDS OF TRANSGENIC PLANTS OVEREXPRESSING *VFAAP1* UNDER CONTROL OF A STORAGE PROTEIN GENE PROMOTER

Overexpression of *VfAAP1* cDNA in *V. narbonensis* plants was driven by the *LegB4* promoter. This promoter confers expression in the storage parenchyma of maturing cotyledons during mid to late developmental stages of seed development, and has been used in several instances for transformation of *V. narbonensis* plants (Weber *et al.*, 1998b; Weber *et al.*, 2000). Northern analyses of seeds at late development (stage VII; see Figure 1) showed that *VfAAP1* is overexpressed in some lines up to 10 fold higher compared to wild type seeds (Figure 26a). *VfAAP1* overexpression is concomitant with an increase in the mRNA level of the endogenous *LegB4* gene (Figure 26b). Expression of the *LegB4* gene, as well as of several other storage protein genes, is up-regulated by amino acids (Figure 20; Weber *et al.*, 1998c) and by sucrose (Weber *et al.*, 1996). It is likely that similar mechanisms are responsible, directly or indirectly, for the effects seen here in regard to the up-regulation of the *LegB4* gene. On the other hand, it is less likely that these effects were caused by the introduction of the foreign *LegB4* promoter alone, since transgenic lines that did not overexpress the *VfAAP1* transgene had normal levels of *LegB4* endogenous mRNA (data not shown), suggesting that

induction of the endogenous *LegB4* gene depends on the overexpression of *VfAAP1*. Total N is positively correlated to sulfur contents in the *VfAAP1* overexpressing seeds, which corresponds well to the properties of this permease seen in the yeast mutant regarding the preferable uptake of cysteine. In plants grown under inadequate sulfur supply, total protein is usually reduced, but the effects of the deficiency are largely confined to the synthesis of sulfur-rich proteins, such as legumins, and less or no effect is seen on sulfur-poor proteins, e.g. vicilins (Evans *et al.*, 1985). In sulfur deficient peas, legumin accumulation is drastically reduced as a direct consequence of reduced level of mRNA (Higgins, 1984; Evans *et al.*, 1985). Similarly, Beach *et al.* (1985) reported that after recovery from sulfur deficiency the level of *legumin* mRNA increased 20-fold. It remains to be shown whether or not the expression of genes encoding sulfur-poor storage proteins has been altered in the *VfAAP1* overexpressing seeds.

Because the plants studied here were of the $F_3$ generation and therefore unlikely to be homozygous lines (see Weber *et al.*, 2000), and because several pertinent parameters remain to be investigated, the following discussion should be considered of preliminary nature. Biochemical analyses of *VfAAP1* overexpressing lines showed that their mature seeds accumulate more total nitrogen, sulfur and globulins, but not albumins (Figure 28). Although it remains to be proven that amino acid uptake in *VfAAP1* overexpressing seeds is indeed more efficient, higher amino acid concentration seems to be a plausible explanation triggering processes that led to a N increase in the mature seeds. This is substantiated by a number of experimental evidences as follows. Synthesis and accumulation of storage proteins in seeds is highly dependent on N compounds, mainly amino acids, made availability of to the seeds (Barneix *et al.*, 1992; Hayati *et al.*, 1996). Higher levels of amino acids in seeds could arise from increased supply (Lohaus and Moellers, 2000) or from more effective uptake (Golombek *et al.*, 2001). In *V. faba* cultivars, significant correlations between protein and free amino acids have been found (Barratt, 1982), which are positively correlated to the capacity of amino acid uptake by the cotyledons (Golombek *et al.*, 2001).

Increases in N and sulfur correlate inversely to starch accumulation (Figure 28). Therefore, the effects of *VfAAP1* overexpression do not seem to be restricted to N accumulation and genes involved in it, but seem to affect also C flow and starch biosynthesis. Surprisingly, total carbon and sugars are not significantly changed, indicating a reduction of C flux to starch and accumulation of other sugars. Starch reduction is coupled to a loss in dry weight and to a wrinkled phenotype. Transgenic *V. narbonensis* seeds with similar appearance and increased

sucrose to starch ratio have been previously reported by Weber *et al.* (1998a; 2000). The wrinkled phenotype may be caused in a similar manner as the *rugosus* character of the peas mutants studied by Mendel. In these mutant seeds, total sugars are kept unaltered, but the proportion of starch is lower and of sucrose is higher, causing a higher uptake of water and thereby an increase in seed size. During seed maturation a greater loss of water occurs, resulting in the wrinkled phenotype (reviewed by Bhattacharyya *et al.*, 1993). Higher uptake of water in response to higher concentration of soluble sugars has been demonstrated following antisense inhibition of an enzyme of the starch biosynthetic pathway in *V. narbonensis* (Rolletschek *et al.*, submitted). The relative proportion of starch and proteins in the seeds, i.e. C/N ratio, is determined by the nutrient supply, the sink demand, and the interaction between them. The biochemical and physiological backgrounds of this relationship are complex and not fully understood (reviewed by Motto *et al.*, 1997). Despite evidence that carbon and nitrogen metabolisms are not tightly linked (Hayati *et al.*, 1996), the level of storage protein accumulated in barley grains is dependent on the C/N ratio exported from the vegetative tissues (Barneix *et al.*, 1993). The molecular mechanisms of this interaction are still unclear, but it has been known that N-limited plants typically show an accumulation of starch and reduction in amino acid contents (Nielsen *et al.*, 1998; Paul and Driscoll, 1997), and that the flow into starch responds inversely to low or high nitrogen conditions (Champigny *et al.*, 1992). Evidence suggests that starch metabolism may be regulated at the transcriptional level by an interaction between the C and N status of the plant (Nielsen *et al.*, 1998). By antisense-inhibition of a key enzyme in the starch biosynthetic pathway, Weber *et al.* (2000) showed that a decrease in carbon flow from sucrose to starch leads to a higher protein accumulation. However, it was unclear whether this increase was related to changes in carbon partitioning, nitrogen uptake and/or duration of seed filling period. Similarly, pea mutants seeds that have increased protein contents show a reduction on starch accumulation (Perez *et al.*, 1993; Boutin *et al.*, 1998). The present results are in general agreement with the presumed role of VfAAP1 in supplying amino acids to build up storage proteins and to previous observations concerning N and C partitioning in seeds. They also suggest that the rate of protein accumulation in cotyledons may be limited, at least in part, by the capacity of the cotyledons to take up amino acids. In addition, is has been shown that amino acid loading and translocation into and through the phloem are decisive factors for protein accumulation is the seeds, and that the total content of amino acids in the phloem correlate positively with the contents of deposited protein (Lohaus and Moellers, 2000). Together these data corroborate

that both amino acid availability and uptake capacity are limiting for final accumulation of proteins. A number of questions remains, however, to be answered in regard to the VfAAP1 overexpressing seeds, for instance whether embryo development was altered, if and how amino acid concentration and/or uptake during embryo development was increased, and whether accumulation of the various storage proteins was equally affected.

# CHAPTER 5

# CONCLUSIONS

As in other higher plants, the present results show that amino acid permeases and oligopeptide transporters of *Vicia faba* are members of gene families. VfAAP1, VfAAP3 and VfOPT1 are functionally active and promote uptake of amino acids and peptides, respectively in a yeast heterologous system. Similar to the *A. thaliana* AAPs, VfAAP1 and VfAAP3 have high substrate affinity and show different substrate specificity, i.e. VfAAP1 transports preferentially cysteine, whereas VfAAP3 has a preference for lysine and arginine. OPTs, on the other hand, are known to have low substrate specificity, being able to transport a wide range of di- and tri-peptides. Different expression patterns indicate that *AAP*s and *OPT*s are distinctly regulated. Accumulation of *VfAAP1* mRNA is highest in developing cotyledons, suggesting that this transporter plays an important role on providing developing embryos with amino acids that will be used for the synthesis of storage proteins. Within the cotyledons, *VfAAP1* transcripts are distributed in the storage parenchyma tissue, but not in the epidermal transfer cell layer. Previous works have demonstrated that transcripts of sugar and amino acid transporters are localized to the transfer cells of legume cotyledons, which implies a role on assimilate uptake from the surrounding apoplastic space. *VfAAP1* mRNA distribution is

peculiar in this sense, once it is found in the storage parenchyma of cotyledons, which is a symplasmically connected tissue, and not presumed to be associated to active transport processes. *VfAAP3* is high expressed in vegetative tissues analyzed and also in seed coats, and may therefore play a 'housekeeping' role on the transport of amino acids. *VfOPT1* transcripts were abundant in vegetative tissues as well as in cotyledons during late stages of development. Similar to other transporters, within the cotyledons, *VfOPT1* mRNA in only detectable in the transfer cells. Another peptide transporter, *VfOPT2*, is also expressed in late developing cotyledons, but at lower levels.

In developing cotyledons, the maximum expression level of *VfAAP1*, the increase in the concentration of free amino acids and the appearance of storage protein gene transcripts happen in a coordinate manner. Because amino acid supply modulates *VfAAP1* and storage protein genes in an antagonistic manner, the present results provide evidence that amino acid concentration may, to some extent, influence the switch between amino acid import and storage protein synthesis. Moreover, it was shown that amino acid supply modulates the activity of the *VfAAP1* promoters, indicating that this gene is regulated, at least in part, at the transcriptional level.

Legume seeds are of great economical importance, and understanding the function and the mode of regulation of amino acid and peptide transporters in relation to protein synthesis in these seeds is of high interest for future genetic manipulation programs. Preliminary results showed that seeds overexpressing the *VfAAP1* cDNA under control of a strong seed specific promoter have increased nitrogen and globulin contents, and a decrease in starch accumulation. These observations suggest that partitioning of C and N assimilates may be manipulated by changing the expression levels of *AAP*s in seeds. Finally, VfAAP1 may be an useful tool for breeding strategies, since this permease has a preference for cysteine, a sulfur containing amino acid, which is considered a desirable nutritional characteristic.

# CHAPTER 6

# SUMMARY

Three amino acid permeases (*AAP*) and two oligopeptide transporters (*OPT*) were isolated from seed-specific libraries of *Vicia faba*. *VfAAP1* (1590 bp), *VfAAP3* (1878 bp), *VfAAP4* (1450 bp) and *VfOPT1* (2028bp) were full length cDNAs and encoded predicted proteins with 486, 475, 481 and 584 amino acid residues, respectively. The second peptide transporter cDNA, *VfOPT2* (1014 bp), carried an incomplete open reading frame. VfAAP1, VfAAP3 and VfAAP4 predicted proteins sharing up to 66% identity among themselves, while VfOPT1 and VfOPT2 were 42.2% identical. AAPs and OPTs share no obvious similarities. Functional characterization in the *plas*23-4B yeast mutant, which is deficient in the uptake of amino acids, showed that VfAAP1 and VfAAP3 have high substrate affinity and transport a broad range of amino acids, whereas VfAAP1 showed a preference for cysteine and VfAAP3 for lysine and arginine. VfOPT1 functionally complemented the mutation in a peptide transporter gene of the *LR2* yeast strain by promoting growth in a medium supplemented with the di-peptide His-Ala. Specific expression profiles were seen for the *AAP*s and *OPT*s. *VfAAP1* was highly expressed in cotyledons at early developmental stages and moderately in gynoecia. 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 to build up storage proteins. *VfAAP3* was most abundantly expressed in maternal tissues, i.e.

in gynoecia, stems, roots, pods, as well as in seed coats at different developmental stages. *VfAAP4* transcripts could be detected by northern hybridizations. *VfOPT1* was strongly expressed in maternal tissues, as well as in cotyledons during late developmental stages. *VfOPT2* transcripts were detected by RT-PCR mainly in cotyledons during late development. Expression of the *OPT*s in cotyledons may be correlated to periods of intensive proteolysis due to leaf senescence. *In situ* hybridization showed that *VfAAP1* mRNA is distributed throughout the cotyledon parenchyma, but not in the epidermal cell layer. It also accumulated in the chlorenchyma and thin-walled parenchyma of seed coats. *VfOPT1* transcripts were localized only in the epidermal cell layer of the cotyledons. To determine whether assimilates can modulate gene expression, cotyledons were cultured *in vitro* revealing that in the presence of sucrose and glutamine, *VfAAP1* and the *vicilin* storage protein gene were, respectively, down- and up-regulated. Genomic analyses through Southern hybridizations suggested that two or more copies of the *VfAAP1* gene may be present, whereas *VfAAP3* seems to be a single copy gene. Differences in the 5' upstream region of *VfAAP1* confirmed the presence of at least two copies of this permease. Two types of *VfAAP1* 5' upstream regions were cloned and fused to the GUS reporter gene and transiently transformed into *Nicotiana plubaginifolia* protoplasts, which showed that these regions are functional promoters. Moreover, these two promoters were down-regulated in the presence of cysteine, suggesting that *VfAAP1* regulation by amino acids may occur at the transcriptional level. Seeds from transgenic *V. narbonensis* plants overexpressing the *VfAAP1* cDNA under control of the *LegB4* promoter had altered contents of nitrogen, proteins, sulfur and starch, implicating that this permease can affect, at least to some extent, the accumulation of storage products in the seeds.

# CHAPTER 7

# ZUSAMMENFASSUNG

Diese Arbeit beschreibt die Isolierung und Charakterisierung von Aminosäure- und Peptidtransportern in sich entwickelnden Samen. Ziel ist es, den Transfer stickstoffhaltiger Assimilate in sich entwickelnde Samen und dessen Regulation besser zu verstehen zu lernen. Drei Aminosäure-Permeasen (AAPs) und zwei Oligopeptid-Transporter (OPTs) wurden aus samenspezifischen Bibliotheken von *Vicia faba* isoliert. *VfAAP1* (1590 bp), *VfAAP3* (1878 bp), *VfAAP4* (1450 bp) und *VfOPT1* (2028bp) sind vollständige cDNAs und kodieren für Proteine mit abgeleiteten Sequenzen von 486, 475, 481 bzw. 584 Aminosäuren. Die zweite Peptid-Transporter-cDNA, *VfOPT2* (1014 bp), besitzt einen unvollständigen offenen Leserahmen. VfAAP1, VfAAPI3 und VfAAP4 sind auf Aminosäureebene bis zu 66% identisch, VfOPT1 und VfOPT2 hingegen zu 42.2%. AAPs und OPTs haben keine offensichtlichen Ähnlichkeiten. Beide Proteinklassen enthalten putative hydrophobe, membranüberspannende Domänen, und sind sehr wahrscheinlich Membranproteine.

Die funktionelle Charakterisierung in der *plas23-4B* Hefe-Mutante mit mangelhafter Aminosäure-Aufnahme zeigte, dass VfAAP1 und VfAAP3 hohe Substrataffinitäten haben und verschiedene Aminosäuren transportieren. VfAAP1 bevorzugt Cystein, dessen Aufnahmerate mehr als zweifach erhöht war gegenüber anderen Aminosäuren. VfAAP3 transportiert mit hoher Aktivität Arginin und Lysin und ist bezüglich Substratspezifität

verwandt mit AtAAP3 und AtAAP5. Weder saure noch aromatische Aminosäuren wurden nennenswert transportiert. VfOPT1 komplementierte funktionell eine Mutation im Peptidtransporter-Gen *PTR2* des Hefestammes *LR2*, gezeigt durch Wachstumsförderung in einem mit His-Ala angereicherten Medium. Dieser Peptid-Transporter ist daher in der Lage, dieses Dipeptid zu transportieren.

Northernanalysen zeigten für *AAP*s und *OPT*s spezifische und entwicklungsabhängige Expressionsmuster. *VfAAP1* ist hoch exprimiert in Fruchtknoten sowie Kotyledonen früherer Entwicklungsstadien. RNA-Mengen in Kotyledonen sind maximal zusammen mit Speicherprotein-Transkripten. Zum Ende der Reifung hin fallen die Gehalte auf ein niedrigeres und konstantes Maß ab. Es wird geschlossen, dass dieser Transporter Aminosäuren bereitstellt zur Speicherproteinsynthese. *VfAAP3* wird stark exprimiert in maternalem Gewebe, z. B. Fruchtknoten, Stengel, Wurzel, Hülse sowie Samenschale zu verschiedenen Entwicklungsstadien. Das impliziert eine "Hausmeisterfunktion" dieser Permease. Die VfAAP4-Isoform konnte durch Northernanalyse nicht nachgewiesen werden. *VfOPT1* wird hoch exprimiert in maternalem Gewebe sowie in Kotyledonen später Entwicklungsstadien. *VfOPT2*-Transkripte wurden durch RT-PCR nachgewiesen und am häufigsten in Kotyledonen später Entwicklungsstadien gefunden. Zusammengefasst zeigen die Expressionsprofile in den Kotyledonen, dass VfAAP1 eine wichtige Rolle während der Vor-Speicherphase spielt und dass beide Peptid-Transporter wichtig für die späte Samenentwicklung sein könnten, wenn Speicherproteine synthetisiert werden. *In situ* Hybridisierungen zeigen, dass *VfAAP1* mRNA im ganzen kotyledonären Parenchym verteilt ist, nicht jedoch in der epidermalen Zellschicht. Das Muster gleicht dem der Speicherprotein-Gene. *VfAAP1* mRNA ist im Chlorenchym und im Dünnwand-Parenchym von Samenschalen angereichert, *VfOPT1*-Transkripte dagegen nur in Transferzellen der Kotyledonen, ähnlich dem Muster von Zucker-Transportern in *V. faba*. Die Verteilung von *VfAAP1* mRNA ist bemerkenswert, da sie nicht mit Transfer- sondern mit Speicherparenchymzellen verknüpft ist. Spekulationen über eine mögliche essentielle Rolle des aktiven Transportsystems in diesen symplastisch verbundenen Zellen würden die Verschiebung von Aminosäuren innerhalb der Kotyledonen und/oder die Rückgewinnung von "ausgelaufenen" Aminosäuren aus Zellzwischenräumen einschließen.

Während der Samenentwicklung von *V. faba* steigt die Konzentration freier Aminosäuren während der Füllphase rapide an und sinkt während der Proteinsynthese drastisch ab. Um herauszufinden, ob Aminosäuren die Genexpression modulieren können, wurden

Kotyledonen *in vitro* in Anwesenheit von Glutamin kultiviert. Transkripte von *VfAAP1* und *Vicilin* wurden durch Glutamin herunter- bzw. heraufreguliert. Das heisst, *VfAAP1*-Expressionsprofil, Konzentration an freien Aminosäuren und Beginn der Speicherprotein-Synthese sind während der Embryonalentwicklung korreliert und Aminosäuren könnten eine regulatorische Kontrolle auf Speicheraktivität ausüben. Um zu untersuchen, ob *VfAAP1* auf Transkriptionsebene reguliert wird, wurde seine 5'-nichtkodierende Region (*5'-UR*) isoliert. Unterschiede in der DNA-Sequenz zwischen den Klonen, die das *VfAAP1 5'-UR* enthielten, wiesen auf mindestens zwei Kopien dieser Permease an. Das bestätigt Ergebnisse aus Southernblotanalysen, wobei *VfAAP1* in einer geringen Anzahl von Kopien vorliegt. Beide Isoformen der *VfAAP1 5'-UR* wurden mit dem *GUS* Reporter-Gen fusioniert und transient in *Nicotiana plubaginifolia* Protoplasten transformiert. GUS-Aktivität in Protoplasten, die eine der *5'-UR*s trugen, wiesen auf Promotor-Aktivität hin. Nach Zugabe von Cystein zum Kulturmedium war die GUS-Aktivität niedriger in Protoplasten, die mit einer der *5'-UR*s transformiert worden waren. Dies deutet daruf hin, dass die VfAAP1 auf Transkriptions-Ebene durch Aminosäuren reguliert wird.

um *VfAAP1* überzuexprimieren wurden *V. narbonensis* Pflanzen mit Konstrukten transformiert, welche die *VfAAP1* cDNA in *sense* Richtung unter Kontrolle des *LegB4*-Promotors trugen. Der Promoter vermittelt eine starke Expression im Speicherparenchym der Kotyledonen. Transgene Samen mit erhöhter *VfAAP1*-mRNA haben höhere Gehalte von Stickstoff, Proteinen und Schwefel. Stärke hingegen war reduziert, Der Gesamtgehalt von Zuckern und Kohlenstoff blieb unverändert, was bedeutet, daß der Fluß von löslichen Zuckern in Stärke reduziert war. Die molekularen Mechanismen dieser Wechselwirkung sind immer noch unklar, doch ist bekannt, dass stickstofflimitierte Pflanzen typischerweise mehr Stärke und weniger Aminosäuren enthalten. Darüberhinaus war das endogene *LegB4*-Gen in diesen Samen ebenfalls heraufreguliert. Synthese und Akkumulation von Speicherproteinen in Samen ist in hohem Maße abhängig von Stickstoff-Verbindungen, hauptsächlich Aminosäuren. Die Änderungen in der Speicherstoffzusammensetzung bestätigt die vermutete Rolle dieser Permease für die Aminosäureversorgung.

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# APPENDIX

## I. CLONE SEQUENCES

### *VfAAP1*

```
ATTAAACACCCTGTAGAAGAATTTGATGACAATGGAGGAGAAGGAAGAACACTCTACAGAAGCTGCAGTTACTTCACACAATGAATCCAA      90
                      M  T  M  E  E  K  E  E  H  S  T  E  A  A  V  T  S  H  N  D  S  K

ATTGTTCGACGATGACGACCGTGTTAAACGAACAGGAACGGTTTGGACAACAAGTTCGCATATAATAACAGCAGTAGTAGGATCAAGAGT     180
  L  F  D  D  D  D  R  V  K  R  T  G  T  V  W  T  T  S  S  H  I  I  T  A  V  V  G  S  G  V

GTTGTCGTTGGCATGGGCGATAGCTCAATTGGGTTGGATAATTGGTTTATCAGTCATGATTTTCTTCAGTCTCATCACTTGGTATACTTC     270
  L  S  L  A  W  A  I  A  Q  L  G  W  I  I  G  L  S  V  M  I  F  F  S  L  I  T  W  Y  T  S

ATCACTCCTATCCGAATGTTATCGAACAGGAGATCCTCATTTCGGGAAAAGAAACTATACTTTCATGGAAGCTGTTCACACCATTCTCGG     360
  S  L  L  S  E  C  Y  R  T  G  D  P  H  F  G  K  R  N  Y  T  F  M  E  A  V  H  T  I  L  G

GGGTTTCTATGACACCCTTTGTGGGATAGTTCAGTACAGCAATCTTTATGGAACTGCAGTAGGATACACAATTGGTGCTTCCATTAGTAT     450
  G  F  Y  D  T  L  C  G  I  V  Q  Y  S  N  L  Y  G  T  A  V  G  Y  T  I  G  A  S  I  S  M

GATGGCAATAAAAAGGTCTAACTGTTTCCATTCCTCGGGAGGAAAAGATGGATGTCGCATTTCAAGCAATCCATACATGATCAGTTTTGG     540
  M  A  I  K  R  S  N  C  F  H  S  S  G  G  K  D  G  C  R  I  S  S  N  P  Y  M  I  S  F  G

AGTAATCCAAATTTTCTTTTCTCAAATTCCAGATTTTCATGAAATGTGGTGGCTCTCAATTGTTGCAGCAATCATGTCTTTCACGTATTC     630
  V  I  Q  I  F  F  S  Q  I  P  D  F  H  E  M  W  W  L  S  I  V  A  A  I  M  S  F  T  Y  S

ATTAATTGGCCTTGGTCTCGCAATTGCCAAAGTTGCAGAAAATGGTTCCTTCAAAGGTAGTATCACAGGAGTAAGCATTGGAACTGTGAC     720
  L  I  G  L  G  L  A  I  A  K  V  A  E  N  G  S  F  K  G  S  I  T  G  V  S  I  G  T  V  T

AGAAGCCCAAAAAGTATGGGGAGTTTTCCAATCTCTTGGCAACATAGCTTTCGCGTATTCATATTCACAAATTCTCATTGAAATTCAGGA     810
  E  A  Q  K  V  W  G  V  F  Q  S  L  G  N  I  A  F  A  Y  S  Y  S  Q  I  L  I  E  I  Q  D

TACCATAAAAAGTCCACCTTCCGAGATGAAAACAATGAAGCAAGCCACAAAGATAAGTATAGGCGTGACAACGATATTTTACATGCTTTG     900
  T  I  K  S  P  P  S  E  M  K  T  M  K  Q  A  T  K  I  S  I  G  V  T  T  I  F  Y  M  L  C

CGGCGGTATGGGCTATGCTGCATTTGGAGACTTGTCACCAGGAAACTTACTCACTGGATTTGGTTTCTATAATCCATATTGGCTCATTTGA     990
  G  G  M  G  Y  A  A  F  G  D  L  S  P  G  N  L  L  T  G  F  G  F  Y  N  P  Y  W  L  I  D

TATTGCTAACGCCGCTCTCATAATTCATCTTGTGGGAGCATACCAAGTTTACGCGCAACCCTTATTTGCTTTCGTCGAGAAAATAAGGAT    1080
  I  A  N  A  A  L  I  I  H  L  V  G  A  Y  Q  V  Y  A  Q  P  L  F  A  F  V  E  K  I  M  I

AAAAAGATGGCCTAAAATAAAAAAGGAATACAAACTTACAATTCCTGGTTTTCGTCCTTACCATCTAAATCTATTTAGATTAATTTGGAG    1170
  K  R  W  P  K  I  K  K  E  Y  K  L  T  I  P  G  F  R  P  Y  H  L  N  L  F  R  L  I  W  R

GACTATATTTGTGATCACAACAACATTTATATCAATGTTGATTCCTTTCTTCAATGATGTTTTGGGATTAATTGGAGCAGCTGGAATTTG    1260
  T  I  F  V  I  T  T  T  F  I  S  M  L  I  P  F  F  N  D  V  L  G  L  I  G  A  A  G  F  W

GCCTTTAACAGTTTATTTTCCAGTGGAGATGTATATCAAACAGAAGAAGATTACAAAATGGAGTTATAAATGGATTAGTATGCAAACTTT    1350
  P  L  T  V  Y  F  P  V  E  M  Y  I  K  Q  K  K  I  T  K  W  S  Y  K  W  I  S  M  Q  T  L

AAGTGTTATATGTTTTGTAGTATCAGTTGTGGCTTTTGTTGGATCAGTGTCAAGTATTGTGGTTGATCTTAAGAAATACAAACGATTCAC    1440
  S  V  I  C  F  V  V  S  V  V  A  F  V  G  S  V  S  S  I  V  V  D  L  K  K  Y  K  P  F  T

AACTGATTATTGAAATCCACTCAACTTTTATTGTATTCTTAAGGGTGTAGAAAGCAACTTTGGGTAGTGATTTATGTGGTTTTTGTTTCA    1530
  T  D  Y

TTATCTATTAATAACAACAGTGCTAGTTAAAAAAAAAAAAAAAAAA                                              1577
```

*VfAAP3*

```
TGCAGCGAGTGGTTTCAATTACAGACGACGACGACTTCAGGCGGCTGGCTGGCGAGGTCGTCGGAGAGGTTTCTCTTGAAG(  90

CTTTCAAATCAATACCACCGAAGATTTTCCGAATCTCTCACTCTGAGATTGAAAATTTTAAGAGAGATTAGGAACCAAATC(  180

GAGCTTTTTCGCACCAGCCACCACTAGCTGCCAGATCTGTAAAAGATGGTAGAAAACATTTCCAGAACAAACCTTAGCTAC(  270
                                                M  V  E  N  I  S  R  T  N  L  S  Y  R  G  D

ACTGGTGGCATCGAGGAAGCCATAGATGACGCACCTTTACAAACTGACTCCAAATTCTATGATGACGATGGCCGTGTTAAA(  360
 T  G  G  I  E  E  A  I  D  D  A  P  L  Q  T  D  S  K  F  Y  D  D  D  G  R  V  K  R  T  G

ACTGTTTGGACTACATGCTCCCACATAATAACAGGTGTGATAGGATCTGGAGTGCTCTCCTTAGCCTGGTCCGTAGCTCAGA  450
 T  V  W  T  T  C  S  H  I  I  T  G  V  I  G  S  G  V  L  S  L  A  W  S  V  A  Q  M  G  W

ATTGCTGGTCCTGCAACCATGATCTTTTTCAGTATTATCACTCTGTATACTTCATCATTTCTCGCTGATTGTTATCGTAGT(  540
 I  A  G  P  A  T  M  I  F  F  S  I  I  T  L  Y  T  S  S  F  L  A  D  C  Y  R  S  G  D  T

GAATTTGGAAAGAGAAACTATACTTTCATGGATGCAGTTCACAATATTCTTGGTGGGCCCAGTGTTAAGATTTGTGGGGTA(  630
 E  F  G  K  R  N  Y  T  F  M  D  A  V  H  N  I  L  G  G  P  S  V  K  I  C  G  V  V  Q  Y

TTGAATCTTTTCGGAAGTGCAATAGGATACAATATTGCGGCTGCCATGAGCATGATGGAAATCAGAAAGTCTTACTGTGTC(  720
 L  N  L  F  G  S  A  I  G  Y  N  I  A  A  A  M  S  M  M  E  I  R  K  S  Y  C  V  H  S  S

CATGGCGAAGACCCGTGTCACGTTTCGGGCAACGCTTACATGATAGCTTTTGGTGTGGCACAACTTTTCTTTTCTCAAATTC  810
 H  G  E  D  P  C  H  V  S  G  N  A  Y  M  I  A  F  G  V  A  Q  L  F  F  S  Q  I  P  D  F

CACAACACGTGGTGGCTCTCAATAGTTGCAGCAGTCATGTCGTTCTTCTATTCTACAATTGCTCTCGCTCTTGGAATTTCCA  900
 H  N  T  W  W  L  S  I  V  A  A  V  M  S  F  F  Y  S  T  I  A  L  A  L  G  I  S  K  V  A

GAAACGGGTACTGTCATGGGTAGCCTCACAGGAATAAGCATCGGGACAGTGACCCCGGCCCAAAAAGTATGGGGGGTTTTC(  990
 E  T  G  T  V  M  G  S  L  T  G  I  S  I  G  T  V  T  P  A  Q  K  V  W  G  V  F  Q  A  L

GGAAATATTGCCTTTGCCTATTCATATTCTTTCATTCTCCTGGAAATTCAGGACACCATCAAATCTCCACCATCGGAAGGAA  1080
 G  N  I  A  F  A  Y  S  Y  S  F  I  L  L  E  I  Q  D  T  I  K  S  P  P  S  E  G  K  A  M

AAGAAGGCTGCAAAGCTAAGTATTGGAGTAACCACCACATTTTATTTGCTCTGTGGCTGCACGGGCTATGCTGCTTTTGGA(  1170
 K  K  A  A  K  L  S  I  G  V  T  T  T  F  Y  L  L  C  G  C  T  G  Y  A  A  F  G  D  A  A

CCAGGTAACCTGCTCGCTGGATTTGGTGTCTCGAAAGCATATATTCTTGTAGATATGGCTAATGCTGCTATTGTAGTTCAC(  1260
 P  G  N  L  L  A  G  F  G  V  S  K  A  Y  I  L  V  D  M  A  N  A  A  I  V  V  H  L  F  G

GCATACCAAGTGTATGCCCAACCCCTCTTTGCCTTCGTTGAGAAAGAGGCAGGAAAAAAAATGGCCCAAAATTGACAAAGGAT  1350
 A  Y  Q  V  Y  A  Q  P  L  F  A  F  V  E  K  E  A  G  K  K  W  P  K  I  D  K  G  F  E  V

AAAATTCCCGGTTTGCCAGTCTACAATCAAAACATATTTATGTTAGTTTGGCGGACAATTTTTGTCATCGTCCCCACATTGA  1440
 K  I  P  G  L  P  V  Y  N  Q  N  I  F  M  L  V  W  R  T  I  F  V  I  V  P  T  L  I  A  M

TTGATTCCCTTTTTCAATGACGTTTTGGGAGTGATTGGAGCATTGGGATTTTGGCCTTTAACTGTTTACTTTCCTGTGGAGA  1530
 L  I  P  F  F  N  D  V  L  G  V  I  G  A  L  G  F  W  P  L  T  V  Y  F  P  V  E  M  Y  I

ATTCAGAAGAAGATCCCAAAATGGAGTAGGAAATGGATTTGTCTGGAAATAATGAGTACTTTCTGCCTCTTCGTTTCTGTTC  1620
 I  Q  K  K  I  P  K  W  S  R  K  W  I  C  L  E  I  M  S  T  F  C  L  F  V  S  V  V  A  G

CTTGGTTCATTGATTGGTGTCTGGATTGACCTGAAGAAATACAAACCATTCAGCTTAGAGAATTGAACACCTCGTAAAATCT  1710
 L  G  S  L  I  G  V  W  I  D  L  K  K  Y  K  P  F  S  L  E  N
```

```
TGTATGTAAATGGCTGTGGGAGGTTCTCAATGGGATGTTGATTTCTTAATTATTCCTAAGATGCAATTAAGAATAATAATCAGTGTTGAA 1800

TATTATGATCCTTTTACTATGCATATTTTAATTGATTTTTGTACCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  1878
```

## *VfAAP4*

```
TACTATGGTTGTTGAGAAGAATGCTAGCAACAATCATCACCAAACATTTTATGTCTCCATTGATCAACAAATAGATTCTAA(  90
     M  V  V  E  K  N  A  S  N  N  H  H  Q  T  F  Y  V  S  I  D  Q  Q  I  D  S  K  F  F  D

CGACGATGGTCGCGTCAAACGAACAGGCACATCATGGACGGCAAGTGCCCATGTAATAACAGCTGTGATAGGATCTGGAGT(  180
  D  D  G  R  V  K  R  T  G  T  S  W  T  A  S  A  H  V  I  T  A  V  I  G  S  G  V  L  S  L

AGCTTGGGCTATAGCTCAGCTTGGATGGATTGCTGGTCCTATTGTCATGTTTCTCTTTGCTTGGGTTACTTATTACACCTC(  270
  A  W  A  I  A  Q  L  G  W  I  A  G  P  I  V  M  F  L  F  A  W  V  T  Y  Y  T  S  V  L  L

CTGTGAATGTTATCGTAATGGCGATCCCGTCAATGGCAAGAGAAACTACACTTACATGGAAGTTGTTCATTCAAATCTCGGT  360
  C  E  C  Y  R  N  G  D  P  V  N  G  K  R  N  Y  T  Y  M  E  V  V  H  S  N  L  G  G  F  Q

AGTCCAATTATGTGGACTAATTCAATATCTCAACCTTGTTGGGGTTGCGATTGGATACACGGTAGCTTCTGCCATAAGCAT(  450
  V  Q  L  C  G  L  I  Q  Y  L  N  L  V  G  V  A  I  G  Y  T  V  A  S  A  I  S  M  M  A  I

TGTAAGGTCAAATTGTTTTCATAGGAGTGGAGGAAAAGATCCATGTCATATGAACAGCAACATTTACATGATTGCATTTGGT  540
  V  R  S  N  C  F  H  R  S  G  G  K  D  P  C  H  M  N  S  N  I  Y  M  I  A  F  G  A  V  Q

AATTATATTCTCTCAAATTCCTGATTTTGATCAATTATGGTGGCTTTCCATCGTTGCTGTTGTTATGTCCTTTACATATTC(  630
  I  I  F  S  Q  I  P  D  F  D  Q  L  W  W  L  S  I  V  A  V  V  M  S  F  T  Y  S  T  I  G

TCTTGGCCTTGGTATTGGAAAAGTTATAGAGAACAAAAAGTTTGCTGGAACCATTACTGGGATAAATGATGTGACAAAAGCT  720
  L  G  L  G  I  G  K  V  I  E  N  K  K  F  A  G  T  I  T  G  I  N  D  V  T  K  A  Q  K  T

TTGGGGGAGTTTGCAAGCTCTTGGTGACATAGCTTTTGCTTACTCATTCTCCATGATTCTTATAGAAATTCAGGATACAATA  810
  W  G  S  L  Q  A  L  G  D  I  A  F  A  Y  S  F  S  M  I  L  I  E  I  Q  D  T  I  K  A  P

ACCTCCATCAGAGTCGAAAACAATGAAGAAGGCTACATTAATCAGTGTTATAGTGACAACATTTTTCTACATGCTTTGCGGT  900
  P  P  S  E  S  K  T  M  K  K  A  T  L  I  S  V  I  V  T  T  F  F  Y  M  L  C  G  C  F  G

ATACGCAGCGTTTGGAAATTCAAGTCCCGGAAATCTTTTAACCGGTTTCGGATTCTATAACCCATTTTGGCTCCTTGACATA  990
  Y  A  A  F  G  N  S  S  P  G  N  L  L  T  G  F  G  F  Y  N  P  F  W  L  L  D  I  A  N  A

TGCCATAGTGATCCACCTTATTGGTGCATACCAAGTTTATTGTCAACCCCTTTTTGCTTTTGTCGAAAATTACACGGCGAAA  1080
  A  I  V  I  H  L  I  G  A  Y  Q  V  Y  C  Q  P  L  F  A  F  V  E  N  Y  T  A  K  R  F  P

AGACAGTGATTTTGTGAACAAGGACGTAAAGATTCCAATTCCCGGTTTAGATAGGTATAAACTCAACCTTTTTAGATTGGTT  1170
  D  S  D  F  V  N  K  D  V  K  I  P  I  P  G  L  D  R  Y  K  L  N  L  F  R  L  V  W  R  T

AGTTTATGTAATACTAACAACTTTGATATCAATGCTCCTTCCATTCTTCAATGATATTGTTGGACTTCTTGGTGCAATTGGA  1260
  V  Y  V  I  L  T  T  L  I  S  M  L  L  P  F  F  N  D  I  V  G  L  L  G  A  I  G  F  W  P

TCTAACTGTTTATTTTCCGGTGGAGATGTATATAATTCAAAAGAAAATACCAAAGTGGAGCACAAAATGGATTTGCCTTCAA  1350
  L  T  V  Y  F  P  V  E  M  Y  I  I  Q  K  K  I  P  K  W  S  T  K  W  I  C  L  Q  L  L  S

TGGTGCGTGTCTTATAATCACTATAGCAGCTACAATTGGTTCTATTGCTGGCCTTATCCTTGATCTTAAAGTTTTTAAGCCA  1440
  G  A  C  L  I  I  T  I  A  A  T  I  G  S  I  A  G  L  I  L  D  L  K  V  F  K  P  F  K  T

TATCTATTA  1450
  I  Y
```

*VfOPT1*

```
GGCACGAGCTATGGGTTCCGTAGAGGACGATTCTTCGCGTTTGGAAGAAGCGCTTATTCAGGATGAAGAAAGTAAGCTATA(  90
          M  G  S  V  E  D  D  S  S  R  L  E  E  A  L  I  Q  D  E  E  S  K  L  Y  T  G  D

TGGCTCGGTCGACTTTAAAGGGAGGCCTGTGCTTAAGAAGAATACTGGCAATTGGAAAGCTTGCCCATTTATCCTAGGCAA1 180
  G  S  V  D  F  K  G  R  P  V  L  K  K  N  T  G  N  W  K  A  C  P  F  I  L  G  N  E  C  C

TGAACGTTTGGCATACTATGGCATTGCAACAAATCTTGTTAAACCTATCTTACTCGCCAAGCTACATGAAGGAAATGTCTC1 270
  E  R  L  A  Y  Y  G  I  A  T  N  L  V  K  P  I  L  L  A  K  L  H  E  G  N  V  S  A  A  R

AAACGTCACCACTTGGCAAGGCACTTGTTACCTTGCACCTCTCATTGGAGCAGTTCTAGCAGATTCTTACTGGGGACGATA(  360
  N  V  T  T  W  Q  G  T  C  Y  L  A  P  L  I  G  A  V  L  A  D  S  Y  W  G  R  Y  W  T  I

TGCCATTTTCTCCATGATTTATTTCATTGGAATGGGTACGTTGACACTTTCTGCTTCTATTCCAGCATTGAAGCCTGCTGA/  450
  A  I  F  S  M  I  Y  F  I  G  M  G  T  L  T  L  S  A  S  I  P  A  L  K  P  A  E  C  L  G

TGCTGTAYGCCCTCCAGCTACTCCTGCACAATATGCTGTGTTCTTCATTGGTCTCTATCTGATTGCGCTTGGGACTGGTGG1 540
  A  V  ?  P  P  A  T  P  A  Q  Y  A  V  F  F  I  G  L  Y  L  I  A  L  G  T  G  G  I  K  P

ATGTGTGTCTTCTTTTGGGGCAGACCAGTTTGATGATACTGATTCGCGGGAAAGGGTTAAGAAGGGATCGTTTTTCAACTG(  630
  C  V  S  S  F  G  A  D  Q  F  D  D  T  D  S  R  E  R  V  K  K  G  S  F  F  N  W  F  Y  F

TTCTATCAACATAGGAGCCCTCATATCAAGCAGTTTTATTGTGTGGATTCAAGAAAATGCAGGCTGGGGTCTTGGATTTGG/  720
  S  I  N  I  G  A  L  I  S  S  S  F  I  V  W  I  Q  E  N  A  G  W  G  L  G  F  G  I  P  A

TTTATTTATGGGATTAGCTATCGGAAGCTTCTTTTTAGGCACACCCCTATATAGGTTTCAAAAACCAGGAGGAAGCCCTCT1 810
  L  F  M  G  L  A  I  G  S  F  F  L  G  T  P  L  Y  R  F  Q  K  P  G  G  S  P  L  T  R  M

GTGCCAGGTTGTGGCTGCATCTTTTCGGAAACGGAATCTTACTGTTCCTGAAGATAGTAGTCTCCTCTATGAGACACCAGA(  900
  C  Q  V  V  A  A  S  F  R  K  R  N  L  T  V  P  E  D  S  S  L  L  Y  E  T  P  D  K  S  S

TGCAATTGAAGGAAGTCGGAAACTACAGCATAGCGATGAATTAAGGTGTCTTGACAGAGCAGCTGTAATCTCTGATGATGA/  990
  A  I  E  G  S  R  K  L  Q  H  S  D  E  L  R  C  L  D  R  A  A  V  I  S  D  D  E  R  K  R

AGGTGACTATTCTAATTTGTGGAGACTTTGCACTGTGACACAGGTGGAGGAATTAAAAATCTTGATCCGCATGTTTCCAGT1 1080
  G  D  Y  S  N  L  W  R  L  C  T  V  T  Q  V  E  E  L  K  I  L  I  R  M  F  P  V  W  A  T

TGGGATAGTTTTTTCTGCTGTCTATGCTCAGATGTCAACAATGTTTGTGGAACAAGGAACTATGATGGACACTAGCGTTGG1 1170
  G  I  V  F  S  A  V  Y  A  Q  M  S  T  M  F  V  E  Q  G  T  M  M  D  T  S  V  G  S  F  K

AATACCAGCAGCTTCCCTCTCAACTTTTGATGTAATCAGTGTTATTTTCTGGGTCCCTGTCTATGACAGGTTTATAGTTCC(  1260
  I  P  A  A  S  L  S  T  F  D  V  I  S  V  I  F  W  V  P  V  Y  D  R  F  I  V  P  I  A  R

GAAATTTACTGGCAAAGAAAGGGGATTTTCAGAGTTGCAAAGAATGGGAATTGGTCTTTTTATTTCAGTCYTGTGCATGTC/  1350
  K  F  T  G  K  E  R  G  F  S  E  L  Q  R  M  G  I  G  L  F  I  S  V  L  C  M  S  A  A  A

TATTGTCGAGATTAAGCGTCTGCAGCTTGCCAAAGAGCTTGACCTTGTTGATAAAGCTGTCCCCGTACCACTTACTATATT1 1440
  I  V  E  I  K  R  L  Q  L  A  K  E  L  D  L  V  D  K  A  V  P  V  P  L  T  I  F  L  Q  I

CCCCCAGTATTTCTTATTGGGAGCAGCAGAAGTATTCACATTTGTGGGGCAGCTTGAGTTCTTCTATGACCAATCTCCAGA1 1530
  P  Q  Y  F  L  L  G  A  A  E  V  F  T  F  V  G  Q  L  E  F  F  Y  D  Q  S  P  D  A  M  R

AAGTTTATGCAGTGCTCTGTCACTTCTGACTACTTCGCTCGGAAATTACTTGAGTTCATTCATTCTCACTGTGGTACTTTA(  1620
  S  L  C  S  A  L  S  L  L  T  T  S  L  G  N  Y  L  S  S  F  I  L  T  V  V  L  Y  F  T  T

ACGAGGGGGAAATCCTGGATGGATTCCGGATAACTTGAACAAAGGTCATCTCGATTACTTTTCTGGCTTAGCTGGACTCAG(  1710
  R  G  G  N  P  G  W  I  P  D  N  L  N  K  G  H  L  D  Y  F  S  G  L  A  G  L  S  F  L  N

TATGTTTCTGTACATAGTTGCTGCCAAAAGATACAAGTCAAAGAAGGCTTCGTAAGGTTTTTCAGGCACTTGTTTGTAGTG(  1800
  M  F  L  Y  I  V  A  A  K  R  Y  K  S  K  K  A  S
```

```
TTTCACAATTCCTTCTGTCATTGTCGTGATTGATCTTTATGTAATATAATATGATATTATTCAATTGCGTTGTAAAGGAAA  1890

ATGCCTTGTTCAAAATAGTCCCTGCCCCCTCTTGTGATGGCAGTTTATGTTCACTCCCAAAATTGCAACTATGACACTTGT  1980

TATCTAAAAGTTTGATACCCTTTGATGTGAAAAAAAAAAAAAA  2028
```

## *VfOPT2*

```
TTTGGCGCTGATCAGTTTGATGAGTTTGAGCCCAAAGAGAGATCCTATAAGCTTTCCTTCTTTAATTGGTGGTTTTTTAGCA  90
 F  G  A  D  Q  F  D  E  F  E  P  K  E  R  S  Y  K  L  S  F  F  N  W  W  F  F  S  I  F  V

GGTACCCTTTTCTCCAACACTTTCCTAATCTACATACAAGACAGAGTGGGTTGGGCTGTAGGGTATGGCCTTCCTACCGCC(  180
 G  T  L  F  S  N  T  F  L  I  Y  I  Q  D  R  V  G  W  A  V  G  Y  G  L  P  T  A  G  L  T

ATTTCAGTATTGGTATTTCTGATAGGAACTCCGTTATATAGGCATAAATTGCCCTCAGGCAGCCCTATAACTAGGATGCTC(  270
 I  S  V  L  V  F  L  I  G  T  P  L  Y  R  H  K  L  P  S  G  S  P  I  T  R  M  L  Q  V  F

GTGGCGTCGATCAGAAAAGTGGAAGGCACGAGTCCAGATGATCCGAAAGAGCTACATGAGTTAAGCATAGATGAGTATGCAT  360
 V  A  S  I  R  K  V  E  G  T  S  P  D  D  P  K  E  L  H  E  L  S  I  D  E  Y  A  Y  N  G

AGAAATCGAATTGATCACAGCTCTTCCTTGAGGTTAAGTCTAATCACTTTTATCTCTTCTAATGTCATGAAAATAGGACAA(  450
 R  N  R  I  D  H  S  S  S  L  R  L  S  L  I  T  F  I  S  S  N  V  M  K  I  G  Q  V  N  N

TTAACTTGCATGCATTACCTCTTCTATGCTTGCAGTTTTCTTGACAAAGCGGCTATGAAGACCAGCCAAACTTCATCATGGA  540
 L  T  C  M  H  Y  L  F  Y  A  C  S  F  L  D  K  A  A  M  K  T  S  Q  T  S  S  W  M  L  C

ACAGTGACACAAGTTGAGGAAACTAAACAAATGACAAAAATGATTCCTATTTTGATTACAACAATTATCCCAAGCACGTTGA  630
 T  V  T  Q  V  E  E  T  K  Q  M  T  K  M  I  P  I  L  I  T  T  I  I  P  S  T  L  I  V  Q

TCAACCACACTCTTCATTAAACAAGGCACCACACTAGACAGGAGTATGGGGCCACATTTTGATATCCCTCCAGCATGTCTCA  720
 S  T  T  L  F  I  K  Q  G  T  T  L  D  R  S  M  G  P  H  F  D  I  P  P  A  C  L  T  A  F

ATAACTATCTTCATGCTAATAAGCATTGTAGTCTACGACCGTGCTTTTGTGCCCATGGTCCGGCGATACACAAAGAATCCCA  810
 I  T  I  F  M  L  I  S  I  V  V  Y  D  R  A  F  V  P  M  V  R  R  Y  T  K  N  P  R  G  I

ACAATGTTGCAGAGATTAGGAATTGGTCTAGTGTTGCACATCGCTATAATGGTCACTGCATGCTTGGCTGAGAGGAAAAGA(  900
 T  M  L  Q  R  L  G  I  G  L  V  L  H  I  A  I  M  V  T  A  C  L  A  E  R  K  R  L  R  V

GCAAGAGAAAACAATCTCTTTGGCCGGCATGATACGATTCCCCTTACAATTTTTATTCTCCTCCCTCAGTTTGCATTGGGA(  990
 A  R  E  N  N  L  F  G  R  H  D  T  I  P  L  T  I  F  I  L  L  P  Q  F  A  L  G  G  V  A

GATAGCTTCGTCGAAATTGCCAAACTAGAGTTCTTCTATGACCAAT(  1041
 D  S  F  V  E  I  A  K  L  E  F  F  Y  D  Q  S  P
```

*VfAAP1 5'-URa*

ACCAAAGAAGTCGAAGAACAACACAAATGTCAAAGTTCATGGATACACTAACTCAAATTTCAGTGGAGATCAAGACGAAAAGAAAAGTAC 90

TGCAAACTACATTTTCATGATCGAAGATGCTCCAATCTATTGGAGTTCGAGGAAGCAAAGAATTGTGACTTTGTCATCATGTGAAGCAGA 180

ATATGTTGCTGCATCATATGATGCATGCCAAGCAGCATGAATCGAAATGCTACTTGAAGAACTCATGATAATGGAACCTAAGAAGATGAA 270

GTTTTTTGTCGACAACAAATCAGCTATTAACCTGGCGAACCACCCAGTGCCATGGTCGAAGTAAACACATAGAAAAAGGTATCATTTTCT 360

GAGGGATCAAGTCAATAAAGGGAAGTTTGAGATAAAGTATTGCAAGACAGAAGTGCAACTAACCGACATATTTATCAAACCTCTGAAGAA 450

AGTCAGATTCGACGAGTTAAAGAAAAGCATCGAGATGAGAAGTCTCGAGAACATGAATTAGGGGGGGGTGTTAGAAGTATAATTCTTGTTC 540

TGTCGAAATGCATGTGTGGTGTATGTCGAATAACTTTTGTATAAGTTATAAGATGTATTCGACAGAGTCGAATACATCAGACATTATAGT 630

ATGTCGAAATCATGTTTTGGGCTTTAGCATATTTTGTCTAAATTAGCTTGGACATTGTTTGCCTATATAAAGGACTGCTGAATTTGTAAT 720

CTCTAAGCAATCAAGAATAACAAACACTCAATTATCTCTTCTCTCTTCTTCTTCTCCTTCTCTTTCATAAATCATCTTTCTTGAATGTTT 810

GTTCCAACGAGATTTAATACGAATCAATCTGAATACTATTACCTAACAATACACACGTGCGGCTGAAAATATAAATTGATTTTAAATTTT 900

TTATTACCTAGGAATAAAAAATCTGTAATGAGTCAAAACAAAATTTTCCATTTCTATTTCATAAAAATCAAATAAATATTAAAATTGTTA 990

TTTTTAGTACTAGTAATTTTATGAAATATGTTTCATCTATTTTAATATTATTGTTTAAACAATAATATTTTTTCGCTATTTTTTAATTTT 1080

CATAAAATTAATAACAAATATTTTTAAAAATATGTCAATGTAATATAACTAATTTATTGCCATCATCGATTTGCATGATTTGCATTACAA 1170

AATTCTTTAAACATTCAACGTCCATAATTTATTGAGGGTAGATGGATAACATATTTCAATTAAATTCTATTTTTAACTAGTATAAAATAA 1260

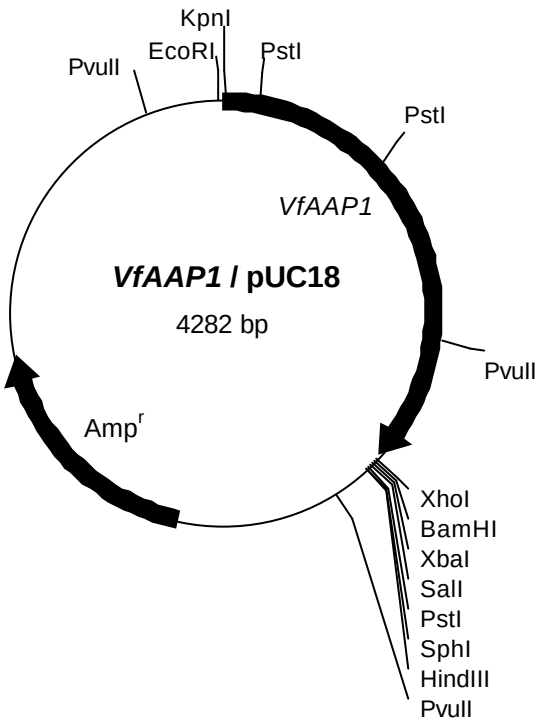
AAAAAATAAAACTCCTATTAATTACCGGTCCCGTCTAAATAAGTAAATAAATATTGTGATCAATATGACAGCGCATACGCCATAATTTTA 1350

AAAACCTTGCTCTGTTGTCAGATGTGTGAAAATGCAATCCAATTGACGGTACAAACAAAATAACAGTAAGGTCATTATCACTACCGAATA 1440

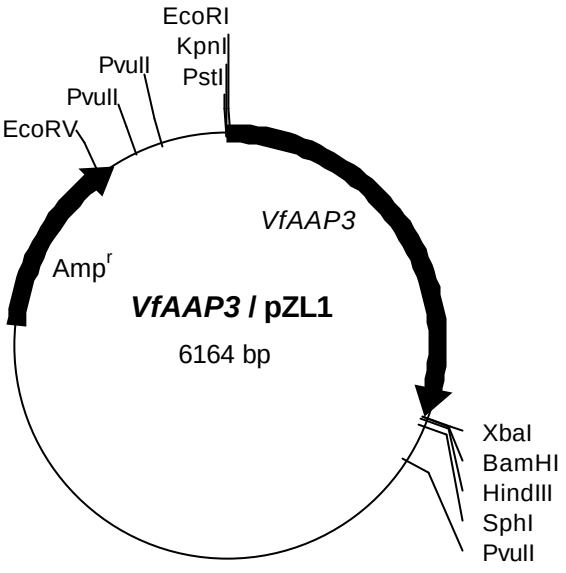
CTCTTTCTCTCAATTATTGTATTTCTTTTTGTCTCTATCTCATAAACCTCGTTTGGAATTAAACACCCTGTAGAAGAATTTG 1522

## II. CLONING MAPS

***VfAAP1* / pUC18**




***VfAAP3* / pZL1**

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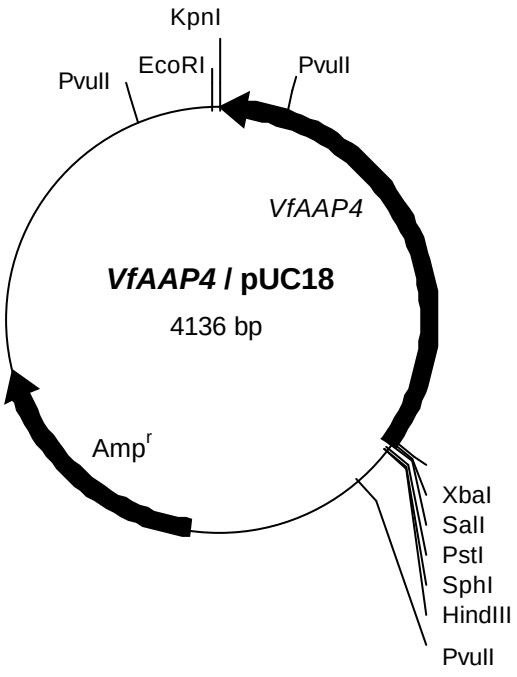
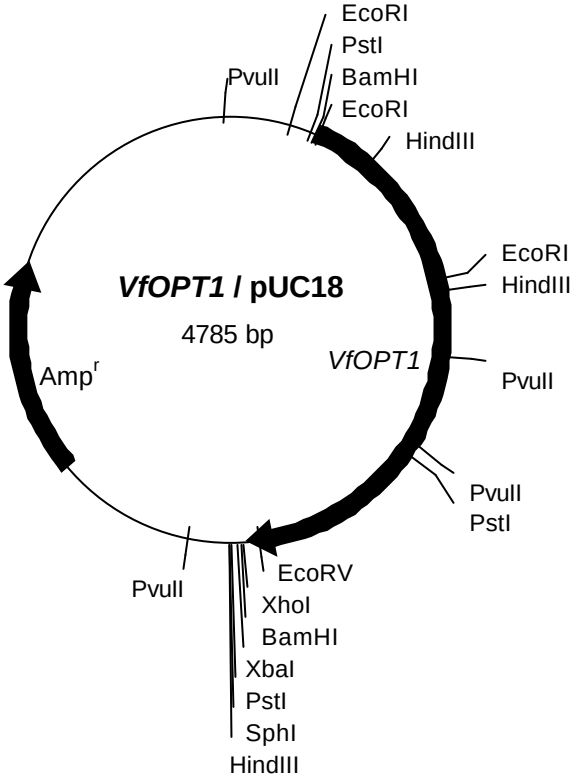



***VfOPT1* / pUC18**



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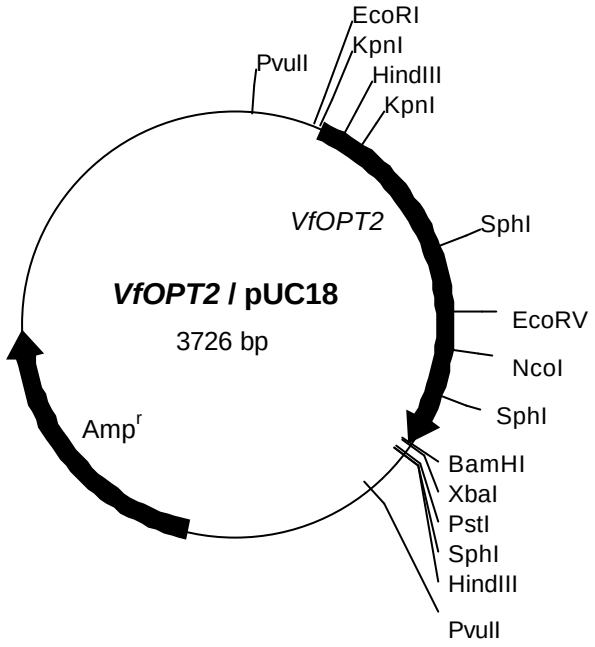
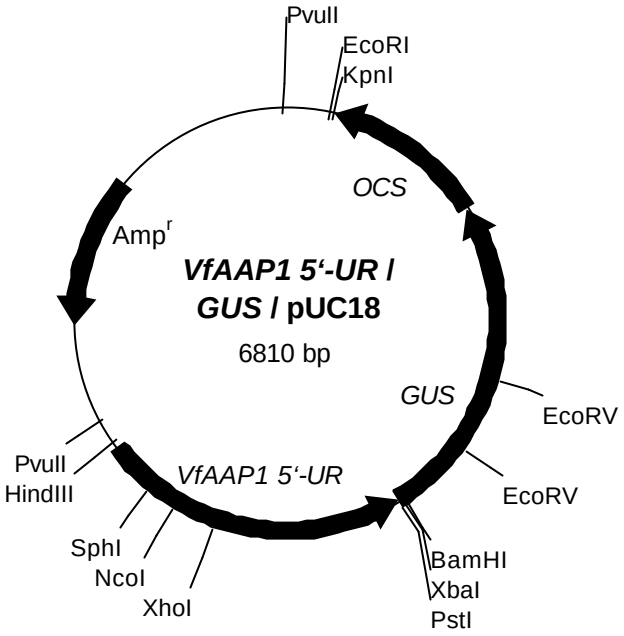
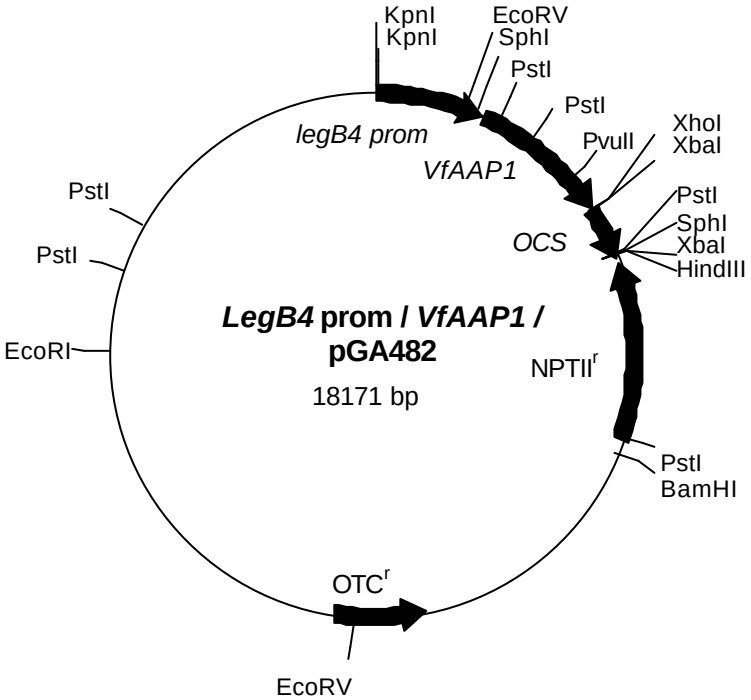



***VfAAP1 5'-UR* / *GUS* / pUC18**




***LegB4* promoter / *VfAAP1* / pGA482**



**ERKLÄRUNGEN**

**GEMÄß § 5 ABS. 2 DER PROMOTIONSORDNUNG VOM 17.09.1998**

Ich habe mich vor Einleitung dieses Promotionsverfahrens noch nie anderweitig um einem Doktorgrad beworben.

Ich habe diese Arbeit selbständig und ohne fremde Hilfe verfaßt und keine anderen als die angegebenen Quellen und hilfsmittel verwendet. Verwendet Quellen und Hilfsmittel sind an den entsprechenden Stellen kenntlich gemacht.

Gatersleben, den 28. Mai 2001

Manoela Miranda

# LEBENSLAUF

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| | |
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| Familienstand: | Ledig |

## II. AUSBILDUNG

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|---|---|
| 1977 – 1982 | Grundschule, Rio de Janeiro und Recife, Brasilien |
| 1983 – 1989 | Gymnasium, Recife, Brasilien |
| 1990 – 1993 | Ciências Biológicas (Studium der Biologie)<br>Universidade Federal de Pernambuco, Recife, Brasilien<br>21. Januar 1994, Abschluß als ‚Bachelor of Science'<br>Dissertation: *Morphological and biochemical characterization of a sweet potato (*Ipomoea batatas *L.) germplasm bank*. |
| 1995 – 1996 | Master Course in Agricultural Science (Agrarwissenschaften)<br>Tokyo University of Agriculture, Tokio, Japan<br>20. März 1997, Abschluß als ‚Master of Science'<br>Dissertation: *Cytogenetial studies in* Citrus, Poncirus *and* Fortunella *species and somatic hybrids.* |
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## III. PUBLIKATIONEN

Die folgende Publikationen worden unhabhandlich von dieser Arbeit veröffentlichen:

MIRANDA M, IKEDA F, ENDO T, MORIGUCHI T, OMURA M (1997) Comparative analysis on the distribution of heterochromatin in *Citrus*, *Poncirus* and *Fortunella* chromosomes. *Chromosome Research* 5: 1-7.

MIRANDA M, IKEDA F, ENDO T, MORIGUCHI T, OMURA M (1997) Chromosome markers and alterations in mitotic cells from interspecfic *Citrus* somatic hybrids analysed by fluorochrome staining. *Plant Cell Reports* 16: 807-812.

MIRANDA M, IKEDA F, ENDO T, MORIGUCHI T, OMURA M (1997) rDNA Sites and heterochromatin in Meiwa kumquat (*Fortunella crassifolia* Swing.) chromosomes revealed by FISH and CMA/DAPI staining. *Caryologia* 50: 333-340.

MIRANDA M, MOTOMURA T, IKEDA F, OHGAWARA T, SAITO W, ENDO T, OMURA M, MORIGUCHI T (1997) Somatic hybrids obtained by fusion between *Poncirus trifoliata* (2x) and *Fortunella hindsii* (4x) protoplasts. *Plant Cell Reports* 16: 401-405.

Gatersleben, den 28. Mai 2001

Manoela Miranda

## PUBLIKATIONEN

Aus den, in dieser Arbeit dargestellten, Ergebnissen sind folgende Publikationen hervorgegangen, die im Text nicht zitiert werden:

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. *The Plant Journal* **28**: 61-71.

MIRANDA M, BORISJUK L, DIETRICH D, RENTSCH D, WOBUS U, WEBER H (in preparation) Characterization of peptide transporters during seed development of *Vicia faba* L.

MIRANDA M, ROLLETSCHEK H, WOBUS U, WEBER H (in preparation) Changes in nitrogen and starch contents in transgenic *Vicia narbonensis* L. seeds overexpressing an amino acid permease.