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Review

Solute pores, ion channels, and metabolite transporters in the outer and inner envelope membranes of higher plant plastids

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Abstract

All plant cells contain plastids. Various reactions are located exclusively within these unique organelles, requiring the controlled exchange of a wide range of solutes, ions, and metabolites. In recent years, several proteins involved in import and/or export of these compounds have been characterized using biochemical and electrophysiological approaches, and in addition have been identified at the molecular level. Several solute channels have been identified in the outer envelope membrane. These porin-like proteins in the outer envelope membrane were formerly thought to be quite unspecific, but have now been shown to exhibit significant substrate specificity and to be highly regulated. Therefore, the inter-envelope membrane space is not as freely accessible as previously thought. Transport proteins in the inner envelope membrane have been characterized in more detail. It has been proved unequivocally that a family of proteins (including triose phosphate-/phosphoenolpyruvate-, and glucose 6-phosphate-specific transporters) permit the exchange of inorganic phosphate and phosphorylated intermediates. A new type of plastidic 2-oxoglutarate/malate transporter has been identified and represents the first carrier with 12 putative transmembrane domains, to be located in the inner envelope membrane. The plastidic ATP/ADP transporter also contains 12 putative transmembrane domains and possesses striking structural similarity to ATP/ADP transporters found in intracellular, human pathogenic bacteria.

Keywords: Plastid envelope membrane; Transport protein; Channel; Porin

1. Introduction

All members of the plastid organelle family conduct vital biosynthetic functions in every plant cell. Plastids are surrounded by two membranes, referred to as the inner and the outer envelope, which sepa-

Abbreviations: AAC, mitochondrial ADP/ATP carrier; AGPase, ADP-glucose pyrophosphorylase; AATP, ATP/ADP transport protein; DHAP, dihydroxyacetone phosphate; FBPase, fructose 1,6-bisphosphate phosphatase; GAP, glyceraldehyde 3-phosphate; Glc1P, glucose 1-phosphate; Glc6P, glucose 6-phosphate; GOGAT, glutamine/2-oxoglutarate aminotransferase; GPT, glucose 6-phosphate/phosphate transporter; OAA, oxaloacetate; OEP, outer chloroplastic envelope protein; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; 3-PGA, 3-phosphoglyceric acid; PPT, phosphoenolpyruvate/phosphate transporter; Toc/Tic, protein translocase of the outer/inner chloroplastic membrane; TPT, triose phosphate/phosphate transporter

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rate spatially the plastid compartment from the cytoplasm. The two envelope membranes are distinguishable by their structure, function, and biochemical properties, but also co-operate, e.g. in the synthesis of lipids and in protein translocation [1].

Chloroplasts are the site of carbon dioxide reduction and its assimilation into carbohydrates, amino acids, fatty acids and terpenoid compounds [1]. Annually about 120×10^9 tons of CO_2 are converted into organic substances by chloroplasts of higher land plants. Furthermore, chloroplasts are the sole site of nitrite and sulfate reduction [2,3], and their conversion into organic compounds. The wide range of biosynthetic functions of the chloroplasts requires the existence of different and effective transport mechanisms across the envelope membranes to provide the cell with carbohydrates, organic nitrogen and sulfur compounds. On the other hand chloroplasts also take up inorganic cations (e.g. K^+, Na^+, Mg^{2+}, Ca^{2+}), anions (e.g. NO_2^{2-}, SO_4^{2-}, PO_4^{2-}), and a variety of other metabolic pathway intermediates such as phosphoenolpyruvate (PEP), dicarboxylic acids, acetate, and various amino acids in order to fulfill their biosynthetic functions. In contrast to chloroplasts, all types of non-photosynthesizing plastids are unable to synthesize ATP or precursors for anabolic reactions. Therefore, these plastids need specific transport proteins to allow uptake of such compounds from the cytosol.

In this review we focus on recent results concerning the presence, molecular structure, biochemical characteristics, and physiological functions of a range of channels and transporter proteins in the two envelope membranes. We consider the molecular characterization of substrate-specific outer membrane solute channel proteins, the discovery of a family of inner membrane transport proteins catalyzing the movement of phosphorylated intermediates, and the identification of a new type of eukaryotic ATP/ADP transporter, as an illustration that the plastid envelope is (and will be) of general interest to the biological community providing new insights into general principles of membrane transport. We hope to demonstrate that plastid membrane transport is a rapidly developing field and will be an exciting scientific area for the future.

2. Transport across the outer envelope membrane

2.1. Chloroplasts contain several regulated solute channels in the outer membrane

Electrophysiological studies using either giant chloroplasts of *Nitellopsis* [4] or isolated envelope membrane vesicles from spinach [5,6] have shown the presence of different voltage-dependent, high conductance channels in the envelope membranes. The outer envelope membrane has been assumed for a long time to be freely permeable for most low molecular mass compounds up to 10 kDa [5]. Correspondingly, it was believed that the osmotic barrier against the cytosol is formed exclusively by the inner envelope membrane, which therefore should contain specific carrier proteins. Several of these were identified at the functional and also at the molecular level (see below). However, recent reports revealing the presence of several specific solute pores in the outer envelope indicate that the intermembrane space is not freely accessible to low molecular mass solutes (see below).

While various inner envelope carrier proteins, e.g. the triose phosphate/phosphate transporter, various dicarboxylic acid transporters, or the hexose phosphate/phosphate transporter show a distinct substrate selectivity and specificity, it is not yet clear to what extent transport through outer membrane channels is regulated. Furthermore, the total number of different channels that are embedded in this membrane and are required for plastid function is largely unknown. In mitochondria, a major solute channel with high conductance is represented by the **v**oltage-**d**ependent **a**nion **c**hannel (VDAC). Although evidence exists that beside VDAC, additional high conductance channels could be present in the mitochondrial outer membrane none has so far been identified at the molecular level [7]. In Gram-negative bacteria, however, several different types of high conductance channels have been identified in their outer membrane [8]: (i) so-called porin-forming water-filled pores that allow the downhill diffusion of solutes, provided the size of the solutes does not exceed the exclusion limit of the channel pore (OmpF, outer membrane protein F [9]), (ii) porin-like channels car-

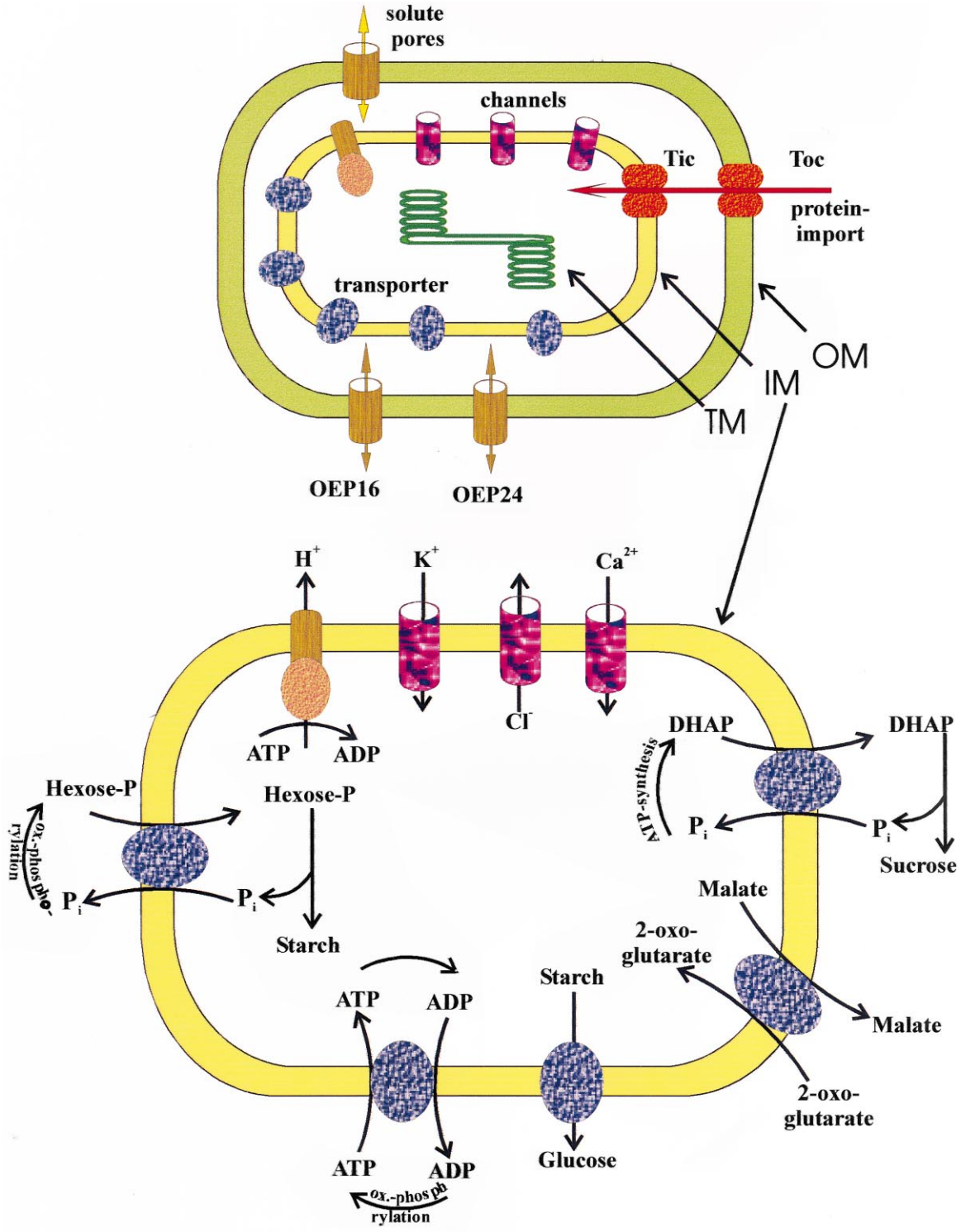

Fig. 1. Channels, porins, and metabolite transporters in the outer and inner envelope membranes of higher plant plastids. IM, inner envelope membrane; OM, outer envelope membrane; TM, thylakoid membrane. For other abbreviations and details see text.

rying specific sites, through which specific diffusion processes are facilitated (LamB, outer membrane maltoporin [9]), and (iii) ligand-gated channels providing energy-dependent uptake of nutrients into bacteria (FePA, ferric enterobactin receptor from *Escherichia coli* [10]).

The ancestral relationship between mitochondria, plastids and Gram-negative bacteria would also suggest the presence of a large number of different solute channel proteins in the organellar outer membrane. In pea chloroplasts, three channel proteins have been identified and functionally characterized so far: (I) Toc75 (**t**ranslocase of the **o**uter **c**hloroplastic membrane 75 kDa), probably consisting of 16 amphipathic transmembrane β-strands, forms the pre-protein conducting channel (Fig. 1) [11]; (II) the **o**uter **e**nvelope **p**rotein of 16 kDa (OEP16) that forms a cation-selective (about 1 nm wide) channel, with a high specificity for amino acids and amines [12] (Fig. 1); (III) the identified channel protein OEP24 also constitutes a high conductance solute channel with a diameter of about 2.5 nm (Fig. 1). This slightly cation-selective OEP24 channel allows the passage of triose phosphates, ATP, inorganic phosphate (P_i), dicarboxylic acid, and positively or negatively charged amino acids [13], thus appearing to be a rather non-selective pore.

The nuclear-encoded OEP21 and OEP16 proteins show no significant sequence homology to each other and are phylogenetically not closely related. However, both proteins reveal mainly β-sheet topology and the voltage-dependent gating of both solute channels closely resembles that observed for β-barrel membrane channels [12–14]. OEP16 allows the passage of molecules containing the amino acid backbone, but excludes C_4 and C_5 sugars and other similar compounds, although the pore size of the channel would be sufficient to allow the passage of these molecules. The current fluxes through the OEP16 channel were also regulated by the redox state of two spatially close cysteine residues [12]. Recently, two additional proteins in the outer membrane of pea chloroplasts have been identified, which form selective and regulated solute pores as well (R. Wagner, J. Soll, in preparation).

In summary, evidence is accumulating that the transport of different solute classes across the outer envelope membrane is facilitated through distinct solute pores in a selective and regulated manner. Although details of these transport systems are only just emerging, the coexistence of selective and non-selective channels – as found in the outer membrane of Gram-negative bacteria (for review see [15]) – might, under limiting rates of metabolic substrates, also be required in mitochondria and chloroplasts.

3. Transport across the inner envelope membrane

3.1. Ion channels in the inner envelope are required for pH homeostasis

Ion channels in the plasma membrane of plant cells and in plant organellar membranes are involved in volume regulation, transepithelial transport, and regulation of the membrane potential. In chloroplasts, ion channels participate in the pH homeostasis across the inner envelope membrane, and across the thylakoid membrane. During photosynthesis, light-driven H^+ gradients are generated between the cytosolic site ($\sim$pH 7), the chloroplast stroma ($\sim$pH 8), and the thylakoid lumen ($<$pH 6). Ion channels in the chloroplast inner envelope membrane and the thylakoid membrane appear to be involved in the development and regulation of H^+ gradients, and membrane potentials across these membranes [16–19].

In the chloroplast stroma concentrations of the physiologically important ions are in the order of 150 mM for K^+ [20], 50 mM for Cl^- [20], and 5 mM for Mg^{2+} [21]. The steady-state membrane potential across the inner chloroplast envelope membrane was found to be in the order of about -100 mV (negative in the stroma [22]), while the steady-state membrane potential across the thylakoid membrane is small ($\sim$10 mV, positive in the lumen [16]).

Due to the pH optimum of the key enzymes of the photosynthetic carbon reduction cycle (around pH 8), the rate of light-dependent CO_2 fixation in chloroplasts depends on the maintenance of a high stromal pH [23,24]. The uphill H^+ flux across the chloroplast envelope into the stroma is probably driven by a H^+-ATPase [18,25] and indirectly coupled to the uptake of potassium ions from the cytosol [26–30]. It has also been shown that the pro-

ton gradient across the inner envelope membrane drives Ca^{2+} uptake, mediated by a uniporter [31,32].

Pharmacological and electrophysiological studies with either intact chloroplasts or proteoliposomes, containing proteins from solubilized chloroplast envelope membranes, indicated the presence of a potassium channel in the inner envelope membrane [6,33]. It has been shown by electrophysiological measurements that the inner envelope K^+ channel is blocked by millimolar concentrations of ATP. The presence of an ATP-dependent K^+ channel in the inner envelope membrane could also be confirmed by flux measurements on intact chloroplasts [6,25]. Recently, a cDNA from *Arabidopsis thaliana* (2082 bp), encoding a 662 amino acid long putative K^+ channel, has been deposited in the EMBL data bank (accession number: Z83202; see [34]). Due to the presence of a putative chloroplast targeting sequence it has been argued that the translation product of this cDNA might represent the inner envelope K^+ channel [34]. However, neither function nor location of the protein encoded by this cDNA has been proven up to now.

The inner envelope membrane is known to be permeable to chloride [29]. Chloride channels have also been detected in the chloroplast envelope of the green algae *Nitellopsis* sp. and *Eremosphera viridis* by the patch-clamp technique and with ion-selective microelectrodes [4,35]. The presence of a low conductance (60 pS) Cl^- channel in the inner membrane of spinach chloroplasts has been confirmed by electrophysiological measurements [6].

It is worth mentioning that in addition to the low conductance channels, electrophysiological measurements revealed the existence of a porin-like channel in the reconstituted inner envelope membranes of spinach chloroplasts. This high conductance channel was slightly more selective for anions than cations, with a main conductance of $\Lambda \cong 525$ pS [36]. In the case of chloroplasts from *Nitellopsis* sp. two cation-selective high conductances were found [4]. However, the molecular identity of these conductance pores remains elusive.

Since it has been shown that the H^+ efflux from the chloroplasts in the light is coupled to K^+ uptake, and Cl^- efflux, it is conceivable that, beside the proton pumping ATPase, both the potassium channel and the chloride channel of the inner membrane represent key elements for the regulation of the stromal pH. However, transport of other cations like Ca^{2+}, Mg^{2+}, and anions like nitrite and inorganic phosphate is energetically coupled to proton transport, and thus adds to balancing the fluxes from and into the plastids.

3.2. A family of transport proteins mediating the exchange of inorganic phosphate and phosphorylated compounds

In chloroplasts, as well as in non-photosynthesizing plastids, the controlled exchange of P_i for various phosphorylated metabolites is crucial for the function of metabolism. One important feature of the transport processes involved is that the import/export of P_i and of phosphorylated compounds is balanced stoichiometrically. Such tightly regulated transport is required for several reasons:

(1) In chloroplasts, photophosphorylation of ADP to ATP depends upon continued supply of P_i (for review see [37]). During CO_2 fixation substantial amounts of triose phosphates leave the chloroplast and a simultaneous uptake of inorganic phosphate has to occur to maintain light-driven ATP synthesis. (2) In non-photosynthesizing plastids – such as starch-storing amyloplasts – the uptake of hexose phosphates has to be coupled to P_i export since the stromal enzyme ADP-glucose pyrophosphorylase (AGPase, a key enzyme of starch biosynthesis) is allosterically inhibited by rising concentrations of P_i [38]. The balanced movement of inorganic phosphate and phosphorylated intermediates is mediated by a family of transport proteins which so far includes the triose phosphate/phosphate, the phosphoenolpyruvate/phosphate, and the glucose 6-phosphate/phosphate transporters.

3.2.1. Triose phosphate/phosphate transporter

Photosynthetic CO_2 fixation leads to the plastidic (stromal) generation of triose phosphates (glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP)) which are mainly exported for sucrose synthesis into the cytosol [37]. This export is mediated by the triose phosphate/phosphate transporter (TPT) representing the best characterized protein of the plastid envelope membrane [39–41] (Fig. 1). The exchange mode of transport [39] – which is characteristic for all members of the plastidic phos-

phate transporter family [42] – ensures that the release and the uptake of phosphate moieties are balanced, and allows continuous ATP synthesis.

For both triose phosphates, GAP and DHAP, similar sub-millimolar apparent affinity constants have been determined [39]. However, as the thermodynamic equilibrium of the triose phosphate isomerase reaction leads to an about 20-fold higher concentration of DHAP over GAP, the former metabolite represents the more rapidly transported substrate under in vivo conditions. By coupling the triose phosphate export to the import of 3-phosphoglyceric acid (3-PGA) (and not to P_i import occurring during sucrose synthesis) the chloroplastic TPT is proposed to be involved in a shuttle system allowing the indirect export of ATP, NADH, and/or NADPH [43]. The extremely low cytosolic NADH/NAD ratio [44] indicates that triose phosphates can be converted to 3-PGA in mesophyll cells. Due to experimental constraints, quantification of fluxes via a triose phosphate/3-PGA shuttle under in vivo conditions has not been possible so far. However, the functionality of a triose phosphate/3-PGA shuttle to generate stromal ATP has been demonstrated in a mutant of *Chlamydomonas reinhardtii*, unable to photosynthetically synthesize ATP [45]. In this case, the shuttle imports DHAP from the cytosol and allows stromal ATP generation by a reversal of the glyceraldehyde 3-phosphate dehydrogenase/3-phosphoglycerate kinase reactions [45]. It remains to be elucidated whether such a shuttle system could be responsible for autotrophic growing of the *Chlamydomonas reinhardtii* Fd50 mutant – which also lacks thylakoid ATP synthase activity. This mutant has a growth rate of 30% of the wild-type cells under photoautotrophic conditions [46].

The TPT of the inner envelope represents about 10% of the total protein of this membrane. According to gel electrophoretic determinations the apparent molecular mass of the TPT from various types of chloroplasts is about 29 kDa [43]. This value, however, is a significant underestimate, since sequencing of corresponding cDNA clones and determination of the N-terminal end of the mature protein reveals a 36 kDa protein [40], with 6–7 predicted transmembrane domains [40,47]. The functional form of the TPT is probably a dimer [48]. Therefore, this transport protein possesses a similar structure as a wide range of metabolite-transporting proteins located in the inner mitochondrial membrane, which also function as dimers of 2×6 transmembrane domains [49].

The nuclear-encoded plastidic phosphate transporter proteins are synthesized as immature pre-proteins possessing N-terminal transit sequences of about 80 amino acids length [40,41]. It is quite remarkable that the transit peptides of the plastidic phosphate transporter proteins resemble features of those proteins found in the mitochondrial compartment (e.g. low serine, high arginine content [50]) while they do not share similarities with the transit peptides required for targeting of soluble stroma- or membrane-bound thylakoid proteins. Indeed, under in vitro conditions, the transit peptide of the TPT allows import into both isolated chloroplasts and isolated mitochondria [51]. Under in vivo conditions, however, the TPT pre-protein specifically enters the chloroplast envelope [51], which is probably due to additional target information in the mature part of the transporter protein [51]. The exact location of this additional target information is controversial; suggested locations are either within the first 23 N-terminal amino acids of the mature protein [51], or in a hydrophobic region of the processed protein between the amino acid positions 24 and 45 [52]. As the latter group analyzed the protein import properties of the TPT from pea plants [52], whereas Silva-Filho et al. [51] examined the protein import properties of the spinach homologue, the reason for these contradictory results remains unclear.

The nucleotide and deduced amino acid sequences of TPT proteins from various species including e.g. spinach, pea, maize, and *Flaveria* have been determined [40,47,41]. Interestingly, the genus *Flaveria* comprises C3 plants such as *F. pringlei* and C4 species such as *F. trinerva* in which chloroplasts are exposed to different physiological constraints. In C4 plants, photosynthetically active chloroplasts are present in two types of cells, bundle-sheath cells and mesophyll cells. In mesophyll cells, primary CO_2 fixation occurs via carboxylation of PEP in the cytosol, previously synthesized in the chloroplasts. Therefore, appreciable rates of PEP transport have to be maintained across the plastidic envelope.

After identification of the corresponding cDNA encoding the protein from either *F. pringlei* (C3) or *F. trinerva* (C4) it was possible to analyze their trans-

port properties [41]. This was allowed because the TPT was the first plastidic transport protein to be functionally expressed in fission yeast without alteration of the biochemical properties [53]. As expected, the TPT from the C4 species *F. trinerva* transports PEP with about a 5-fold higher velocity than the TPT from the C3 species *F. pringlei* [41]. The two *Flaveria* TPTs share about 87% identity at the level of amino acid sequence. Only one single amino acid exchange is present in one transmembrane domain and occurs throughout all TPTs from C3 and C4 plants [41]. These observations tempted the authors to speculate that this single amino acid (threonine 139 is exchanged for serine in the *F. trinerva* TPT) might be responsible for the altered substrate specificity [41]. To prove this hypothesis it would be interesting to conduct site-directed mutagenesis of the TPT protein from the C3 species *F. pringlei* at this position (Thr139→Ser139), and to study subsequently the biochemical properties of the recombinantly expressed protein.

3.2.2. Phosphoenolpyruvate/phosphate transporter

Most chloroplasts, and several types of non-photosynthesizing plastids, do not possess the enzymatic equipment required for the conversion of 3-PGA to PEP [54–56]. However, the presence of stromal PEP is strictly required for various metabolic pathways including the Shikimate pathway [57], and the synthesis of various amino acids [58]. For a long time it has remained uncertain how these pathways could be provided with the required carbon skeletons as the TPT protein from C3 plants does not exhibit a sufficiently high affinity for PEP [39].

The recent discovery of a new class of plastidic phosphate transporters with high specificity for PEP (phosphoenolpyruvate/phosphate transporter, PPT) filled the gap in our knowledge about the interconnection between metabolites of primary and secondary metabolism. Surprisingly, the PPT exhibits not more than about 30% identity to the TPT at the level of the amino acid sequence [59] and shows – as a recombinantly expressed protein – about 11-fold higher affinity for PEP than the chloroplastic TPTs [59]. Such high affinity obviously ensures that under in vivo conditions plastids are able to import PEP to supply the specific anabolic reactions.

Northern blot analysis has shown that the expression of the PPT structural gene is not restricted to leaves, but significant amounts of transcript also accumulate in non-green plant tissues. Therefore, it is likely that the PPT represents a housekeeping protein necessary in all types of plant cells. In the case of the TPT and the PPT it has been clearly demonstrated that the mRNAs encoding both transporters accumulate in the same tissue [59]. From this observation the question arises as to why one type of plastid requires the presence of both a TPT and a PPT at the same time. A possible answer to this is that under in vivo conditions the uptake of PEP in exchange for P_i (and catalyzed by the TPT) is most likely hampered by the comparably high cytosolic P_i concentrations and the low affinity of the TPT for PEP.

At this point it is not known whether photosynthetically active chloroplasts import the required PEP via the PPT in counter exchange to stromal P_i, triose phosphates, or 3-PGA. Under photosynthetic conditions the stromal P_i concentration is low and the affinity of the PPT for P_i (0.8 mM [59]) is not substantially different from the affinity of the TPT for P_i, which of course does not export P_i. On the other hand, the PPT possesses an affinity for triose phosphates and 3-PGA which is about 5–8-fold lower than the affinity of the TPT for these compounds [59].

3.2.3. Glucose 6-phosphate/phosphate transporter

Starch synthesis and the oxidative pentose phosphate pathway in non-photosynthesizing plastids depend upon the uptake of energy and/or carbon from the cytosol. In general, the chloroplastic TPT is able to facilitate export and import of triose phosphate, since the driving forces for transport are the concentration gradient and the ratio of the putative substrates. With a few exceptions, however, non-photosynthesizing plastids lack the enzyme fructose 1,6-bisphosphate phosphatase (FBPase [60]). This enzyme is a highly active enzyme in photosynthetic chloroplasts and is strictly required for the conversion of triose phosphates to hexose phosphates in plastids.

With these considerations in mind it is not surprising to find that metabolites other than triose phosphates, namely hexose phosphates, serve as the major carbon source for starch biosynthesis, and for the oxidative pentose phosphate pathway in non-photo-

synthesizing plastids [61,62]. Enriched endosperm amyloplasts from wheat were the first non-photosynthesizing plastids to be isolated that showed high rates of starch biosynthesis that was driven by exogenous glucose 1-phosphate (Glc1P) [63]. This observation was later proven and extended by Emes and coworkers [64]. The corresponding transport protein was reconstituted in proteoliposomes and it was shown that Glc1P transport occurred in a 1:1 stoichiometry to P_i transport [65]. In contrast to this, isolated plastids from pea embryos or cauliflower buds preferentially use exogenously supplied glucose 6-phosphate (Glc6P) – instead of Glc1P – for starch biosynthesis [66,67] (Fig. 1). The observation of Glc6P-driven starch synthesis in pea embryo plastids was in agreement with data determined using proteoliposomes containing the envelope proteins from pea root plastids [68]. After preloading of the proteoliposomes with inorganic phosphate it was possible to demonstrate a Glc6P/P_i antiport [68], similar to the characteristics of the triose phosphate/P_i antiport catalyzed by the TPT [39]. From these biochemical and physiological data it was clear that besides a chloroplastic TPT another group of phosphate transport proteins exists mediating hexose phosphate/P_i exchange across the envelope of non-photosynthesizing plastids.

After the first physiological and biochemical characterization of hexose phosphate transport [68] it took about 9 years to identify a corresponding carrier protein at the molecular level. The enrichment of membrane fractions from developing maize endosperm allowed the reconstitution of Glc6P/P_i antiport in proteoliposomes [69]. Moreover, it was only the second time (after the identification of the PPT [59]) that a plastidic transport protein – representing only a minor component of the total membrane proteins – was purified and subsequently usable for partial sequencing of small internal peptides. On the basis of these peptide sequences, deduced oligonucleotides were generated for amplification of a specific probe suitable for a successful screen of a corresponding cDNA library [69]. The full-length cDNA clone that was identified exhibited a length of 1647 bp and encoded a protein of 387 amino acids (40.2 kDa) [69].

The highly hydrophobic protein possesses six predicted transmembrane α-helices leading to the conclusion that in vivo the transporter functions as a dimer of 2×6 transmembrane domains. Homologues to the maize transporter have been identified in pea and cauliflower plants [69]. As the maize cDNA clone could not be expressed in fission yeast the pea root homologue was recombinantly synthesized and analyzed for the competence of Glc6P/P_i exchange. The recombinantly expressed transporter from pea roots exhibited an apparent affinity constant of 0.7 mM for Glc6P (named glucose 6-phosphate/phosphate transporter, GPT [69]) which is close to values reported for the authentic transporter in isolated pea root plastids [56].

Interestingly, the recombinant plastidic GPT exhibits a high affinity not only for Glc6P, but also for the triose phosphates ($\sim$0.4 mM for DHAP) and 3-PGA (1.3 mM) [69]. These data indicate that besides Glc6P the other two phosphorylated carbon compounds might be of some importance for metabolism in non-photosynthesizing plastids. Indeed, the uptake of one Glc6P molecule into isolated pea root plastids leads to the release of CO_2 and DHAP [56,68,70] catalyzed by the set of enzymes comprising the oxidative pentose phosphate pathway. Therefore Glc6P import from the cytosol in exchange for DHAP mediated by the GPT balances the carbon–phosphate pools during the oxidative pentose phosphate pathway. On the other hand, 3-PGA is – due to the allosteric action on AGPase [38] – a positive effector for starch biosynthesis in isolated amyloplasts [67]. Therefore, the biochemical properties of the GPT fulfill everything required for a controlled starch metabolism in storage tissues. Recently, it has been shown that non-photosynthesizing plastids possess in addition to the GPT a TPT protein [71]. The physiological function of a TPT in non-photosynthesizing plastids, however, is still unclear.

3.2.4. General comments on the family of phosphate transporters

It appears remarkable that the three types of plastidic phosphate-transporting proteins, the TPTs, the PPTs, and the GPTs, represent three independent classes of transporters [69]. The PPTs exhibit about 30% amino acid identity to the TPTs [59], and the GPTs exhibit only about 33% identity to the other two. However, all three types of plastidic phosphate transporters share five highly conserved domains of

13–31 amino acids [69], indicating that they may have evolved from a common ancestor. This hypothesis is further strengthened by the presence of two highly conserved amino acid residues (Lys-273 and Arg-274 of the spinach TPT) which are present in every type of plastidic phosphate transporter and thought to be part of the substrate binding center [72,41].

In the future, it should be possible to analyze the structure/function relationships of all plastidic phosphate transporters in more detail. For example, up to now it is entirely unknown which domains in the particular transporters determine the altered substrate specificity. It would also be very interesting to compare the structure of the PEP-transporting TPT from *F. trinerva* with the PEP-transporting PPT in more detail. Such analyses might give insight into structure/function relationships and suggest experiments to increase our knowledge about these important transporter proteins.

Another open question is how the activity and amount of a specific type of plastidic phosphate transport protein is regulated in a tissue. A detailed analysis of the promoters controlling the expression of these proteins is required in the near future. This analysis is important for several reasons: (1) from the biological point of view we need to understand the expression of the corresponding structural genes; (2) these transporter fulfill important metabolic functions, e.g. the PPT provides the plastid with PEP required for synthesis of compounds involved in pathogen defense reactions. Therefore, questions arise whether the PPT activity alters after pathogen infections or whether the transporter activity might limit the degree of defense. Similar scenarios and questions come up concerning the function of the TPT and the GPT.

3.3. Transport of unphosphorylated carbohydrates

During photosynthesis a large portion of the newly synthesized sugar phosphates is exported from the chloroplast via the TPT (see above). The remaining assimilated carbon is used for starch synthesis in the stroma. This starch is mobilized during the night (accordingly named transitory starch) allowing considerable amounts of carbohydrate to leave the chloroplast (for review see [73]). A controlled degradation of transitory starch is a prerequisite for plant development [74,75].

About 20 years ago a glucose transporter located in the inner plastid envelope was initially characterized in isolated spinach chloroplasts [76] (Fig. 1). This transporter exhibits a relatively poor apparent substrate affinity for glucose (K_M 20 mM), and catalyzes a diffusion-driven transport [76]. Maltose was also shown to permeate the plastidic envelope [77], and a detailed biochemical analysis of glucose and maltose movement across the chloroplastic envelope indicated that two types of transporter – a glucose and a maltose transporter – are present in spinach chloroplasts, which both facilitate diffusion [78]. The latter transporter seems to be specific for maltose as glucose does not inhibit uptake, and as maltodextrins, like maltotriose, and higher polydextrins are not transported [78].

The physiological function of a plastidic glucose transporter remained unclear until physiological, biochemical, and molecular approaches were adopted. Using isolated high starch-containing chloroplasts from pea or spinach leaves the release of substantial amounts of glucose and maltose was demonstrated. The export of neutral sugars is, however, not restricted to chloroplasts because cauliflower bud amyloplasts are also capable of starch degradation, leading to the export of glucose [79]. Further evidence for an export of neutral sugars during degradation of transitory starch recently came from elegant experiments using nuclear magnetic resonance (NMR) analysis of the incorporation of a deuterium-labeled H_2O into glucose during starch/sucrose conversion in darkened tomato or bean leaves [80]. In combination with metabolite quantifications it was estimated that more than 75% of all carbon released from the chloroplast during the night enters the cytosol as neutral sugars [80]. The activity of the plastidic glucose transporter is therefore clearly required for the release of starch degradation products into the cytosol.

That the glucose transporter indeed facilitates an important step in dark metabolism is further highlighted by analysis of an *Arabidopsis thaliana* mutant. The *A. thaliana* mutant TC26-5 is unable to degrade transitory starch at sufficient rates [81]. Since all en-

zymes required for starch degradation are present in this plant [74], the question arose whether the mutant might lack a transport protein involved in the export of degradation products. Indeed, by comparison of the glucose transport properties of chloroplasts, isolated from either the wild-type or mutant plants, it was found that the latter organelles are unable to transport glucose [81]. As this phenotype correlates with strongly increased glucose concentrations in freshly isolated chloroplasts, and since chloroplast envelopes from mutant plants lack a protein exhibiting an apparent molecular mass of about 40 kDa [81], it was postulated that *A. thaliana* mobilizes transitory starch predominantly by an amylolytic attack and that the resulting glucose is subsequently exported into the cytoplasm [81].

Very little is known about the activity of the glucose transporter in other plastid types from different tissues. A biochemical approach to furthering our understanding of this transporter will require purification of the corresponding transport protein, reconstitution of transporter activity into proteoliposomes, and partial sequencing of the transporter. However, this approach will depend upon identification of a plant tissue where their plastids exhibit high glucose transport activity. A second approach at a molecular level might be a differential display of cDNA libraries made from potato wild-type leaves and from leaves of transgenic plants with reduced plastidic fructose 1,6-bisphosphate phosphatase (FBPase) activity. Koßmann et al. [82] created such transgenic potato plants by introducing the endogenous FBPase cDNA in the antisense orientation. Darkened leaves from antisense lines with strongly reduced plastidic FBPase activity exhibited increased ability to convert radioactively labeled sucrose into starch [82]. As such conversion does not involve the plastidic FBPase, the authors speculated that the amount of the plastidic glucose transporter protein is pleiotropically increased in such antisense plants. In addition, the analysis of the metabolic and physiological changes connected to a decrease of the plastidic TPT protein in transgenic tobacco plants revealed that the mutant exhibits increased glucose transport activity [83]. Therefore, this transgenic tobacco plant might also be an alternative source for the generation of a cDNA library displaying specifically expressed structural genes.

3.4. Transport of dicarboxylic acids across the plastidic envelope membranes

Besides the carbon ~ P/phosphate exchange involved in photosynthesis and anabolic reactions in storage plastids, both chloroplasts and non-photosynthesizing plastids harbor important metabolic functions requiring the uptake and release of organic acids. Here we will not consider the movement of organic acids such as acetate or pyruvate, but focus on the transport of dicarboxylic acids across the envelope, since impressive results have been generated in recent years.

The first detailed characterization of dicarboxylate transport was conducted using isolated spinach chloroplasts [84]. From the observation that a variety of dicarboxylic acids competitively inhibit each other it was generally assumed that compounds like malate, aspartate, fumarate, and succinate were transported by the same carrier [84]. A very detailed biochemical analysis led to the conclusion that at least two dicarboxylate transport proteins are present in the inner envelope membrane. One transporter mediates the import of 2-oxoglutarate in exchange for stromal malate (2-oxoglutarate/malate transporter), and the other one catalyzes glutamate export in exchange for cytosolic malate (glutamate/malate transporter) [85]. This work was a breakthrough since it was the first time that chloroplasts were shown to possess several translocators working in a cascade-like mechanism as had previously been observed in mitochondria.

In addition, the presence of these two types of transport proteins allows a deeper insight into the process of ammonia assimilation, occurring during nitrite reduction and during photorespiration [85]. Due to the stromal couple of the enzymes glutamine synthase and glutamine/2-oxoglutarate aminotransferase (GOGAT) the free ammonia is first of all used for synthesis of glutamine which acts directly to generate glutamate from 2-oxoglutarate. To export glutamate a malate molecule from the cytosol is required as an exchange substrate (via the glutamate/malate translocator). Malate is then directly re-exported for uptake of 2-oxoglutarate (via the 2-oxoglutarate/malate translocator), subsequently used for glutamine synthesis (two translocator model [85]).

Besides the transport processes discussed above another important dicarbonic acid transport occurs in leaf chloroplasts. In chloroplasts the NADP-malate dehydrogenase forms part of a system named the malate valve [86]. This system uses excess reducing equivalents to reduce oxaloacetate (OAA) to malate within the chloroplasts, and exports this metabolite subsequently into the cytosol. The two dicarbonic acid transporters discussed above exhibit an affinity for OAA which is too low to catalyze substantial OAA import under in vivo conditions. Therefore, they most likely do not participate in this system. However, the discovery of a highly specific OAA carrier in chloroplasts from maize and spinach mesophyll chloroplasts exhibiting micromolar apparent affinity for OAA has provided an explanation how this metabolite is imported into photosynthetically active plastids [87]. Later, the uptake of OAA and the concomitant release of malate was combined to the OAA/malate shuttle [88], which in fact does not mean that malate export is catalyzed in a counter exchange mode by the high affinity OAA transporter. It is still uncertain whether the OAA importer acts as an exchanger, or catalyzes an OAA uniport, followed by a unidirectional malate export mediated by another, yet unknown transporter.

Due to the low yield of plastid envelope membranes obtained during purification a biochemical approach towards purification of transporter proteins is generally limited. Menzlaff and Flügge [89] have been able to purify the 2-oxoglutarate/malate transporter biochemically from highly enriched spinach envelopes using chromatographic techniques, combined with monitoring of transport activity in proteoliposomes [89] (Fig. 1). The apparent molecular mass of the pure protein is 45 kDa [89]. This result concurs with previous observations on an *A. thaliana* mutant unable to perform photorespiration. This mutant lacks a 45 kDa protein in the chloroplastic envelope [90], but possess all the enzymes required for the photorespiratory C/N cycle.

A peptide sequence from the purified 2-oxoglutarate/malate transporter protein allowed the deduction of a specific oligonucleotide for hybridization screening of a spinach cDNA library. The resulting cDNA clone encodes a highly hydrophobic protein of 569 amino acids [91] exhibiting 12 putative transmembrane domains. As the in vitro translated protein was imported into the envelope of isolated chloroplasts and processed to the mature protein, and as the recombinantly expressed cDNA product showed transport characteristics similar to the authentic, biochemically purified protein, a first plastidic transporter with 12 predicted transmembrane domains – which does not conform the 2×6 α-helices forming dimers typically observed in plastid transport proteins – was identified. It will be very challenging to conduct physiological experiments with transgenic plants possessing altered expression of the plastidic 2-oxoglutarate/malate transporter. In addition, we know nothing about homologous proteins in different plant species, and nothing about the expression pattern of the corresponding structural genes in other plant groups, such as C4 and CAM plants.

Recently, another very interesting type of malate transporter was identified at the biochemical level in leukoplast envelopes enriched from castor seed endosperm [92]. This malate transporter imports malate into isolated plastids in a 1:1 stoichiometry to P_i export [92]. Such a transport characteristic makes good biochemical sense insofar as malate is the major carbon source for fatty acid synthesis in this type of plastids [93]. During the conversion of malate to fatty acids the synthesis of malonyl-CoA is required, which needs one ATP molecule per malate imported. Therefore, for each malate molecule that is imported, one P_i is liberated in the stroma and has to be removed during steady-state synthesis. The molecular nature of the plastidic malate/phosphate transporter is so far unknown.

3.5. Plastidic ATP/ADP transporter

Adenylate transport occurs across the membrane of various cellular organelles. The most prominent and best characterized adenylate transporter is the mitochondrial ADP/ATP carrier (AAC; for review see [94]). In addition, adenylate transport is also required across the Golgi and ER membranes [95], and across the inner plastidic envelope membrane [96]. Up to now, the mitochondrial and plastidic adenylate transport systems have been identified at the molecular level.

The mitochondrial ADP/ATP carrier functions as a dimer [94] consisting of 2×6 predicted transmem-

brane domains. In general, the protein is highly conserved between evolutionarily widely distant organisms. For example, the AACs from baker's yeast and from animals exhibit 50% identity at the amino acid sequence level [49]. The plant AAC is also similar in structure but possesses an N-terminal transit peptide required for correct targeting into the mitochondria [97].

Biochemical, physiological, and immunological evidence indicates that the plastidic adenylate transporter must differ at the molecular level from the AAC. Firstly, the direction of ATP and ADP transport is reciprocal between mitochondria and plastids. Under in vivo conditions the mitochondrial ADP/ATP carrier strictly catalyzes ADP import from the cytosol in exchange for matrix ATP. This mode of transport is due to the electrogenic gradient across the inner mitochondrial membrane [94]. In contrast to this, the chloroplastic adenylate transporter catalyzes ATP uptake from the cytosol in exchange for stromal ADP [98]. This import of energy in the form of ATP is necessary to energize anabolic reactions (including e.g. starch and fatty acid synthesis) in storage plastids [66,67], or to allow high rates of starch degradation in chloroplasts at night [99]. Secondly, the mitochondrial AACs from all sources are specifically inhibited by the naturally occurring compounds bongkrekic acid, and carboxyatractylate [100]. In contrast to this, the plastidic adenylate transporter hardly reacts with these inhibitors either as the native protein in intact plastids or after reconstitution in proteoliposomes [101]. Thirdly, polyclonal antisera raised against the mitochondrial ADP/ATP carrier from the yeast *Neurospora crassa* specifically cross-react with corresponding proteins in mitochondria from a variety of sources but do not immunodecorate peptides in highly enriched plastidic envelope membranes [101,102].

Because of these differences it was inappropriate to use degenerate oligonucleotides deduced from conserved domains of the mitochondrial AAC to screen a plant cDNA library for the functional homologous protein in plastids. During screening of an *A. thaliana* cDNA library for a copper transporter a fragmentary cDNA clone was isolated which coded for a protein with substantial similarity to the ATP/ADP transporter from the bacterium *Rickettsia prowazekii* [103] (Fig. 1). The identified cDNA comprised 2221 bp and encodes a highly hydrophobic protein of 589 amino acids [103].

The plastidic protein exhibited about 66% sequence similarity to the ATP/ADP transporter from the evolutionary widely distant bacterium *R. prowazekii* [103,104]. *R. prowazekii* is a strictly intracellular parasite of mammalian and human cells, and the causative agent of epidemic typhus. This bacterium was the first of only a few bacteria characterized to possess an adenylate uptake system [105]. In addition to *Rickettsia*, the intracellular bacterium *Chlamydia trachomatis* also possesses an ATP/ADP uptake system [106], which has recently been identified at the molecular level and shown to be homologous to the rickettsial and plastidic adenylate transporters [107]. The rickettsial and one of the two chlamydial ATP/ADP transport systems import ATP from the host cell in exchange for bacterial ADP [105–107]. Therefore, no net adenylate import occurs but these systems represent a link to connect the energy pool of the host cell to anabolic reactions in the intracellular bacterium. In this respect, *R. prowazekii*, *C. trachomatis*, and plant plastids behave quite similarly; they all exploit the cell by uptake of ATP in exchange for internal ADP.

The plastidic adenylate transport system (named AATP1(At); **A**TP/**A**DP **t**ransport **p**rotein *Arabidopsis thaliana* [103]) is a highly hydrophobic membrane protein of 12 predicted α-helical transmembrane domains [103], and possesses a N-terminal extension – beyond the region of high homology to the rickettsial transporter – of about 100 amino acids. Similar to the transit peptides of the plastidic phosphate and the dicarboxylic acid transporters this transit peptide resembles features of transit sequences of mitochondrial preproteins. The radioactively labeled AATP1(At) protein is incorporated into envelopes from leaf chloroplasts in an ATP-dependent manner, and the transit sequence is subsequently proteolytically processed [102], confirming the subcellular location of this membrane protein.

The plastidic ATP/ADP transporter AATP1(At) was heterologously expressed in baker's yeast and after reconstitution into proteoliposomes the exchange nature of transport was proven [102]. Latterly, AATP1(At) was one of the first plant membrane proteins to be functionally expressed in *Escherichia coli*. By transforming the *E. coli* strain

C43, which has improved properties for the expression of hydrophobic membrane proteins (which are often toxic to the bacterium [108]), it was possible to show ATP or ADP uptake into the intact bacterial cell [109].

This bacterial expression system represents a very powerful tool to study the properties of the plastidic ATP/ADP transporters because the basic biochemical properties of the transporter have not been altered [109,110]. It could be shown that the recombinantly synthesized plastidic ATP/ADP transporter exhibits affinity constants (K_M) for ATP and ADP of 17 and 12 μM respectively [109], which are close to data from adenylate uptake experiments on isolated plastids from various sources [67,101]. The specificity of interaction between the transporter and the substrates resides in the base (only *adenine* nucleoside phosphates are accepted [109]), the sugar (no deoxy-derivatives are accepted; Neuhaus et al., unpublished observation), and the number of phosphate moieties (AMP is not a substrate [109]). In addition, the terminal phosphate group has to be freely accessible since ADP-glucose is also not a substrate [109].

Southern hybridization indicated that *A. thaliana* harbors an isoform to AATP1(At) [103] which was subsequently identified at the molecular level. This protein (named AATP2(At)) comprises 569 amino acids and the mature part exhibits about 78% identity to AATP1(At) [110]. As the biochemical properties of the recombinantly expressed AATP2(At) protein do not differ from the first plastidic ATP/ADP transporter it remains to be elucidated why this plant contains two isoforms. As mentioned for the plastidic phosphate transporter proteins, promoter/reporter gene fusions are required to gain deeper insight into the physiological function of both isoforms.

The provision of non-photosynthesizing plastids with energy in the form of ATP is crucial for various reactions, including RNA, protein, starch, and fatty acid synthesis. Without exogenous ATP nearly no starch synthesis occurs in plastids from pea embryos or cauliflower buds [66,67]. Fatty acid synthesis is also restricted to the plastidic compartment in plant cells and leads to extremely high lipid yields in various storage tissues. Using rape seed embryo plastids as a model system it has be shown that exogenous ATP is also required for fatty acid synthesis [111]. This observation holds true also for plastids from cauliflower buds, and wheat and maize endosperm [112,113]. Moreover, experiments on isolated amyloplasts from cauliflower buds revealed that starch and fatty acid biosynthesis compete for ATP and that the activity of the plastidic ATP/ADP transporter limits the rate of endproduct synthesis [112].

However, this approach has concentrated on a largely artificial experimental system as the plastids have been removed from the natural environment, the cell cytoplasm. Therefore, another experimental attempt was required where the activity of the plastidic ATP/ADP transporter was altered in vivo. For this purpose transgenic potato plants with either increased or decreased activity of this transporter were created. To increase the activity the plants were transformed with the heterologous plastidic ATP/ADP transporter from *A. thaliana* (AATP1(At) [114]). To decrease the activity the endogenous potato homologue AATP1(St) was introduced in antisense orientation. Both cDNAs carried the constitutive cauliflower mosaic virus S35 promoter. After screening the transgenic plants, various lines with either increased or decreased activity were identified [114]. Interestingly, transgenic plants with increased transporter activity showed also significantly higher levels of starch per gram fresh weight [114]. In contrast, a reduction of the transporter activity correlated tightly with a marked decrease of tuber starch [114].

Very remarkably, not only the total amount starch was altered but also its composition since the amylose/amylopectin ratio was changed. Starch consists of two molecules namely amylose (unbranched linear α1,4-glycosidic-linked glucose moieties) and amylopectin, representing a branched polymer which in addition to the α1,4-glycosidic linkages carries an additional α1,6 linkage every 15–30 glucose moieties. Starch from antisense plants exhibited a significantly reduced amylose to amylopectin ratio, whereas starch from sense plants is characterized by an increased ratio [114]. At least two isoforms of starch synthases are involved in starch biosynthesis, a high-affinity soluble enzyme (SSS, **s**oluble **s**tarch **s**ynthase) responsible for amylopectin synthesis, and a granular bound starch synthase (GBSS) with low affinity [115,116]. As obviously a decrease in the plastidic ATP/ADP transporter activity correlates with lowered total starch we have to assume that the concen-

tration of the starch precursor ADP-glucose is also decreased. Therefore, the low affinity GBSS is more strongly affected than the soluble isoforms. It remains to be elucidated whether the reduction of the plastidic ATP/ADP transporter activity in other storage tissues, e.g. rape seed embryo tissue, affects the accumulation of corresponding metabolic endproducts.

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