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Altered plastidic ATP/ADP-transporter activity influences potato (*Solanum tuberosum* L.) tuber morphology, yield and composition of tuber starch

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Summary

The metabolic function of the plastidic ATP/ADP transporter (AATP) in heterotrophic plastids was examined in transgenic potato plants that exhibited increased or decreased amounts of the protein. Altered mRNA levels correlated with activities of the plastidic ATP/ADP transporter. Potato tubers with decreased plastidic ATP/ADP transporter activities exhibited reduced starch contents whereas sense lines accumulated increased amounts of tuber starch. Starch from wild-type tubers had an amylose content of 18.8%, starch from antisense plants contained 11.5–18.0% amylose, whereas starch from sense plants had levels of 22.7–27.0%. The differences in physiological parameters were accompanied with altered tuber morphology. These changes are discussed with respect to the stromal ATP supply during starch biosynthesis.

Introduction

Starch is the most abundant plant storage product and as such important for plant development (Preiss, 1997), human and animal diet (John, 1992), and biotechnological processing (Preiss, 1997). Starch consists of amylose, a linear nearly unbranched α1.4-glucan, and amylopectin which possesses in addition to the linear α1.4 linkages branched α1.6 linkages (Smith *et al.*, 1997). Plant starch biosynthesis takes place exclusively in plastids that are the sole location of starch synthases and starch-branching enzyme (Preiss, 1997).

In heterotrophic storage tissues it has been proposed that two different metabolic pathways can lead to starch accumulation. In cereal storage, tissues such as maize or barley endosperm a cytosolic ADPglucose pyrophosphorylase (AGPase) (Denyer *et al.*, 1996; Thorbjørnsen *et al.*, 1996) synthesises ADPglucose which is subsequently imported into the amyloplasts (Möhlmann *et al.*, 1997). Starch synthesis in amyloplasts from dicotyledonous plants is driven by the uptake of hexose phosphates as shown for plastids from pea embryos (Hill and Smith, 1991), cauliflower buds (Batz *et al.*, 1994), or rapeseed embryos (Kang and Rawsthorne, 1994). The major difference between starch biosynthesis in cereal endosperm tissue and dicotyledonous plants is the location of AGPase. In the latter case, AGPase is exclusively located in the amyloplastic stroma (Emes and Neuhaus, 1997) making it necessary to import ATP in addition to hexose phosphates for synthesis of ADP-glucose. ATP uptake is mediated by a plastidic adenylate transporter (Heldt, 1969; Schünemann *et al.*, 1993) which was identified at the molecular level in an *Arabidopsis thaliana* cDNA library and named *AATP1* (At) (Kampfenkel *et al.*, 1995). Alignments of the deduced amino-acid sequence of AATP1 (At) indicated that the transporter exhibits more than 66% similarity to the ATP/ADP transporter from the Gram-negative, intracellular bacterium *Rickettsia prowazekii* (Kampfenkel *et al.*, 1995).

To verify whether, or to what extent, the plastidic ATP/ADP transporter contributes to the rate of starch biosynthesis in potato tubers under *in vivo* conditions we have altered the activity of the transporter in the developing tissue. Increasing activities of the plastidic ATP/ADP transporter were produced by transforming plants with a corresponding cDNA cloned in a sense orientation, whereas a reduction of the plastidic ATP/ADP transporter activity was achieved by transforming plants with endogenous cDNA cloned in antisense orientation.

The analysis of corresponding metabolic and morphologic differences between transgenic and wild-type plants allowed us to address the following questions: (i) does a decrease of the plastidic ATP/ADP transporter activity correlate with reduced levels of tuber starch; (ii) does an increase of the plastidic ATP/ADP transporter activity induce higher levels of starch indicating a limiting function of this transporter; and (iii) does a potential change in starch content induced by altered activities of the plastidic ATP/ADP transporter correlate with other metabolic or morphologic characteristics?

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Table 1. Biochemical properties of the heterologously expressed AATP1 (St) gene product

	K_m (μM)		Vmax (nmol mg^{-1} protein/h)	
Transporter	ATP	ADP	ATP	ADP
AATP1 (St)	25	20	100	200

Uptake of radioactively labelled adenine nucleotides was quantified after induction of *AATP1* (St) expression in *E. coli.* For synthesis of radioactively labelled ADP and details see Tjaden *et al.* (1998). Data are the mean of three individual experiments. Standard error was always below 5%.

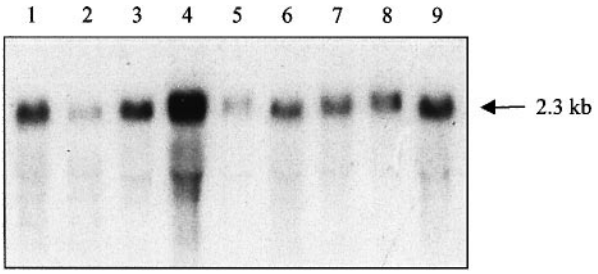

Figure 1. Northern blot analysis of total RNA extracted from various potato tissues.
Total RNA (10 μg each) was extracted from young (sink) leaves (lane 1), fully developed (source) leaves (lane 2), stems (lane 3), tubers (lane 4), roots (lane 5), sepals (lane 6), petals (lane 7), anthers (lane 8), or ovaries (lane 9) and probed with radioactively labelled *AATP1* (*S. tuberosum*) cDNA.

Results

Identification of a potato plastidic ATP/ADP-transporter

To reduce the endogenous level of the plastidic ATP/ADP transporter in potato it was necessary to transform plants with a potato cDNA cloned in an antisense orientation. Since no potato homologue had been identified, we first screened a potato cDNA library for the presence of the plastidic ATP/ADP transporter (see Experimental procedures) and named the resulting homologue *AATP1* (*Solanum tuberosum*, EMBL accession number Y10821). The deduced amino-acid sequence of AATP1 (St) exhibits 68% identity to the plastidic ATP/ADP transporter AATP1 from *A. thaliana* (Kampfenkel *et al.*, 1995).

Transport properties of the heterologously expressed AATP1 (St) cDNA product

The identification of a cDNA with a high degree of identity to a plastidic ATP/ADP transporter does not necessarily mean that a functional homologue has been identified. To verify whether AATP1 (St) transports adenine nucleotides we expressed the entire pre-protein as an His_{10}-AATP1 (St)-fusion protein in *Escherichia coli* and analysed ATP and ADP uptake after induction.

After expression of *His_{10}-AATP1* (St) intact *E. coli* cells imported both ATP and ADP. ATP import occurred at an apparent K_m of 25 μM, ADP import occurred at an apparent K_m of 20 μM (Table 1). ADP import was significantly faster than ATP import (Table 1). Uninduced *E. coli* cells did not exhibit adenine nucleotide import (data not shown; see Tjaden *et al.*, 1998).

Northern blot analysis of AATP1 (St) mRNA accumulation

To get insight into the function of AATP1 (St) in potato tissues we analysed the accumulation of the corresponding mRNA in various heterotrophic and autotrophic tissues (Figure 1). The highest accumulation of *AATP1* (St) mRNA occurred in tubers of wild-type plants and, to a lower degree, also in other heterotrophic tissues like embryo or petal and sepal tissues, and also in young leaves (Figure 1).

Presence of AATP1 (At) mRNA induced by sense transformation of potato

We used the *AATP1* (At) cDNA to create transgenic potato plants with increased activity of the plastidic ATP/ADP transporter (sense plants). The heterologous *AATP1* (At) cDNA was chosen for the generation of sense plants to prevent co-suppression.

The accumulation of *AATP1* (At) mRNA in various lines of transgenic plants was monitored by Northern blot experiments. The DNA-construct used is a chimeric gene that encodes *AATP1* (At)-sense cDNA under the control of the constitutive cauliflower-mosaic virus 35S promoter (Figure 2a). Several lines of transgenic potato plants exhibited strong accumulation of *AATP1* (At) mRNA in both leaves and tubers (Figure 2a). In contrast to this, no *AATP1* (At) mRNA accumulated in leaf or tuber tissues from wild-type plants (Figure 2a) demonstrating the specificity of the probe used for Northern blot analysis. From about 100 lines the strongest accumulation was detectable in lines 62, 78 and 98. These lines were used for subsequent analyses. In addition, various other sense lines also exhibited significant amounts of *AATP1* (At) mRNA (e.g. lines 1, 17, 173, data not shown).

Altered levels of endogenous AATP1 (St) mRNA induced by antisense transformation

Plants transformed with *AATP1* (St) cDNA in antisense orientation (antisense plants) were used to reduce the endogenous activity of the plastidic ATP/ADP transporter. About 100 lines of transgenic potatoes were screened for differences in mRNA accumulation. Some lines exhibited a dramatic reduction of the endogenous *AATP1* (St) mRNA in both leaves and tubers (Figure 2b). In lines 594, 595, 654 and 676 the *AATP1* (St) mRNA was hardly detectable,

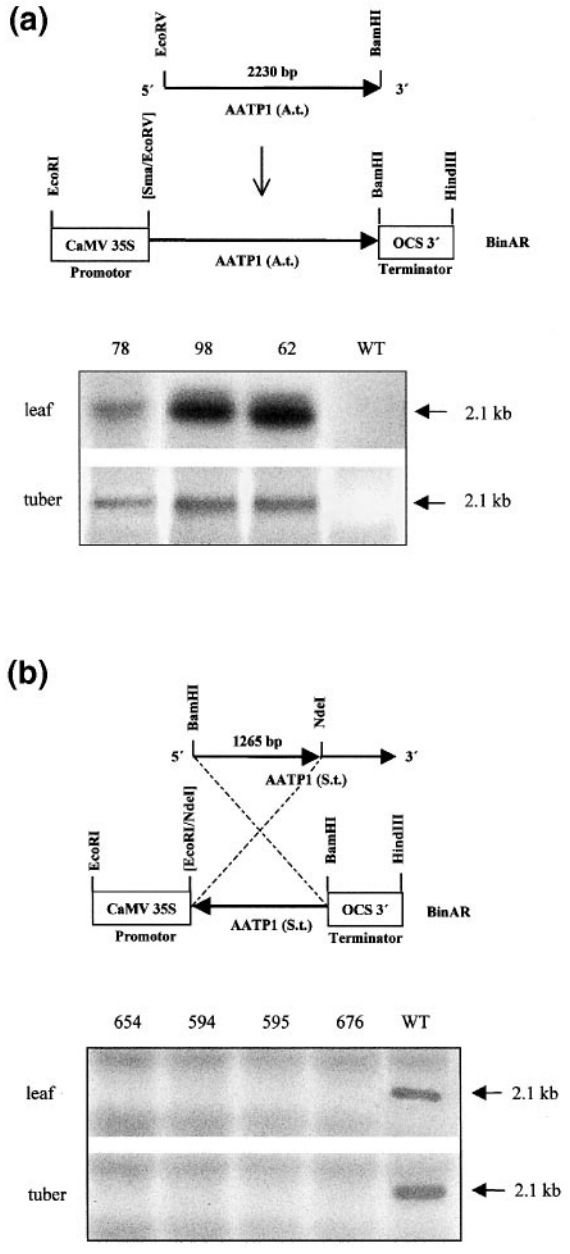

Figure 2. Structure of the chimeric plastidic ATP/ADP-transporter cDNAs and accumulation of corresponding mRNAs.
(a) *AATP1* (At) cDNA cloned in sense orientation and accumulation of *AATP1* (At) mRNA in leaves and tuber tissue.
(b) *AATP1* (St) cDNA cloned in antisense orientation and accumulation of *AATP1* (St) mRNA in leaves and tuber tissue. Number of individual lines are given; WT = wild-type.

whereas the endogenous levels in tissues from wild-type plants resulted in strong radioactive labelling of mRNA (Figure 2b).

Altered levels of plastidic ATP/ADP transporter activity in transgenic plants

To quantify the activity of the plastidic ATP/ADP transporter in various lines, membrane proteins from enriched tuber amyloplasts were reconstituted in proteoliposomes and [α^{32}P]ATP uptake analysed. We minimised the residual mitochondrial ADP/ATP carrier activity by the addition of bongkrekic acid. This compound is known as an extremely specific inhibitor of adenylate transport in animal and plant mitochondria (Stubbs, 1981), but hardly affects plastidic adenylate transport (Schünemann *et al.*, 1993). An optimal bongkrekic acid concentration was determined in an inhibitor saturation experiment using proteoliposomes harbouring only the mitochondrial adenylate carrier. Bongkrekic acid at about 5 μM inhibited more than 87% of mitochondrial adenylate transport activity (data not shown). The data given in Table 2 have been corrected for the mitochondrial contribution to adenyate transport.

Table 2. Rates of ATP uptake into proteoliposomes containing enriched amyloplastic envelope proteins

Line/Genotype	ATP import (nmol mg^{-1} protein h^{-1})	ATP import % of wild-type activity
Wild-type	44.3 ± 3.1	100
1 (sense)	66.1 ± 5.7	149
62 (sense)	69.1 ± 7.7	156
98 (sense)	65.1 ± 10.2	147
595 (antisense)	28.5 ± 4.8	64
654 (antisense)	34.9 ± 1.8	79
676 (antisense)	29.5 ± 2.1	67

Enriched amyloplastic envelope proteins were incorporated into proteoliposomes. ATP uptake was quantified in the presence of 5 μM bongkrekic acid to inhibit mitochondrial adenylate carrier activity. Data are the mean of three independent experiments and given plus standard error (SE).

Proteoliposomes containing the enriched amyloplast membrane protein from wild-type plants imported [α^{32}P]ATP at a rate of 44.3 nmole per mg protein/hour (Table 2). All sense lines examined exhibited increased rates of ATP import into corresponding proteoliposomes (Table 2) reaching about 156% of ATP import compared to proteoliposomes prepared from wild-type plants (Table 2). In contrast, all antisense lines examined exhibited decreased ATP import rates. The reduction of uptake is most pronounced in proteoliposomes containing the amyloplastic membrane proteins from antisense line 595, where ATP import is reduced to around 65% of the corresponding wild-type (Table 2).

Altered morphology of tubers and altered number of tubers in transgenic plants

Alterations in potato tuber starch metabolism can cause phenotypic changes in this organ (Frommer and Sonnewald, 1995). Figure 3a illustrates a typical tuber yield from a wild-type potato plant. A typical yield from antisense

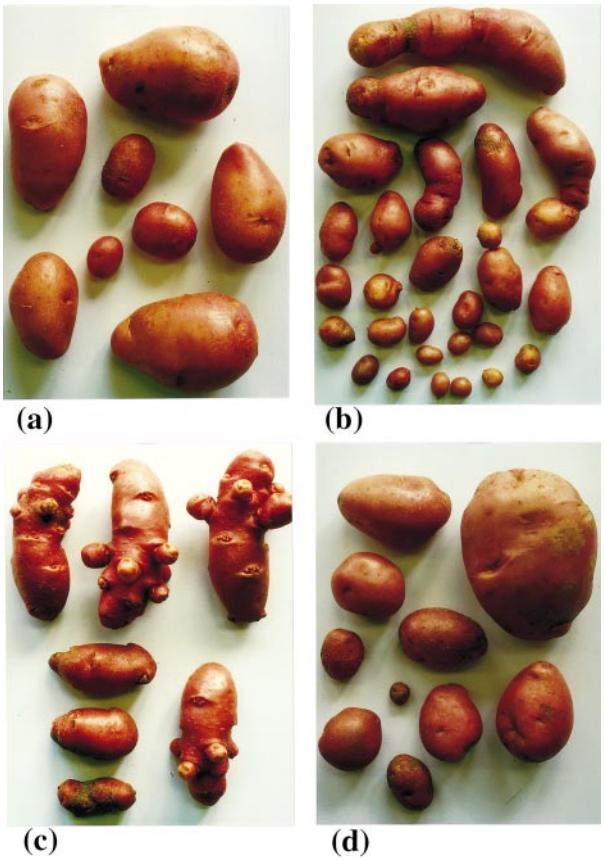

Figure 3. Morphology and number of wild-type and transgenic potato tubers.
(a) Wild-type, (b) antisense line 676, (c) antisense line 654, and (d) sense line 62.

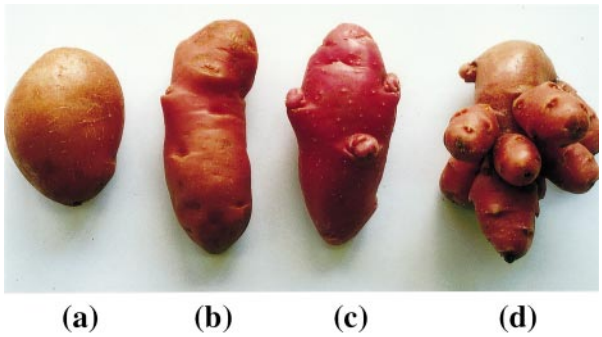

Figure 4. Growth and adventitious tuber budding of tubers harvested from antisense plants.
(a) Wild-type, (b) antisense line 676, (c) antisense line 594, and (d) antisense line 654. Note the pronounced elongation and adventitious budding.

plants is represented by tubers from line 676 (Figure 3b). The antisense tubers are more abundant and smaller in size than the tubers from the wild-type plants (Figure 3a,b). Several antisense lines were characterised not only by an increased number of individual tubers, but also by the presence of adventitious tubers budded from main tubers (e.g. line 654, Figure 3c). The progressive development of this phenotype is illustrated in Figure 4. A clear phenotypic change in the fully developed antisense tubers was a more elongated shape (Figure 4b–d), with the tendency to bud-off additional tubers. Several lines, illustrated by a tuber from line 654 (Figure 4d), possessed highly branched tubers that appeared similar to ginger tubers.

In contrast, the tuber phenotype from sense plants was not so significantly changed. However, in some lines (e.g. sense line 62) one or two major tubers contributed disproportionately to the total fresh weight (Figure 3d).

Altered physiological parameters in tubers from transgenic plants

Total fresh weight of tubers from antisense plants were between 70 and 95% of wild-type tuber fresh weights, whereas tubers harvested from sense plants were between 64 and 87% of wild-type fresh weights (Table 3).

Starch represents the major storage product in potato tubers and contributes the highest amount to the dry weight of that organ (John, 1992). Therefore, it can be anticipated that changing levels of starch correlate with altered volumetric weights of the organs, which can be estimated when tuber weights are measured under standard conditions and compared to the tuber weight measured when tubers have been suspended in water. The ratio between such under-water weight and the standard weight gives the volumetric weight (given in percentage of fresh weight). By this, the determination of the volumetric weight represents a rapid method of screening for altered starch levels.

Tubers from all antisense lines exhibited strongly reduced volumetric weight (Table 3). For wild-type tubers, the volumetric weight represents 8.6% of the fresh weight whereas in antisense line 654 the volumetric weight was 5.76%. In the sense plants, two of three lines examined exhibited increased volumetric weight, reaching 10.5% for tubers harvested from plants of line 98 (Table 3).

To get deeper insight into starch content and distribution of starch in representative tubers we stained tuber discs with iodine. Discs from wild-type tubers stained strongly with iodine although less iodine bound to the centre of the tuber (Figure 5b). Discs from sense plants (e.g. line 62) accumulated iodine but, in contrast to discs from wild-type tubers (Figure 5b), also stained strongly in the centre (Figure 5a). Discs from antisense lines (e.g. line 654) clearly bound less iodine (Figure 5c).

As both the volumetric weights and the iodine staining indicated that the amount of starch was changed in the transgenic plants we quantified starch spectrophotometrically. In wild-type tubers starch accumulated at about 1100 μmole C6 units g^{-1} Fwt (Table 3), whereas in all antisense lines a significant reduction of starch was evident. In line 595 starch was reduced to about 530 μmole C6 units g^{-1} Fwt, which was less than 50% of the content in wild-type plants (Table 3). In contrast, two of three sense lines

Table 3. Tuber weight, volumetric weight, soluble sugar and starch levels, amylose content, and number of tubers in wild-type and transgenic potato plants

Line/Genotype	Total Tuber Fwt (g)	Volumetric Weight (% of Fwt)	Soluble Sugars (μmol gFwt^{-1})	Starch (μmolC6 units gFwt^{-1})	Amylose %	Number of tubers
Wild-type	391 ± 14	8.60 ± 0.15	26.49 ± 2.2	1094 ± 67	18.8 ± 0.9	9.6 ± 1.6
654 (antisense)	279 ± 15	5.76 ± 0.71	42.52 ± 6.2	574 ± 102	15.5 ± 1.3	7.6 ± 1.1
594 (antisense)	297 ± 24	5.56 ± 0.32	48.76 ± 6.5	630 ± 74	14.3 ± 1.0	15.4 ± 2.2
595 (antisense)	334 ± 27	6.74 ± 0.58	45.92 ± 7.3	531 ± 43	18.0 ± 1.7	15.0 ± 2.6
676 (antisense)	373 ± 29	6.38 ± 0.59	40.60 ± 8.7	883 ± 189	11.5 ± 0.6	27.2 ± 2.1
62 (sense)	334 ± 55	9.00 ± 0.70	30.65 ± 4.8	1485 ± 132	27.0 ± 2.2	13.5 ± 3.5
98 (sense)	252 ± 42	10.50 ± 0.82	18.28 ± 2.9	1269 ± 81	22.7 ± 1.8	13.2 ± 1.2
78 (sense)	342 ± 13	8.42 ± 0.40	20.50 ± 1.2	995 ± 178	24.5 ± 2.3	13.2 ± 2.3

The data given are the mean of five independent plants from each line. From every single plant three samples were taken and analysed individually. Data are the mean and given plus standard error (SE).

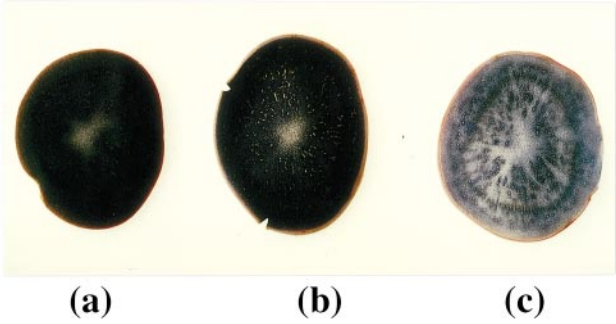

Figure 5. Iodine staining of tuber discs from wild-type and transgenic plants.
(a) Sense line 62, (b) wild-type, and (c) antisense line 654. Tuber discs from wild-type and transgenic plants were incubated in Lugol's solution.

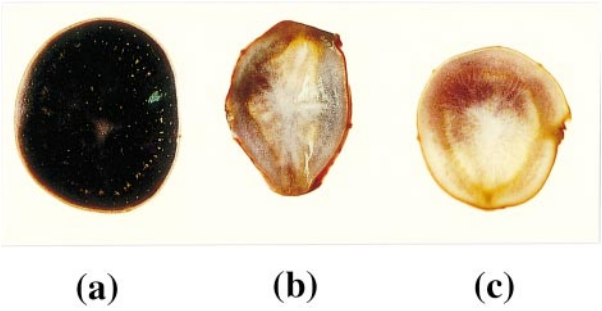

Figure 6. Destaining of iodine-stained tuber discs from wild-type and antisense plants.
(a) Wild-type, (b) antisense line 654, and (c) antisense line 594. Discs from wild-type and transgenic plants were incubated in Lugol's solution and subsequently destained for about 30 min in water.

examined exhibited increased starch levels (Table 3). In line 62 starch was increased by 36% compared to wild-type tubers, in tubers from line 98 an increase in starch of about 16% was observed (Table 3).

When iodine-stained tuber discs were incubated in water for 15–60 min, considerable destaining occurred in discs from antisense lines, e.g. lines 654 and 594 (Figure 6b,c). Interestingly, destaining was significantly less in discs taken from wild-type tubers (Figure 6a) or sense plants (data not shown). As iodine staining of starch is due to the incorporation of iodine into the helical α1.4 linked amylose molecules, we examined the amylose/amylopectin ratio in isolated starch using a colorimetric assay (Hovenkamp-Hermelink *et al.*, 1988). The amylose content in starch from wild-type tubers was 18.8% (Table 3). Antisense lines contained less amylose (Table 3). Starch from antisense line 676 contained only 11.5% amylose, corresponding to a 40% reduction of amylose (Table 3). The opposite observation was true for sense plants as in all sense lines an increased amylose content was measured. The highest amylose content (27%) was observed in tubers from line 62 (Table 3).

Lowered levels of starch correlated with increased contents of soluble sugars (sum of glucose, fructose and sucrose). In wild-type tubers about 26.5 μmole soluble sugars g^{-1} Fwt accumulated whereas nearly twice of this sugar concentration was present in tubers of antisense line 594 (Table 3). In the sense plants, the lines 78 and 98 exhibited the lowest concentrations of soluble sugars in all tuber tissues measured (Table 3).

Discussion

In heterotrophic cells, the bulk of ATP is synthesised in mitochondria, subsequently transported into the cytosol, and from there into various organelles. There have been no reports on how changing activities of a plastidic ATP/ADP transporter might influence metabolism in heterotrophic tissues. Potato was chosen as a model system since this species is relatively easy to transform with chimeric genes (Rocha-Sosa et al., 1989) and because this plant possesses a large heterotrophic storage organ (the tubers) that is a strong sink for carbon (Frommer and Sonnewald, 1995).

The introduction of a chimeric gene containing the coding sequence of the plastidic ATP/ADP transporter *AATP1* (At) from *A. thaliana* leads to the formation of the appropriate

mRNA in both green and heterotrophic tissues (Figure 2a). As in proteoliposomes (containing amyloplast membrane proteins from sense lines) an increased ATP uptake was detectable (Table 2), the newly introduced *AATP1* (At) mRNA obviously induced increased amounts of plastidic ATP/ADP transporter in tuber amyloplasts. This observation demonstrated that the heterologously synthesised transporter protein from *A. thaliana* (AATP1, At) is functionally integrated into the plastid envelope.

AATP1 (St) represents the first non-Arabidopsis plastidic ATP/ADP transporter, joins the small group of this carrier proteins identified at the molecular level (Kampfenkel *et al.*, 1995; Möhlmann *et al.*, 1998), and exhibits biochemical properties similar to two plastidic ATP/ADP transporters from *A. thaliana* (Möhlmann *et al.*, 1998; Tjaden *et al.*, 1998).

The introduction of *AATP1* (St) cDNA, cloned in antisense orientation, leads to reduced levels of mRNA encoding the protein (Figure 2b), and correlated with lowered rates of ATP uptake into proteoliposomes containing amyloplastic membrane proteins from antisense plants (Table 2). Proteoliposomes, harbouring membrane proteins from antisense plants import radioactively labelled ATP with a rate of 65–79% compared to proteoliposomes harbouring wild-type proteins (Table 2). The comparison of the pronounced decrease of mRNA coding for *AATP1* (St) (Figure 2b) and the reduction of transporter activity (Table 2) revealed that there is no strong proportionality between mRNA and corresponding protein levels. Interestingly, such weak proportionality between the reduction of mRNA and transporter activity has also been shown for potato plants with reduced levels of the chloroplastic triose phosphate transporter (Riesmeier *et al.*, 1993).

The observation for wild-type plants that the endogenous *AATP1* (St) mRNA accumulated most strongly in tuber tissues (Figure 1, lane 4) firstly indicates that the provision of ATP into amyloplasts during starch synthesis is important for starch yield and secondly that the rates of ATP synthesis via glycolysis in tuber amyloplasts is not sufficient for anabolic reactions in this organelle. The latter assumption is supported by observations made on other heterotrophic plastids enriched from various sources (Hill and Smith, 1991; Kang and Rawsthorne, 1994; Naeem *et al.*, 1997).

Both Glc6P or Glc1P have been proposed as precursors for starch biosynthesis in isolated potato tuber amyloplasts (Naeem *et al.*, 1997; Schott *et al.*, 1995). In both cases starch biosynthesis requires ATP to energise the stromal AGPase reaction. The large changes in tuber starch levels in transgenic plants exhibiting altered activities of the plastid ATP/ADP transporter (Tables 2, 3) is striking evidence for the involvement of this transporter in the regulation of starch content in potato tubers.

Starch biosynthesis in several sense lines was stimulated and inhibited in all antisense lines (Table 3). From these results it can be concluded that *in vivo* the plastidic ATP/ADP transporter limits starch biosynthesis in potato tuber amyloplasts and exerts considerable control strength. Indeed, the data given in Figure 7 clearly indicate the correlation between the amyloplastic ATP/ADP transporter activity and the starch content in potato tubers, and allowed us to calculate a flux control coefficient (Kacser and Burns, 1973) of the amyloplastic ATP/ADP transporter of about 0.78 for starch biosynthesis. These calculations explain previous observations made on various storage tissues. For example, the carbohydrate metabolism in transgenic potato tuber was unchanged unless more than 50% of the total AGPase activity was reduced (Müller-Röber *et al.*, 1992). Denyer *et al.* (1996) estimated a flux control coefficient of pea embryo AGPase of only 0.1 demonstrating that about 90% of the control of starch biosynthesis in this tissue is exerted by other proteins involved in the pathway. The plastidic ATP/ADP transporter may contribute significantly to such control in potato tuber. In this context, it is noteworthy to mention that in isolated cauliflower amyloplasts a stimulation of starch biosynthesis induces a reduction of the rate of fatty-acid synthesis as both pathways compete for ATP available (Möhlmann *et al.*, 1994).

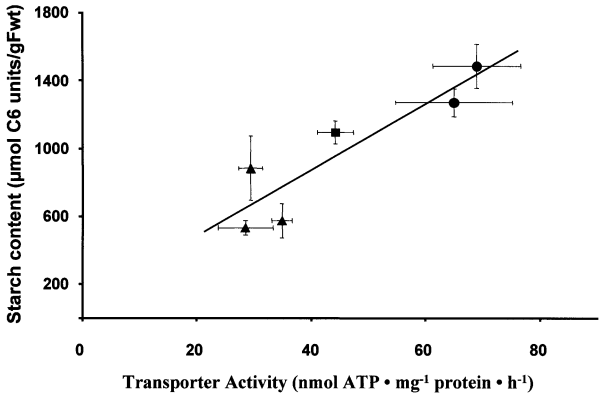

Figure 7. Correlation between the plastidic ATP/ADP transporter activity and starch content in tubers.
ATP/ADP transporter activity has been taken from Table 2; starch content has been taken from Table 3. ●, sense lines, ■, wild-type, ▲, antisense lines. The correlation coefficient r^2 is 0.91. $y = 19.4x + 96.3$. The flux control coefficient (C) of the plastidic ATP/ADP transporter is 0.78. This value has been calculated from the gradient of the fitted line (19.4) which has been scaled relative to the wild-type activity and starch content: 19.4 ● 44.3/1094.

However, we have to keep in mind that altered plastidic ATP/ADP transporter activity might also induce changes in the concentration of other metabolites known to be involved in starch synthesis. For example, reduced or increased levels of cytosolic hexose phosphates or 3-PGA would ultimately lead to reduced or increased rates of starch biosynthesis. Hexose phosphates have been identified as the precursor for starch biosynthesis in potato tuber amyloplasts (see above), and 3-PGA is known to activate allosterically the plastidic AGPase (Preiss, 1997) and is, for

example, required for Glc6P-dependent starch biosynthesis in isolated cauliflower bud amyloplasts (Batz *et al.*, 1994). The metabolic and enzymic changes connected to altered plastidic ATP/ADP transporter activity in potato tubers will be analysed in the near future.

Our observations indicate that the increased starch content in tubers from sense plants is due to an increased rate of ATP import from the cytosol into the ATP-consuming stroma. Such an increased flux depends on a sufficient cytosolic ATP supply. The subcellular adenylate levels in potato tubers are not known. However, in plant leaf tissues it is demonstrated that, even during photosynthetic ATP production, the cytosolic ATP/ADP ratio is several times higher than in corresponding plastids (Stitt *et al.*, 1982; Stitt *et al.*, 1989). In heterotrophic tissues, such a high cytosolic ATP/ADP ratio would permit an increased ATP import into the stroma after increasing the activity of the plastidic ATP/ADP transporter.

Altered starch levels in transgenic plants also correlated with changing amylose levels (Figure 6). Quantitations of amylose in starch from wild-type tubers revealed an amylose content of 18.8% (Table 3), a value reported previously for wild-types from two other potato cultivars (Hovenkamp-Hermelink *et al.*, 1988; Kuipers *et al.*, 1994). Interestingly, the amylose content in low starch antisense lines is decreased (to 11.5% in line 676), whereas in sense lines the amylose content increased up to 27% (line 62, Table 3). One explanation for these changes is the presence of several isoforms of starch synthases. In general, starch synthases can be classified in granule-bound and soluble isoforms (Smith *et al.*, 1997). In potato, the granule-bound isoforms are responsible for amylose synthesis (Kuipers *et al.*, 1994; Visser *et al.*, 1991) and exhibit lower affinities for ADPglucose than the soluble isoforms, which are mainly involved in amylopectin synthesis (Smith *et al.*, 1997). As ATP import in amyloplasts is required for ADPglucose synthesis one can assume that increased ATP import rates correlate with higher stromal ADPglucose concentrations. On the other hand, reduced activities of the plastidic ATP/ADP transporter would lead to reduced concentrations of ADPglucose. By these changes, not only the total starch content would be altered (Figure 5, Table 3) but, due to the different affinities of both starch synthase isoforms, decreased ADPglucose concentrations would limit the activity of the granule-bound isoform more than the activity of the soluble isoforms. The assumption that the kinetic properties of both isoforms of starch synthases are responsible for changing amylose/amylopectin ratios suggests that other mutations that reduce ADPglucose levels would cause similar changes. In peas, the wrinkled seeded mutants (caused by alterations in the *rb* locus) are characterised by a substantially decreased amylose/amylopectin ratio (Kooistra, 1962). As the *rb* mutation led to a decreased AGPase activity in the developing embryo (Hylton and Smith, 1992), a further indication that altered ADPglucose concentrations specifically affects granule-bound starch synthase is given.

The observation that the amylose content in starch from sense lines increased by up to 27% (Table 3) was surprising since it was previously concluded that the amylose/amylopectin ratio in potato tubers is limited by the amylopectin content and does not increase above 20% (Flipse *et al.*, 1996). Our data (Table 3) indicate that, at least in the potato variety used in this study (Désirée), amylose contents of more than 20% are possible. Previously, it has been speculated that the availability of ADPglucose within the highly crystalline starch grain may limit granule-bound starch synthase activity (Martin and Smith, 1995). If true, an increased provision of substrate (induced by an increased ATP/ADP transporter activity) might allow higher activities of the granule-bound starch synthase. This assumption would explain why increased activities of granule-bound starch synthases in transgenic potato plants do not lead to higher amylose levels than those observed in wild-type tubers (Flipse *et al.*, 1996) when the ADPglucose concentration is limiting.

From the data presented in Figure 3 and Table 3 it is evident that decreased plastidic ATP/ADP transporter activity correlates with a significantly increased number of tubers and with tubers exhibiting stimulated growth of adventitious tubers (Figure 3,4). In all antisense lines the reduction of tuber starch was accompanied by a substantial increase of the soluble sugar concentration (Table 3). The increased sugar concentration is most likely responsible for stimulation of tuberisation since, generally, conditions that lead to increased photosynthate concentration in the stolon tips promote tuberisation in potato plants (Moorby and Milthorpe, 1975). Interestingly, transgenic potato plants with reduced AGPase activities and increased sugar concentrations in the tuber also exhibit significantly increased numbers of tubers (Müller-Röber *et al.*, 1992).

In some antisense lines (e.g. line 654, Figure 3c), the total number of individual tubers was not increased but the main tubers tended to bud adventitious tubers on the surface (Figure 4d). A similar morphology was observed on various former Andean potato cultivars and it is believed that this phenotype is controlled by three genes (Ortiz and Huaman, 1994). The longitudinal morphology evident in every antisense line analysed (Figure 3,4) is thought to be regulated by only one gene (Ortiz and Huaman, 1994). Therefore, it will be interesting to examine the expression level of the gene encoding the plastidic ATP/ADP transporter in such cultivars.

Experimental procedures

Plants

Wild-type and transgenic potato plants (*Solanum tuberosum* L., cv. Désirée) used for metabolite quantitation, tuber yield analysis

and RNA extraction were grown in a greenhouse in soil at 22°C and were watered daily. All experiments were conducted on tubers derived from the second vegetative plant generation. The ambient light period was extended to 16 h/day with Philips Sont-Agro lights (200 μmol quanta m^{-2} s^{-1}). Potato tissues required for analysis of the accumulation of mRNA encoding the potato plastidic ATP/ADP transporter were collected between 12 and 16 weeks after sprouting. Transgenic leaf tissue required for Northern blot analyses was collected 10 weeks after sprouting, transgenic tuber tissue used for Northern blot analyses was harvested 14 weeks after sprouting when tubers reached about 80% of their final size. Tubers used for morphologic and physiologic analyses were collected at the end of the vegetative period of the plants.

Preparation of total RNA, synthesis of first-strand cDNA and Northern blot analysis

Total RNA was purified according to Logemann *et al.* (1987) and first-strand cDNA prepared as given in Tjaden *et al.* (1998). Northern blot analyses were performed according to Sambrook *et al.* (1989) using radioactively labelled entire *AATP1* (At)-or *AATP1* (St)-cDNAs as probes.

Library screening and cDNA sequencing

The potato leaf-cDNA library (a gift from Prof. Dr Uwe Sonnewald, IPK-Gatersleben, Germany) was screened with a gene-specific probe generated by PCR. The probe was obtained by PCR of first-strand cDNA from leaves prepared as described above using the degenerated oligonucleotides TJ1 (deduced from the conserved amino acid sequence FANQIT; 5′-TT(C/T) GCN AA(C/T) CA(A/G) AT(A/T/C) AC-3′), and TJ2 (deduced from the conserved amino acid sequence LGKSGGA; 5′-(A/C/G/T)GC (A/C/G/T)CC (A/C/G/T)CC (A/C/G/T)(G/C)(A/T) (C/T)TT (A/C/G/T)CC (A/C/G/T)A-3′, which correspond to the amino acid positions 259–264 and 529–523, respectively, of the AATP1 (At) protein (Kampfenkel *et al.*, 1995). Screening was carried according to Möhlmann *et al.* (1998). The resulting plasmid with the cDNA insert in T3-orientation was named pTM1. The *AATP1* (*S. tuberosum*) cDNA was sequenced on both strands using specific cDNA primers which were synthesised by Eurogentec.

Construction of the bacterial expression vector pJT128 encoding His-AATP1 (St) and transformation of E. coli

To analyse the functionality of the *AATP1 (St)* cDNA product we decided to express the cDNA heterologously in *Eschericha coli* and to quantify ATP and ADP uptake into the intact bacterial cells after induction of *AATP1 (St)* expression. Recently, we have shown that the presence of a histidine tag at the N-terminus of two plastidic ATP/ADP transporters from *A. thaliana* allows the highest rates of adenine nucleotide transport in *E. coli* (Möhlmann *et al.*, 1998; Tjaden *et al.*, 1998). Therefore, AATP1 (St) protein was also constructed as a chimeric protein possessing a N-terminal histidine tag (His10-AATP1 (St)).

The cDNA coding for the entire AATP1 (St) pre-protein was generated by PCR from a full-length cDNA clone with Pfu-DNA polymerase (Stratagene, Heidelberg, Germany). A sense primer (bp positions 110–143, cagagagaggctaactcgagggtgttttacaaac) with a *Xho*I restriction site and an antisense primer (bp positions 2071–2042, cgatgcaaccaaggatccacctcagtcttg) with a *Bam*HI restriction site were used to conduct the PCR reaction. The PCR product was subsequently gel purified, digested with *Xho*I/*Bam*HI, and in frame inserted into the plasmid pET16b (Novagene, Heidelberg, Germany) leading to the presence of a N-terminal histidine tag of 10 amino acids. The sequence of the PCR amplified *AATP1* (St) DNA was confirmed by chain termination sequencing of both strands using specific cDNA primers synthesised by Eurogentec. Transformation of *E. coli* was carried out according to standard protocols. Induction of *His-AATP1* (St) expression and uptake experiments have been carried out as described in Tjaden *et al.* (1998).

Construction of the AATP1 *(At) sense and* AATP1 *(St) antisense gene*

DNA manipulations were performed essentially as described by Sambrook *et al.* (1989). For construction of the *AATP1* (At)-sense gene, the 2.230 bp *Eco*RV/*Bam*HI cDNA insert of the plasmid pET1 (Neuhaus *et al.*, 1997) encoding the *A. thaliana* AATP1 was introduced into *Sma*I-*Bam*HI sites of the plant expression vector pBinAR (Höfgen and Willmitzer, 1990) which contained the cauliflower mosaic virus 35S promoter and the polyadenylation signal of the T-DNA octopine synthase gene.

pBinAR was also used to construct the chimeric *AATP1* (St) antisense gene. The pTM1 plasmid encoding the *AATP1* (St) was digested with *Nde*I and blunt ended by a fill-in reaction with T4 polymerase. After *Bam*HI digestion, the resulting 1.265 bp cDNA-fragment was cloned in the reverse orientation into *Sma*I-*Bam*HI sites of the pBinAR.

Transformation of potato tissue, cell culture and regeneration of plants

Transformation of potato leaf tissue, cell culture and regeneration of transgenic plants were conducted as given in Rocha-Sosa *et al.* (1989).

Purification of potato tuber mitochondria

Potato tuber mitochondria were purified according to Neuburger *et al.* (1982).

Enrichment of potato tuber amyloplast membranes

To establish whether the activity of the plastidic ATP/ADP transporter in transgenic potato plants was altered we employed a proteoliposome system (Neuhaus *et al.*, 1997). Amyloplastic membrane proteins were enriched as follows: 50–60 g Fwt of potato tubers were cut into slices (about 1–1.5 mm) and incubated on ice for 30 min in extraction medium containing 50 mM Hepes-NaOH pH 7.5, 1 mM EDTA, 1 mM KCl, 1 mM $MgCl_2$, and 1 M sorbitol (Naeem *et al.*, 1997). Subsequently, tuber discs and the extraction medium were homogenised for 3 × 4 sec in a Waring blender (200 ml vessel). The homogenate, filtered through muslin and collected in a glass beaker, was incubated for 5 min on ice to allow sedimentation of starch granules. The supernatant was centrifuged for 5 min at 4000 ***g*** and the sediment resuspended in 2 ml of 100 mM tricine-NaOH (pH 7.5), and 30 mM potassium gluconate. The preparation was subsequently homogenised in a tight-fitting glass homogeniser and stored on ice for 5 min to allow sedimentation of starch. An aliquot of the homogenate was frozen at –80°C for quantification of marker enzyme activities. The supernatant was centrifuged for 30 min at 100 000 ***g*** in an

ultracentrifuge and the final sediment resuspended in tricine-gluconate buffer. Marker-enzyme activity measurements revealed that tuber amyloplasts were enriched 1.8-fold compared to mitochondria (data not shown).

Uptake of radioactively labeled ATP in proteoliposomes

Proteoliposomes containing mitochondrial membrane proteins or membrane proteins from enriched amyloplasts were prepared as described previously (Möhlmann *et al.*, 1997). Proteoliposome pre-loading and uptake experiments were carried out as given in Neuhaus *et al.* (1997). All data were corrected for ATP uptake in the presence of an inhibitor mix (5 mM DIDS, 10 mM pyridoxal phosphate) or on ice. Both treatments gave similar background results (6–9% of the corresponding samples). The data presented are the means of three independent experiments.

Quantitative starch determination

Starch was quantified spectrophotometrically as glucose (C6) units after hydrolysis. Potato tubers were sliced in discs (1–1.5 mm). A representative disc from the middle of a tuber was weighed (2–3 g Fwt) and homogenised with liquid nitrogen in a mortar. The powder was resuspended in 10 ml of 50 mM Na-acetate-HCl buffer (pH 4.7) and autoclaved for 2 h at 135°C. After cooling, 3 U per ml of each, α-amylase and amyloglucosidase, were added and starch was digested for 3 h (37°C). After boiling (3 min) and subsequent centrifugation for 5 min at 16 000 ***g*** the glucose in the supernatant was quantified according to Lowry and Passonneau (1972). The sediment was resuspended, autoclaved and digested for at least three additional times until no further glucose release was detectable.

Qualitative starch determination

Qualitative determination of starch was conducted by iodine staining according to Visser *et al.* (1991).

Determination of the amylose/amylopectin ratio in potato tuber starch and quantitation of mono- and disaccharides

The amylose/amylopectin ratio was determined using a colorimetric method specifically developed for potato starch (Hovenkamp-Hermelink *et al.*, 1988). To standardise the test, artificial mixtures of potato amylose and amylopectin (Sigma) were used. The concentration of glucose, fructose and sucrose were determined in a coupled assay as given in Lowry and Passonneau (1972) after extraction of these compounds according to Hatzfeld and Stitt (1990).

Protein quantification

Protein in samples was quantified after solubilization of membrane proteins in 2% TritonX100 using a detergent-insensitive bicinchoninic-acid copper II-sulphate assay (Sigma, Deisenhofen, Germany).

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