

RESEARCH PAPER

# Decreased expression of plastidial adenylate kinase in potato tubers results in an enhanced rate of respiration and a stimulation of starch synthesis that is attributable to post-translational redox-activation of ADP-glucose pyrophosphorylase

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## Abstract

Adenine nucleotides are of general importance for many aspects of cell function, but their role in the regulation of biosynthetic processes is still unclear. It was previously reported that decreased expression of plastidial adenylate kinase, catalysing the interconversion of ATP and AMP to ADP, leads to increased adenylate pools and starch content in transgenic potato tubers. However, the underlying mechanisms were not elucidated. Here, it is shown that decreased expression of plastidial adenylate kinase in growing tubers leads to increased rates of respiratory oxygen consumption and increased carbon fluxes into starch. Increased rates of starch synthesis were accompanied by post-translational redox-activation of ADP-glucose pyrophosphorylase (AGPase), catalysing the key regulatory step of starch synthesis in the plastid, while there were no substantial changes in metabolic intermediates or sugar levels. A similar increase in post-translational redox-activation of AGPase was found after supplying adenine to wild-type potato tuber discs to increase adenine nucleotide levels. Results provide first evidence for a link between redox-activation of AGPase and adenine nucleotide levels in plants.

Key words: Adenylate kinase, ADPglucose pyrophosphorylase, plastid, redox-regulation, potato, respiration, starch.

## Introduction

Much recent research attention has been focused on the manipulation of plant heterotrophic carbohydrate metabolism (Stitt and Sonnewald, 1991; Kruger, 1997) with the potato (*Solanum tuberosum* L.) tuber proving a useful model system in the understanding of the regulation of sink function (Fernie and Willmitzer, 2001; Viola *et al.*, 2001). Our understanding of the mechanism by which photoassimilates are unloaded from the phloem (Kühn *et al.*, 1999; Viola *et al.*, 2001) and the structure of the key pathways of carbohydrate metabolism in the potato tuber are relatively mature (Geigenberger, 2003; Lytovchenko *et al.*, 2007). That said, with the possible exception of the pathway of starch synthesis (Geigenberger *et al.*, 2004) our current understanding of the control and regulation of these pathways is, at best, fragmentary. Given its biotechnological importance much work in the potato tuber has concentrated on the sucrose–starch transition (reviewed in Biemelt and Sonnewald, 2006). A battery of transgenic plants have been created with alterations in the expression levels of the enzymes and membrane transporter proteins that constitute this pathway (Geigenberger *et al.*, 2004; Davies *et al.*, 2005) and their analysis has contributed greatly to the understanding of the control of starch synthesis in this organ (Fernie *et al.*, 2002; Geigenberger *et al.*, 2004; Lytovchenko *et al.*, 2007). Moreover, they illustrated the interdependence of these pathways, for example, lines expressing increased sucrolytic activity exhibited an increased rate of respiration and an impaired starch

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Abbreviations: ADK, adenylate kinase; AGPase, ADPglucose pyrophosphorylase.

synthesis (Trethewey *et al.*, 1998, 1999), and further analysis revealed that an increased rate of sucrose cycling and a less energy-efficient way of breaking down sucrose contributed to the complex changes observed (Fernie *et al.*, 2002; Bologna *et al.*, 2003).

Despite the fact that many attempts to increase starch accumulation produced the exact opposite result, several transgenic lines have been documented to exhibit elevated starch levels at maturity (Stark *et al.*, 1992; Tjaden *et al.*, 1998; Jenner *et al.*, 2001; Regierer *et al.*, 2002; Geigenberger *et al.*, 2005a). Two of these successful approaches were directly targeted at pathway enzymes/proteins—the overexpression of a bacterial ADPglucose pyrophosphorylase (AGPase) (Stark *et al.*, 1992) and the overexpression of an amyloplastidial adenylate transporter from *Arabidopsis* (Tjaden *et al.*, 1998)—while others were targeted at co-factors or effectors of this pathway. These alternative approaches included the antisense inhibition of the NAD-malic enzyme, UMPsynthase and the plastidial isoform of adenylate kinase (ADK) (Jenner *et al.*, 2001; Regierer *et al.*, 2002; Geigenberger *et al.*, 2005a). Whilst plants exhibiting an antisense inhibition of the UMPsynthase reaction of the *de novo* pathway of uridine biosynthesis have been relatively comprehensively characterized, work concerning those plants exhibiting decreased expression of NAD-malic enzyme and the plastidial ADK was mainly limited in focus towards the pathway of starch synthesis. Given that ATP supply to the plastid is largely furnished by respiratory processes occurring in the cytosol and mitochondria it was decided to evaluate the effect of reducing the plastidial ADK on these processes.

For this purpose, a detailed metabolic and physiological characterization of the previously described plastidial ADK lines has been carried out in this study. The major objectives addressed were to examine the rates of respiration in these transgenic lines and to elucidate the factors responsible for the observed increase in starch synthesis. Results obtained are discussed in the context of current models of tuber carbohydrate metabolism.

## Materials and methods

### Plant material

Wild-type potato *Solanum tuberosum* L. cv. Desiree (Saatzucht Lange AG, Bad Schwartau, Germany) and StpADK transgenic lines (Regierer *et al.*, 2002) were grown as previously described (Geigenberger *et al.*, 1998). Growing tubers aged 9–10 weeks were used for all analyses. For enzyme, metabolite, RNA, and non-aqueous fractionation analyses, tuber cores were immediately frozen in liquid nitrogen, ground into a fine powder using a ball-mill (Retsch, Haan, Germany), and stored at  $-80^{\circ}\text{C}$  until further analysis.

### Enzyme activities and metabolite measurements

Proteins were extracted and desalted according to Trethewey *et al.* (1998). Enzyme activities were measured according to the following: ADK (Kleczkowski and Randall, 1986), hexokinase (Renz

*et al.*, 1993), phosphoglucomutase, ATP-dependent phosphofructokinase, and fructokinase (Sweetlove *et al.*, 1996), pyrophosphate-dependent phosphofructokinase (Burrell *et al.*, 1994), and pyruvate kinase (Plaxton, 1990). For nucleotide measurements, tuber material was extracted with trichloroacetic acid and subjected to high performance liquid chromatography (HPLC) according to Geigenberger *et al.* (1997). Soluble sugars and starch were measured according to Geigenberger *et al.* (1996).

### Northern blot analysis

Total RNA extractions from tuber material were performed according to the Trizol method (Invitrogen, Carlsbad, CA). Northern blots were prepared and hybridized under standard conditions using an antisense fragment of the *StpADK* gene as a probe as described in Regierer *et al.* (2002).

### Non-aqueous fractionation

Non-aqueous fractionation of tuber material was performed essentially according to Farre *et al.* (2001) and Tiessen *et al.* (2002) with some modifications: 3 g ground tuber material was dried for 3 d in a lyophilizer and then extracted in 15 ml tetrachlorethylene/heptane (66:34 v:v), sonicated for 2 min on ice and filtered through a 20  $\mu\text{m}$  polyester net, which was then washed with 10 ml tetrachlorethylene/heptane (66:34 v:v). After addition of 5 ml heptane, the solution was centrifuged for 10 min at 750 g and the pellet was resuspended in 4 ml of the tetrachlorethylene/heptane mix. Aliquots were taken to determine total recovery, and then the sample was loaded onto a density gradient. The 30 ml gradient was prepared using a peristaltic pump and a needle as a 25 ml underlayered linear gradient starting from 73% tetrachlorethylene/27% heptane to 100% tetrachlorethylene, with a final 5 ml of 100% tetrachlorethylene. The sample gradient was centrifuged for 1 h at 55 400 g in a swing out rotor, and then 5–7 fractions were taken, dried overnight under vacuum, and stored at  $-20^{\circ}\text{C}$  until further analysis. Proteins were extracted and desalted as described for whole tissue measurements above. AGPase activity was measured according to Tiessen *et al.* (2002) as a marker for the plastid, Pyrophosphate-dependent phosphofructokinase was measured according to Burrell *et al.* (1994) as a marker for the cytosol, and alpha-mannosidase was measured according to Geigenberger *et al.* (1997) as a marker for the vacuole. ADK activity was measured as described above. Subcellular distribution calculations were carried out according to Tiessen *et al.* (2002).

### Tuber disc respiration measurements

Tuber discs (8 mm $\times$ 2 mm) were prepared from growing plants and briefly rinsed in buffer (10 mM MES–KOH pH 6.5) to remove dead cells, then two discs were immediately transferred to the temperature-controlled chamber of a Clark-type electrode (Hansatech, Norfolk, UK) containing 1 ml buffer. Respiration was measured as oxygen consumption at 25  $^{\circ}\text{C}$ .

### Mitochondrial respiration and yield measurements

Mitochondria were isolated from growing tubers according to Jenner *et al.* (2001) but with at least 100 g tuber material ground in 200 ml buffer in a Waring blender, and thereafter according to the published protocol (Jenner *et al.*, 2001). Protein was quantified using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Munich). Mitochondrial respiration was measured as oxygen consumption using a Clark-type electrode using 300 mg protein in 1 ml reaction buffer (Sweetlove *et al.*, 2002) with the addition of succinate (10 mM), NADH (1 mM), KCN (1 mM), ADP (0.5 mM), and salicylhydroxamic acid (SHAM) (10 mM) to determine mitochondrial respiration rates.

Cytochrome *c* oxidase enzyme activity was measured spectrophotometrically according to Millenaar *et al.* (2002) and mitochondrial yield was calculated based on the level of cytochrome *c* oxidase activity in isolated mitochondria compared to whole tissue extracts (Jenner *et al.*, 2001; Millenaar *et al.*, 2002).

#### Calculation of flux control coefficient

The flux control coefficient of plastidial ADK for respiration was calculated according to Geigenberger *et al.* (2004) using the line of best fit in Sigmaplot.

#### Incubation of tuber discs with adenine or orotate

Tuber discs (8 mm×2 mm) were freshly prepared from growing plants and briefly rinsed in buffer (10 mM MES–KOH pH 6.5) to remove damaged cells, then immediately transferred to 100 ml Erlenmeyer flasks containing 4 ml of 2 mM adenine or orotate in buffer, using 10 discs per flask, before subsequent incubation for 2 h at 25 °C with shaking at 90 rpm. Discs were frozen in liquid nitrogen and stored at –80 °C until further analysis.

#### <sup>14</sup>C glucose feeding

Tuber discs were prepared from growing plants and U-<sup>14</sup>C labelling experiments were performed as described by Geigenberger *et al.* (1997). Tuber discs were incubated in 10 mM glucose in 10 mM MES–KOH buffer (pH 6.5) containing U-<sup>14</sup>C labelled glucose (10 discs per 4 ml in 100 ml Erlenmeyer flasks; specific activity 18.5 kBq ml<sup>-1</sup>) for 2 h with shaking at 90 rpm, then briefly rinsed with 10 mM MES–KOH buffer (pH 6.5) and frozen in liquid nitrogen. Tissue extractions and fractionation of soluble components were carried out according to Geigenberger *et al.* (1997) and fractionation of insolubles into starch, protein, and cell wall was carried out according to Merlo *et al.* (1993).

#### TCA cycle flux

TCA cycle flux analysis was performed based on Nunes-Nesi *et al.* (2005). Tuber discs were prepared from growing plants and incubated in 0.2 mM glucose in 10 mM MES–KOH buffer (pH 6.5) containing 1-<sup>14</sup>C-, 3,4-<sup>14</sup>C-, or 6-<sup>14</sup>C-labelled glucose (10 discs per 4 ml in 100 ml Erlenmeyer flasks; specific activity 1.85 kBq ml<sup>-1</sup>) with shaking at 90 rpm. Released CO<sub>2</sub> was captured in 10% KOH every 2 h and quantified by liquid scintillation counting.

#### Western blot analysis of AGPase

Proteins were rapidly extracted from tuber material according to Tiessen *et al.* (2002, 2003) in 4× Laemmli buffer (Laemmli, 1970) and subjected to Western blot analysis according to standard procedures. Membranes were probed with a primary rabbit antibody raised against the B subunit of AGPase (Tiessen *et al.*, 2002). The secondary antibody was an affinity-purified IRDye800-conjugated goat anti-rabbit antibody (catalogue number 611-132-003, Rockland Immunochemicals Inc, Gilbertsville, PA). Signal was quantified using the Odyssey Infrared Imager system (Li-Cor Biosciences, Lincoln). The activation state of AGPase was determined by measuring the ratio of the 50 kDa active (monomer) to 100 kDa inactive (covalently-linked dimer) protein according to Tiessen *et al.* (2002).

#### Statistical analysis

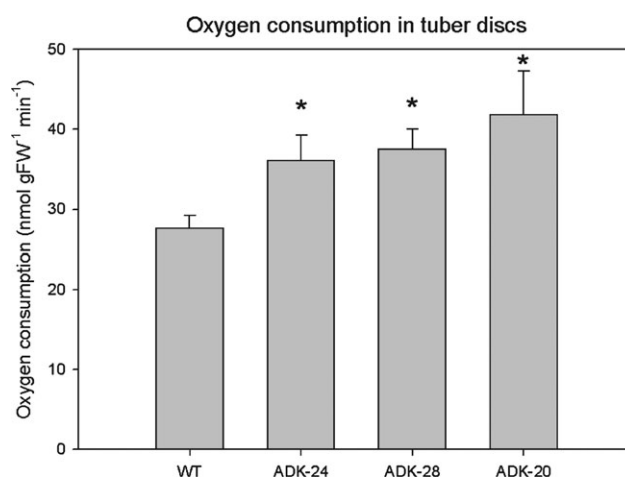
Data were analysed using Student's *t* test and deemed significant if *P* < 0.05.

## Results

### *StpADK* transgenic tubers have a higher respiration rate compared to wild type

The transgenic plants used in this study contain a construct with an antisense fragment of the *StpADK* plastidial ADK gene under the control of the 35S promoter and have previously been described (Regierer *et al.*, 2002). Northern blot analysis confirmed that three lines (ADK-20, -24, and -28) have maintained the *StpADK* antisense phenotype, showing reduced *StpADK* gene expression (data not shown). ADK activity in tubers was reduced to 90% (±5), 79% (±7), and 70% (±11) of wild-type activity for lines ADK-24, -28, and -20, respectively (significant for ADK-28 and ADK-20, *P* < 0.05). Non-aqueous fractionation of tuber tissue from wild type and line ADK-20 showed that ADK activity was significantly reduced in the plastidial fraction in ADK-20 by 40% compared with wild type (*P* < 0.05, *n* = 3 ADK-20 fractions and *n* = 4 wild-type fractions), whereas ADK activity was not significantly different in the cytosol-mitochondria fraction (data not shown). These results confirm that the reduction in tuber ADK activity in the *StpADK* transgenic lines occurs specifically in the plastid.

This study focused on the analysis of respiration in actively growing *StpADK* tubers aged 9–10 weeks. The rate of oxygen consumption was measured in tuber discs immediately after harvest (Fig. 1). These results showed that respiration was significantly increased by 31–51% in *StpADK* transgenic tubers. As reported previously (Regierer *et al.*, 2002), the plant lines used in this study showed only a slight and inconsistent increase in tuber ATP levels

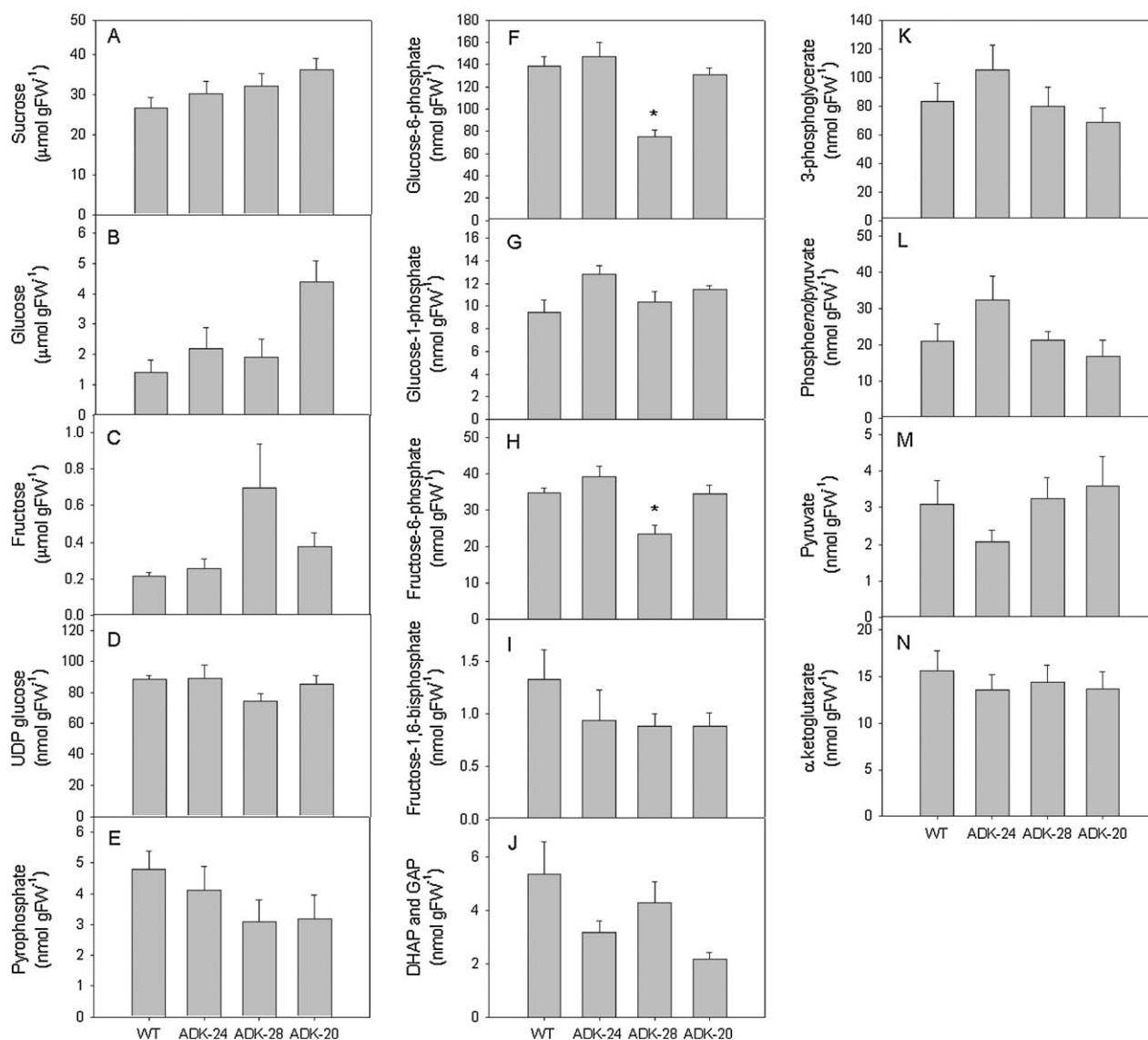


**Fig. 1.** Respiration rates in tuber discs from wild-type and *StpADK* transgenic tubers at 9 weeks. Respiration was measured as the rate of oxygen consumption by two tuber discs (8 mm×2 mm prepared immediately after harvest) in 1 ml of 10 mM MES–KOH pH 6.5 using a Clark electrode. The graph shows the average and standard error of 12 replicate measurements per line, with each measurement taken from a single tuber, from six plants per line. An asterisk denotes significant difference from wild type (*P* < 0.05).

(see Supplementary Fig. S1A at *JXB* online), but significantly higher ADP levels compared to wild-type tubers (see Supplementary Fig. S1B at *JXB* online). Also AMP levels were increased, the increase being significantly different from the wild-type in line ADK-20 (see Supplementary Fig. S1C at *JXB* online), and all transgenic lines showed a significantly higher total adenylate nucleotide sum (see Supplementary Fig. S1D at *JXB* online). The ATP/ADP ratio was reduced in StpADK tubers, which is consistent with a higher respiration rate (see Supplementary Fig. S1E at *JXB* online).

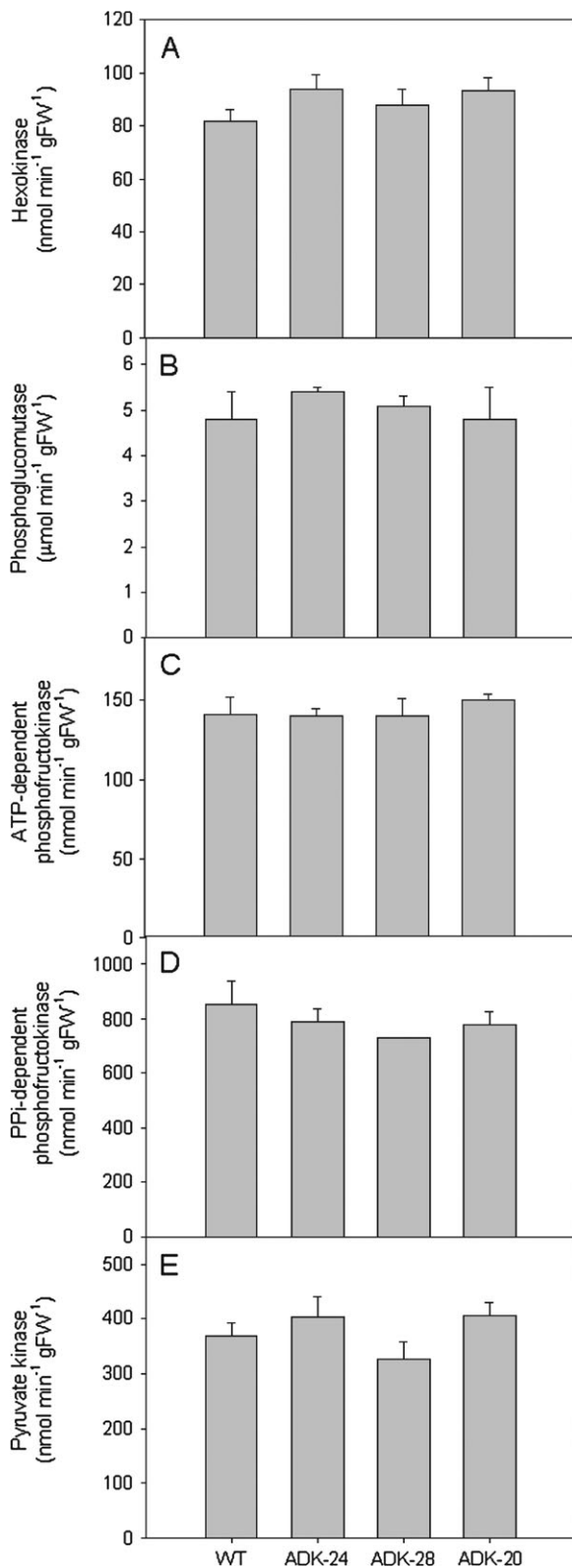
To determine whether the increased respiration rate could be due to pleiotropic effects on glycolysis or mitochondrial respiration and not directly due to the reduced plastidial ADK activity, measurements of metabolites, glycolytic enzymes, mitochondrial respiration, and mito-

chondrial yield were performed. The levels of sucrose, glucose, fructose, UDP-glucose, glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, DHAP and GAP, glycerate-3-phosphate, phosphoenolpyruvate, pyruvate, alpha keto-glutarate, and pyrophosphate are shown in Fig. 2. Most metabolites had similar concentrations in the StpADK tubers compared to the wild type. The only significant differences were a decrease in glucose-6-phosphate and fructose-6-phosphate in ADK-28. Maximal activities of key glycolytic enzymes in tubers showed no significant difference to the wild type (Fig. 3). Analysis of mitochondria isolated from tubers showed that the mitochondrial respiration rates and mitochondrial yield in the StpADK transgenics were not significantly different from the wild type (Fig. 4). Taken together, these results indicate that the higher respiration



**Fig. 2.** Metabolite levels in 10-week-old tubers from the StpADK transgenic plants. Data are shown as the average of six plants per line. An asterisk denotes significant difference from wild type ( $P < 0.05$ ).





**Fig. 3.** Maximal enzyme activities in 10-week-old tubers from the StpADK transgenic plants. Data are shown as the average of six plants per line. An asterisk denotes significant difference from wild type ( $P < 0.05$ ).

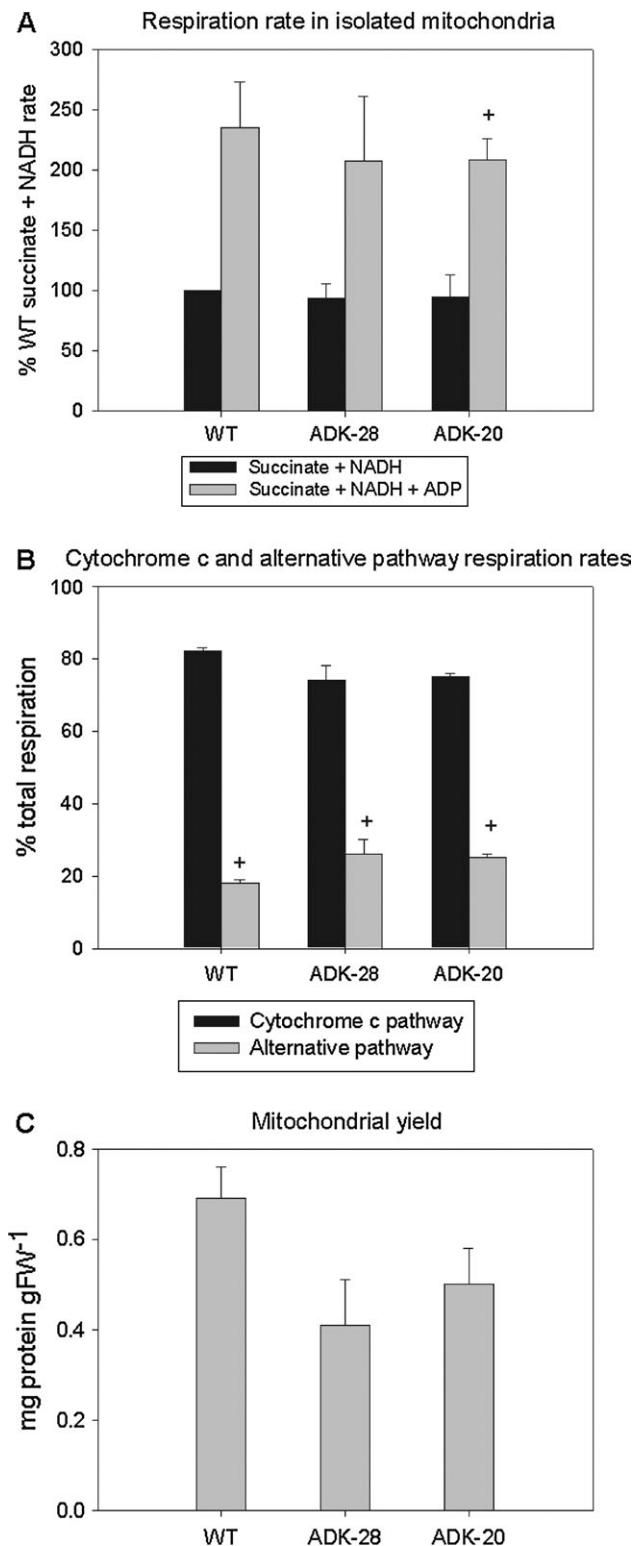
rate in the StpADK transgenic tubers is not due to an increase in maximal glycolytic or mitochondrial capacity, but rather is primarily due to the reduction in plastidial ADK activity. The control coefficient of plastidial ADK for respiration was calculated to be  $-1.5$  ( $R^2=0.90$ , Fig. 5), which suggests that plastidial ADK has a strong control over the rate of respiration in tubers.

*Increased respiration in the StpADK transgenic tubers is associated with increased flux to starch synthesis*

Incubation of tuber discs with  $U-^{14}C$  glucose was performed to measure fluxes through the major carbon metabolic pathways in tubers. Tuber discs were incubated for 2 h and the tissue was fractionated to determine the label distribution in tuber metabolites. No significant difference was detected in total label uptake or amount of label metabolized in the StpADK tuber discs compared to the wild type (Fig. 6A, B). A significant increase in label incorporation into starch was detected in all StpADK transgenics compared to the wild type (Fig. 6D), and this was reflected in an increased flux into starch synthesis (Fig. 6M). ADK-28 showed a significant decrease of label incorporation into sucrose, while ADK-24 showed a significant reduction of label incorporation into organic acids. Flux into cell wall, glycolysis, and sucrose was similar between the wild type and the StpADK lines (Fig. 6N–P).

The increased flux into starch synthesis in the StpADK transgenic tubers is consistent with a previous analysis of these plants which showed that the level of starch in tubers from fully senescent plants is more than 60% higher in StpADK tubers than the wild type (Regierer *et al.*, 2002). Moreover, analysis of plants under field conditions showed a yield increase to between 65% and 85% above that found in the wild type, and estimates of starch content per plant were found to be double that found in wild-type lines (Regierer *et al.*, 2002). Analysis of 9-week-old plants revealed only a marginal increase in starch content in the StpADK transgenics when assessed on a per gram fresh weight basis, but due to the significantly higher biomass exhibited by StpADK tubers, starch levels were significantly higher when assessed on a per plant basis (data not shown). These findings indicate that starch synthesis is already enhanced at a relatively early stage of tuber development and dramatic increases in starch content are only observed at later stages. Wild-type tubers exhibit a linear increase in starch content during development, and tubers with different growth rates show larger differences in starch content at later stages of development than at earlier stages (Engels and Marschner, 1986).

In a second experiment, analysis of the flux through the TCA cycle was performed by incubating tuber discs in positionally-labelled  $^{14}C$  glucose. For the StpADK tuber discs, the ratio of label detected in  $CO_2$  arising from incubation in C1 glucose versus C3,4 glucose was lower



**Fig. 4.** Analysis of mitochondrial respiration rates and mitochondrial yield in StpADK transgenic lines. Respiration rate was measured in the presence of succinate and NADH with and without ADP (A); a cross denotes significant difference after ADP addition ( $P < 0.05$ ). The percentage of respiration attributed to the cytochrome and alternative pathways was measured as the rate of KCN-sensitive and KCN-insensitive respiration, respectively (B); a cross denotes significant difference between cytochrome and alternative pathways ( $P < 0.05$ ).

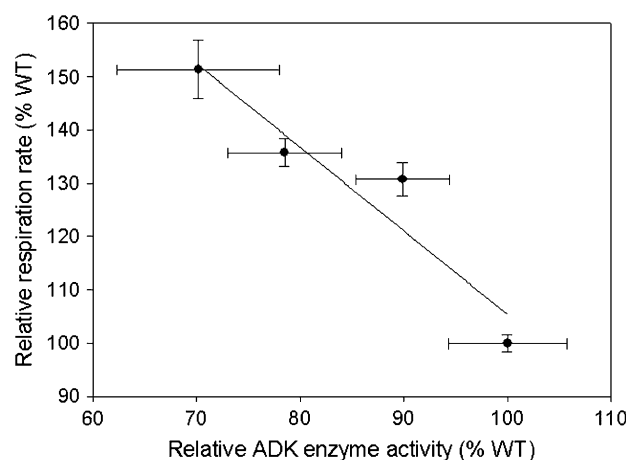
than wild type at all time points from 2–8 h, averaging 1.01 ( $\pm 0.09$ ), 0.81 ( $\pm 0.09$ ), 0.85 ( $\pm 0.08$ ), and 0.96 ( $\pm 0.06$ ) for wild type, ADK-24, ADK-28, and ADK-20, respectively (significant for ADK-24,  $P < 0.05$ ) indicating that the StpADK transgenic tubers had a higher rate of flux through the TCA cycle. The ratio of label detected in  $\text{CO}_2$  arising from incubation in C1 glucose versus C6 glucose was similar in wild-type and StpADK tuber discs, indicating no difference in the rate of the oxidative pentose phosphate pathway (data not shown).

#### *Reduced plastidial ADK activity results in increased activation of AGPase*

AGPase is a key regulatory enzyme for starch synthesis in tubers, and the activation of AGPase closely reflects the level of starch synthesis (Preiss, 1988; Stark *et al.*, 1992; Tiessen *et al.*, 2002; Geigenberger *et al.*, 2004). AGPase is a heterotetrameric enzyme consisting of two small (AGPB) and two slightly larger (AGPS) subunits (Morell *et al.*, 1987). The enzyme is sensitive to allosteric regulation (Preiss, 1988; Stark *et al.*, 1992) and most recently was found to be subject to post-translational redox regulation, which involves reversible disulphide-bond formation between the two small subunits (Fu *et al.*, 1998; Ballicora *et al.*, 2000; Tiessen *et al.*, 2002). The change in redox state can be detected from a change in the electrophoretic mobility of AGPB in non-reducing SDS gels, with AGPB running as a dimer in the oxidized form and as a monomer in the reduced form. The redox-activation state of AGPase in the StpADK transgenic tubers was therefore measured by determining the monomer/dimer ratio of AGPB (Fig. 7A, B). The results showed that the redox-activation state of AGPase was increased in the transgenic tubers by 1.6–2.8-fold over wild type, while the overall protein level of AGPB was not substantially changed (Fig. 7A, B). These results indicate that the increased starch synthesis in the StpADK tubers is due to an increased redox-activation state of AGPase.

To investigate further the reason for the increased activation of AGPase, the effect of feeding adenine on the AGPase activation state was studied. Previous studies showed that endogenous adenine nucleotide levels are increased when wild-type tuber discs were supplied with 2 mM adenine, while uridine and guanidine nucleotide levels remained unchanged (Loef *et al.*, 1999, 2001). Therefore wild-type tuber discs were incubated with 2 mM adenine to analyse the redox-activation state of AGPase. As seen in previous studies, adenine feeding led to a significant increase in the total adenylate pool of the

Mitochondrial yield was calculated as the ratio of cytochrome *c* oxidase activity in isolated mitochondria and in whole tissue (C). The graphs show the averages of two replicate mitochondrial isolations using mitochondria with respiratory control ratios  $> 2$ .



**Fig. 5.** Flux control coefficient of plastidial ADK for respiration. The respiration rates in wild type and StpADK transgenic tubers (data from Fig. 1, shown as a percentage of wild-type levels) was plotted against tuber ADK activity, and the flux control coefficient was calculated as the slope of the line of best fit.

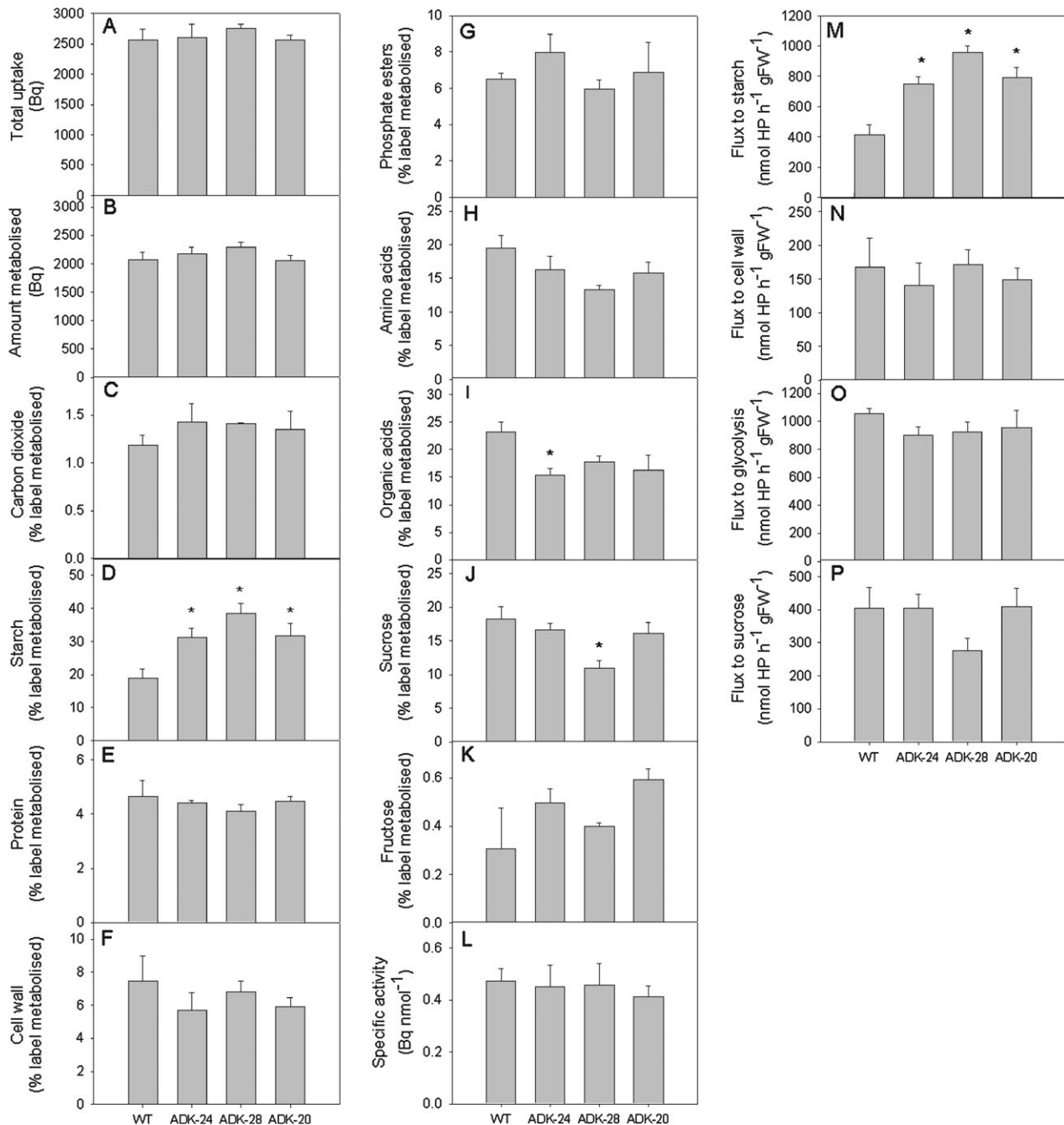
discs rising from  $56.5 \pm 9.8$  in the control to  $124.1 \pm 10.8$   $\text{nmol g}^{-1}$  fresh weight after adenine feeding in the present study (mean  $\pm$  SE,  $n=3$ ). This was accompanied by a significant increase in the activation state of AGPase by 32% (Fig. 7C). To confirm that the rise in AGPase activation was specific for adenine nucleotides rather than uridine nucleotides, discs were supplied in a separate experiment with 2 mM orotate which is an intermediate of *de novo* uridine nucleotide biosynthesis. Orotate feeding led to a slight decrease (17%) rather than an increase in the redox-activation state of AGPase (Fig. 7D). The different levels in the controls of the orotate (Fig. 7D) and adenine feeding experiments (Fig. 7C) were due to the use of tuber material from different batches of plants. Previous studies showed that orotate feeding leads to an increase in uridine nucleotide levels, while adenine nucleotide remain unchanged, which leads to a strong stimulation in the rate of sucrose degradation (Loef *et al.*, 1999), the resulting low sugar levels providing a likely explanation for the slight decrease in AGPase activation state in response to orotate. Overall results indicate that an increased level of adenine nucleotides, but not uridine nucleotides, is able to increase the AGPase activation state. Taken together with the above results, it is probable that the increased activation state of AGPase in the StpADK tubers is due to the higher endogenous level of adenylate nucleotides.

## Discussion

There is extensive evidence documented in the microbial and mammalian literature that ATP demand controls the rate of respiration (Hofmeyer and Cornish-Bowden, 2000; Koebmann *et al.*, 2002), and the mechanisms by which this is achieved are fairly well defined and include the allosteric regulation of the phosphofructokinase reaction

by ATP (Fornie *et al.*, 2004). In plants, the situation is less clear. Feeding adenine to increase the levels of adenine nucleotides (Loef *et al.*, 2001) and certain transgenic modifications that create an enhanced demand for ATP were also found to result in elevated rates of respiration (Fornie *et al.*, 2002; Bologna *et al.*, 2003). However, plant phosphofructokinase is not allosterically affected by ATP (Fornie *et al.*, 2004). That said, both theoretical and experimental studies suggest that the rate of respiration is controlled at steps downstream of the reaction catalysed by pyruvate kinase (Thomas *et al.*, 1997; Thomas and Fell, 1998) and control by adenylate status remains a distinct possibility. In a previous study, it was demonstrated that transgenic potato plants exhibiting decreased expression of a plastidial ADK exhibited increased growth and elevated levels of starch, amino acids, and a minor increase in the level of ATP (Regierer *et al.*, 2002). Given these changes, it is hypothesized that these lines were also altered in their rates of respiration. Direct analysis of respiratory parameters revealed this indeed to be the case. Interestingly, the increased rate of respiration was only observed in intact tuber tissue (Fig. 1), with no change observed in isolated mitochondria (Fig. 4A, B), and there was a tendency towards a decreased mitochondrial density per cell in the transformants (Fig. 4C). This suggests that the effect on respiration is probably due to fine control mechanisms and is clearly not the consequence of a pleiotropic effect on the mitochondrial respiratory machinery. Further experiments revealed that there was little change in either the maximal catalytic activities of the enzymes of glycolysis (Fig. 3) or the levels of intermediates of primary metabolism in the StpADK transgenic lines (Fig. 2). When the rate of respiration is plotted against the residual activity of the plastidial ADK in the transformants, a very clear negative correlation is apparent, suggesting that the plastidial ADK exhibits significant control over the rate of respiration (Fig. 5). Whilst we are currently unable to define the exact mechanism underlying this regulation it appears to be largely unrelated with glycolysis. In keeping with this fact the estimated flux through glycolysis is unaltered in the transformants, however, the flux to starch is dramatically elevated (Fig. 6M).

In contrast to the observations concerning respiration, it was possible to provide mechanistic insight into the elevated flux to starch displayed by the transformants. Antisense repression of plastidial ADK led to an increase in post-translational redox-activation of AGPase (Fig. 7A), which represents a key regulatory mechanism of starch synthesis in potato tubers (Tiessen *et al.*, 2002; Geigenberger *et al.*, 2005b). There were no significant changes in the levels of glycolytic intermediates in all lines (Fig. 2), including hexose-phosphates and 3PGA, which represent the substrate and allosteric effector of AGPase, respectively, making it unlikely that the increase in starch synthesis was due to increased metabolite levels

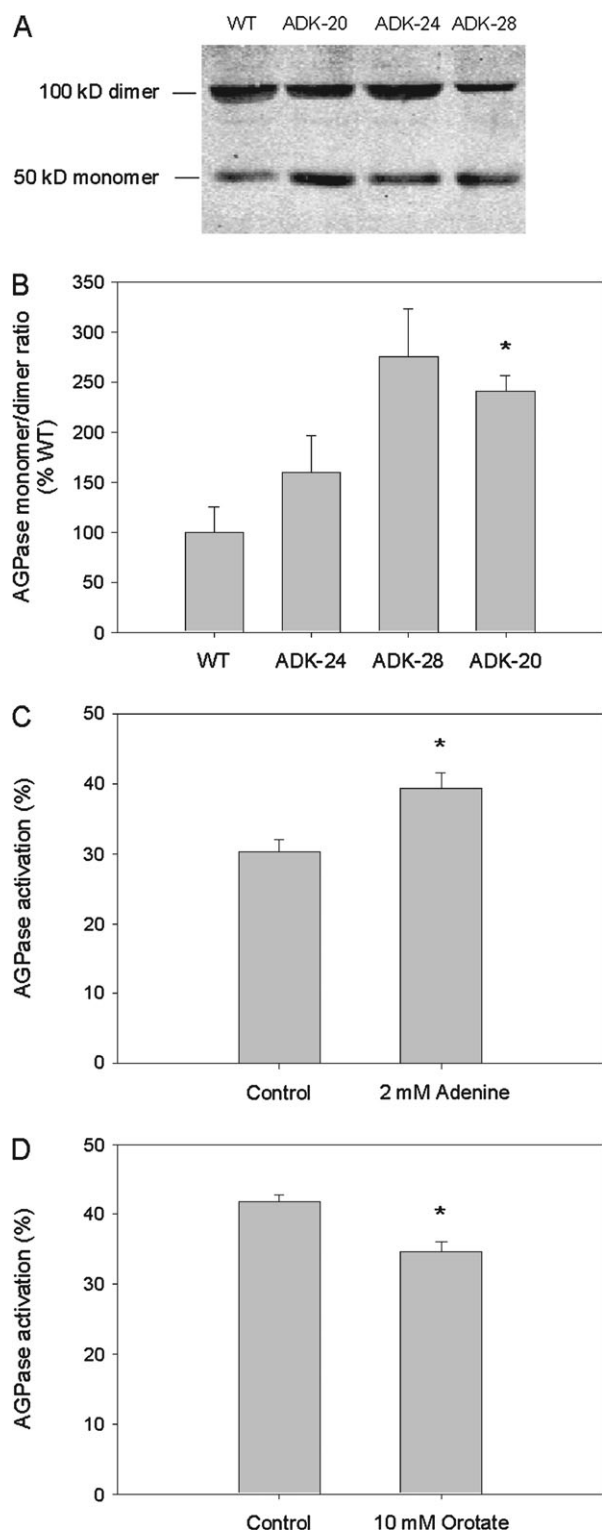


**Fig. 6.** Metabolism of  $^{14}\text{C}$  glucose in tuber discs from StpADK tubers. Ten tuber discs (prepared immediately after harvest) were incubated in 4 ml of 10 mM MES–KOH pH 6.5 containing 10 mM  $\text{U-}^{14}\text{C}$  glucose ( $0.5 \mu\text{Ci ml}^{-1}$ ) for 2 h, and the redistribution of label was measured in fractionations of ethanol extracts. The graphs show the total label uptake (A), amount of label metabolized (B), label incorporated into carbon dioxide (C), starch (D), protein (E), cell wall (F), organic acids (G), amino acids (H), phosphate esters (I), sucrose (J), and fructose (K). Specific activity (L) was calculated from the amount of label in the hexose phosphate pool. Fluxes of  $^{14}\text{C}$  glucose into starch (M), cell wall (N), glycolysis (O), and sucrose (P) were calculated as label incorporation divided by the specific activity. Label into glycolysis was calculated as the sum of label incorporation of carbon dioxide, protein, organic acids, and amino acids. Data show the mean and standard error from three replicate incubations per line. An asterisk denotes significant difference from wild type ( $P < 0.05$ ); HP, hexose phosphates.

and subsequent allosteric regulation. Moreover, the overall protein level of AGPase was not substantially changed in the transgenic tubers in comparison to wild-type, as seen from the immunoblots in Fig. 7. Consistent with this, the previous study by Regierer *et al.* (2001) showed that there

were no substantial changes in the maximal activities of AGPase, phosphoglucosmutase, soluble starch synthase, granule-bound starch synthase, and starch branching enzyme in tubers after repression of ADK. It is therefore concluded that increased starch synthesis in tubers with





**Fig. 7.** Activation state of AGPase in StpADK transgenic tubers and after incubation in adenine or orotate. Western blots of tuber protein were performed using an antibody against the B subunit of AGPase (A). The amount of 50 kDa monomer and 100 kDa dimer was quantified and the monomer/dimer ratio of AGPase was used to determine the activation state of the enzyme in tubers (B) ( $n=3$  tubers from separate plants). (C, D) The effect of adenine and orotate, respectively, on the activation state of AGPase. Tuber discs (prepared immediately after

decreased expression of plastidial ADK was not due to allosteric regulation of AGPase or changes in the maximal activities of enzymes involved in the pathway of starch synthesis in the plastid. This underlines the importance of post-translational protein modification, with redox-activation of AGPase being the most likely mechanism responsible for the increased starch synthesis.

Having said this, alternative explanations such as regulation of starch biosynthetic enzymes by reversible protein phosphorylation or protein-protein interactions cannot be ruled out. Excellent studies by Tetlow and colleagues provide convincing evidence that protein phosphorylation and protein-protein interaction indeed occur and are functionally significant with respect to starch synthases or starch branching enzymes in developing wheat seeds (Tetlow *et al.*, 2004a, b). More studies are needed to investigate whether similar mechanisms are also involved in regulating starch synthesis in other tissues, such as potato tubers. Investigating the link between changes in adenylate nucleotide pools and protein phosphorylation in the plastid is an interesting avenue of future research.

The reason for the increased redox-activation of AGPase in lines with decreased plastidial ADK still needs to be clarified. Previous studies showed that redox-activation of AGPase responds to changes in sugar supply, with high levels of sucrose promoting activation of AGPase in leaves and tubers via signals involving SNF-like protein kinases and trehalose-6-phosphate (Hendriks *et al.*, 2003; Tiessen *et al.*, 2003; Kolbe *et al.*, 2005). However, decreased expression of ADK did not lead to significant changes in the levels of sucrose (Fig. 2A), making it unlikely that redox-activation of AGPase has been promoted by sugar signalling in these tubers.

An alternative explanation would be that redox-activation of AGPase in the ADK lines is due to the increase in adenine nucleotide levels. Evidence for a link between adenine nucleotide levels and redox-activation of AGPase has been provided by feeding nucleotide precursors to wild-type potato tuber discs. Adenine feeding led to increased adenine nucleotide levels (see above), which were accompanied by increased redox-activation of AGPase (Fig. 7C). Stimulation of AGPase was due to an increase in adenine rather than uridine nucleotide levels, since orotate feeding led to a slight decrease in AGPase redox-activation (Fig. 7D). Previous studies by Loeff *et al.* (1999) showed that orotate feeding leads to an increase in uridine nucleotide levels, without changing adenine nucleotide levels, resulting in a marked increase in the

harvest) were incubated in buffer only (control, 10 mM MES-KOH pH 6.5), or buffer with 2 mM adenine or 10 mM orotate for 2 h. After incubation, proteins were extracted and the AGPase activation state was measured as the ratio of activity without DTT ( $V_{red}$ ) to activity with DTT (fully activated,  $V_{sel}$ ) and is expressed as a percentage. Data represent the mean and standard error of three separate incubations. An asterisk denotes significant difference from control treatment ( $P < 0.05$ ).

rate of sucrose degradation which will lead to decreased sucrose levels. Decreased sugars have previously been shown to lead to a decrease in the redox-activation of AGPase (Tiessen *et al.*, 2002, 2003).

Adenylates might act directly on AGPase since they are binding as direct substrates. *In vitro* experiments showed that DTT- or thioredoxin-induced activation of potato AGPase is dependent on the presence of ATP or ADP-glucose substrates, which are needed to attain the active conformation after reduction (Fu *et al.*, 1998; and data not shown). Moreover, it has been found for photosynthetic enzymes that thioredoxin-dependent redox-activation is modified by pH, Mg<sup>2+</sup>, and the levels of substrates and products, which lead to a change in the mid-point redox-potential of the regulatory cysteines (Scheibe, 1991). It is tempting to speculate that a similar mechanism could be involved in promoting redox-activation of AGPase in response to increased adenine nucleotide levels. More experiments are underway to investigate the link between adenine nucleotide levels and redox-activation of AGPase, and the underlying signalling pathways.

In conclusion, decreased expression of plastidial ADK leads to a stimulation of respiration and starch synthesis, which resembles the response of adenine feeding to wild-type potato tuber discs. In both experimental systems, there was an increase in adenylate pool levels which was accompanied by post-translational redox-activation of AGPase, the key enzyme of starch synthesis. This provides first evidence for a possible link between AGPase redox-activation and adenine nucleotides that requires further investigations in the future.

## Supplementary data

Supplementary data are available at *JXB* online.

**Fig. S1.** Adenine nucleotide levels in 10-week-old tubers from the StpADK transgenic plants: (A) ATP, (B) ADP, (C) AMP, (D) total adenylates (sum ATP, ADP, and AMP), and (E) ATP/ADP ratio. Data are shown as the average of six plants per line. An asterisk denotes significant difference from the wild type ( $P < 0.05$ ).

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