The relevance of adenylate levels and adenylate converting enzymes on metabolism and development of potato (*Solanum tuberosum L.*) tubers

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Dissertation

zur Erlangung des Grades Doktor der Naturwissenschaften (Dr. rer. nat.) der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Potsdam

vorgelegt von

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Potsdam, Mai 2008

2. Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und unter Verwendung keiner anderen als den von mir angegebenen Quellen und Hilfsmitteln verfasst habe. Ferner erkläre ich, dass ich bisher weder an der Universität Potsdam noch anderweitig versucht habe, eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen.

3. Summary

Adenylates are metabolites with essential function in metabolism and signaling in all living organisms. As Cofactors, they enable thermodynamically unfavorable reactions to be catalyzed enzymatically within cells. Outside the cell, adenylates are involved in signalling processes in animals and emerging evidence suggests similar signaling mechanisms in the plants' apoplast. Presumably, apoplastic apyrases are involved in this signaling by hydrolyzing the signal mediating molecules ATP and ADP to AMP. This PhD thesis focused on the role of adenylates on metabolism and development of potato (*Solanum tuberosum*) by using reverse genetics and biochemical approaches.

To study the short and long term effect of cellular ATP and the adenylate energy charge on potato tuber metabolism, an apyrase from *Escherichia coli* targeted into the amyloplast was expressed inducibly and constitutively. Both approaches led to the identification of adaptations to reduced ATP/energy charge levels on the molecular and developmental level. These comprised a reduction of metabolites and pathway fluxes that require significant amounts of ATP, like amino acid or starch synthesis, and an activation of processes that produce ATP, like respiration and an immense increase in the surface-to-volume ratio.

To identify extracellular enzymes involved in adenylate conversion, green fluorescent protein and activity localization studies in potato tissue were carried out. It was found that extracellular ATP is imported into the cell by an apoplastic enzyme complement consisting of apyrase, unspecific phosphatase, adenosine nucleosidase and an adenine transport system. By changing the expression of a potato specific apyrase via transgenic approaches, it was found that this enzyme has strong impact on plant and particular tuber development in potato. Whereas metabolite levels were hardly altered, transcript profiling of tubers with reduced apyrase activity revealed a significant upregulation of genes coding for extensins, which are associated with polar growth.

The results are discussed in context of adaptive responses of plants to changes in the adenylate levels and the proposed role of apyrase in apoplastic purinergic signaling and ATP salvaging.

In summary, this thesis provides insight into adenylate regulated processes within and outside non-photosynthetic plant cells.

4. Deutsche Zusammenfassung

Adenylate haben essentielle Funktionen in Stoffwechselprozessen und fungieren als Signalmoleküle in allen Organismen. Als Cofaktoren ermöglichen sie die Katalyse thermodynamisch ungünstiger Reaktionen innerhalb der Zelle, und außerhalb der Zelle wirken sie als Signalmoleküle in Tieren und nach neueren Forschungsergebnissen wohl auch in Pflanzen. Vermutlich wird die Signalwirkung von ATP und ADP durch Hydrolyse zu AMP unter Beteiligung apoplastische Apyrasen terminiert. Diese Arbeit behandelt den Einfluss der Adenylate auf Stoffwechsel- und Entwicklungsprozesse in der Kartoffelpflanze (*Solanum tuberosum*) mittels biochemischer und revers-genetischer Ansätze.

Um kurzfristige und langfristige Einflüsse zellulären ATPs und der Energieladung auf den Stoffwechsel von Kartoffelknollen zu untersuchen, wurde eine mit einem plastidären Transitpeptid fusionierte Apyrase aus *Escherichia coli* induzierbar und dauerhaft exprimiert. Beide Ansätze führten zur Identifizierung von Anpassungen an eine reduzierte ATP Verfügbarkeit bzw. verringerte Energieladung. Die Anpassungen beinhalteten eine Reduzierung von ATP-verbrauchenden Stoffwechselaktivitäten und Stoffwechselprodukten, wie die Aminosäure- oder Stärkesynthese, und eine Aktivierung von Prozessen, welche die ATP-Bildung oder eine effizientere ATP-Bildung ermöglichen, wie Zellatmung und die Vergrößerung des Oberfächen/Volumen-Verhältnisses der Kartoffelknolle.

Extrazelluläre Adenylat-umsetzende Enzyme wurden mit Hilfe des grün fluoreszierenden Proteins und Aktivitätsmessungen identifiziert und charakterisiert. Es wurde ein potentieller ATP Bergungsstoffwechselweg gefunden, der ATP über die Enzyme Apyrase, unspezifische Phosphatase und Adenosin-Nukleosidase zu Adenin umsetzt, welches über eine Purin-Permease in die Zelle transportiert wird. Transgene Manipulation der Aktivität der kartoffelspezifischen Apyrase zeigte, dass dieses Enzym einen großen Einfluss auf die Pflanzen-, insbesondere die Knollenentwicklung hat. Obwohl sich Stoffwechselaktivitäten kaum verändert hatten, führte die Verringerung der Apyrase Aktivität in den Knollen zur übermäßigen Expression von Extensin-Genen, die eine Funktion im polaren Wachstum von Pflanzenzellen besitzen.

Die Ergebnisse wurden mit Hinblick auf Anpassungen der Pflanze an veränderte Adenylat-Spiegel und der potentiellen Beteiligung der endogenen Apyrase an einem apoplastischen ATP-Signalweg bzw. ATP-Bergungsstoffwechselweg diskutiert.

Zusammengefasst, präsentiert diese Arbeit neue Einsichten in Adenylat-regulierte Prozesse in- und außerhalb nicht-photosynthetischer Pflanzenzellen.

5. Abbreviations

(m)U	(mili)Units (μmol·min^{-1})
3-PGA	3-Phosphoglycerate
aa	amino acid
AATP	ATP/ADP-transporter
ACR	apyrase conserved regions
ADH	alcohol hehydrogenase
ADK	adenylate kinase
Adn	adenine
Ado	adenosine
ADP	adenosine triphosphate
AGPase	ADP-glucose pyrophosporylase
AMP	adenosine monophosphate
ANase	adenosine nucleosidase
ANT	= AATP
ATAMT2	*Arabidopsis thaliana* ammonium transporter 1
AtAPY	*Arabidopsis thaliana* apyrase
ATP	adenosine triphosphate
AtPUP	*Arabidopsis thaliana* purine permease
AWF	apoplastic washing fluids
CCCP	carbonyl cyanide chlorophenyl hydrazone
cDNA	copy DNA
CS	citrate synthase
Db-LNP	*Dolichos biflorus* lectin/nucleotide phosphohydrolase
EF1-α	elongation factor 1 alpha
EST	expressed sequence tag
FADH	flavin adenine dinucleotide
FDR	false discovery rate
FNR	feredoxin NADP$^+$ reductase
GABA	gamma amino butyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GBSS	granule bound starch synthase
GCMS	gas chromatography mass spectometry
gDNA	genomic DNA
GDP	guanosine diphosphate
GFP	green fluorescent protein
GMP	guanosine monophosphate
GS52	*Glycine max* 52 kD apyrase
GTP	guanosine triphosphate
HPLC	high pressure liquid chromatography
kb	kilo base
kD	kilo Dalton
K_m	Michalis-Menten Constant
LDH	lactate dehydrogenase
MDR1	multi drug resistance
mRNA	messenger RNA
MS1	manuscript 1
n	number of replicates/observations
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NAF	non aqueous fractionation
NOS	nopaline synthase
nt	nucleotide
OCS	octopine synthase
ORF	open reading frame
p	p-value
P1	publication 1
P2	publication 2
PAGE	polyacryl gel electrophoresis
PDC	pyruvate decarboxylase
PEP	phospho*enol*pyruvate
P_i	inorganic phosphate
PNP	p-nitro phenylphosphate
PP_i	pyrophosphate
PUP	purine permease
SE	standard error
SSS	soluble starch synthase
StAPY	*Solanum tuberosum* apyrase
TC	tentative consensus sequence
TCA-cycle	tricarboxylic acid cycle
TIGR	The Institute for Genomic Research
UDP	uridine diphosphate
UDPG	UDP-glucose
UGPase	UDP-glucose pyrophosphorylase
UMP	uridine monophosphate
UTP	uridine triphosphate
UTR	uridine triphosphate
Zea	zeatin

6. Table of contents

7. General introduction

Adenosine triphosphate (ATP) is a ubiquitous metabolite with essential function in all living cells. It is involved in various enzymatic reactions where its hydrolysis drives thermodynamically unfavorable reactions. The product of this hydrolysis, ADP (and also AMP), is converted into ATP again by respiratory, fermentatory or photosynthetic activity. Consumption and production of the adenylates have to be intensely balanced, since its turnover takes place within seconds. It has thus been proposed that the ratios of ATP, ADP and AMP are the central regulators of many biochemical pathways, affecting enzymatic activity *in vivo* by allosteric regulation or signaling leading to translational and postranslational regulation.

In plants, regulation of energy metabolism is of particular importance in heterotrophic tissues that experience hypoxic or anoxic conditions like roots, rice kernels or potato tubers. Naturally, hypoxic or anoxic conditions are caused by flooding, which leads to a dramatically reduced oxygen availability of the flooded parts of a plant. Changes of the energy charge are thus often associated with changes in the oxygen availability. According to the law of supply and demand, generation of ATP has to be adjusted to the metabolic demand. In particular storage pathways leading to starch, oil or protein, are a huge sink of ATP within a cell, and the production of these compounds requires a balanced production of ATP depending on assimilate supply.

In the past, many studies proved the relevance of oxygen supply and adenylate energy balance on metabolic performance in potato tubers. Reduction of oxygen supply to potato tuber slices to subambient concentrations resulted in a reduced adenylate energy charge, reduced glycolysis, reduced synthesis of starch, amino acids, protein and lipids and a switch to energy conserving pathways (Geigenberger et al., 2000). A further reduction of oxygen to a level below 1% led to an increase in glycolytic flux to produce ATP by fermentation. These data, however, do not allow differentiation between changes that are caused by oxygen sensing from those that are caused by the adenylate energy charge directly.

By feeding adenine to tuber slices, it was possible to increase the ATP content and the ATP/ADP-ratio in potato tubers (Loef et al., 2001). This, in opposite to hypoxia, led to an increase in starch production. An interpretation of these results in terms of the adenylate energy charge was not possible, because this approach also led to an increase of the total adenylate pool. Reduction of the activity of plastidial adenylate kinase (ADK) in potato tubers led to comparable results (Regierer et al., 2002), namely an increase in ATP and the total adenylate content concomitant with increased starch production an elevated levels of plastidially synthesized amino acids. Although the specific mechanism, by which the

reduction of ADK alters the adenylate pools has not been revealed yet, the authors hypothesized that increased biosynthetic activity is most likely a consequence of higher ATP-availability or a higher adenylate pool in the amyloplast or the whole cell. These findings were in accordance with previous results showing that the starch production rate is dependent on the activity of the plastidial ATP/ADP-translocator (AATP) (Tjaden et al., 1998; Geigenberger et al., 2001). Crucially, reduction of AATP did not only lead to a slight reduction of overall ATP and adenylate levels and starch synthesis, but also to tubers with a higher surface-to-volume ratio and increased respiration. Although these studies underline the importance of adenylates for regulation of metabolic pathways in the model system potato tuber, they were not conducted to unravel the direct influence of changes in the energy state on metabolism.

ATP has also been shown to have an important function in the apoplast. Manipulation of extracellular ATP (eATP) by either application of ATP or ATP-consuming enzymes led to responses that suggest eATP having a function in stress and wound response (Jeter et al., 2004; Song et al., 2006), gravitropic growth (Tang et al., 2003) and regulation of cell viability (Chivasa et al., 2005). It is believed that eATP is transmitting a signal either via an as yet unidentified plasma membrane receptor (Jeter et al., 2004; Roux and Steinebrunner, 2007) or by binding to a soluble receptor or protein phosphorylation (Chivasa et al., 2005). This model is further supported by the existence of apoplastic apyrases in plants. These phosphatases specifically hydrolyze both the γ- and ß-phosphates of ATP and ADP without being coupled to any other biochemical function. It has been suggested that apoplastic apyrases terminate eATP signaling in the apoplast (Steinebrunner et al., 2000; Song et al., 2006), as it has been proven for mammal systems, where apyrase terminates signaling events controlling platelet aggregation, synaptic signaling or vasodilation/-constriction by hydrolyzing the signal mediating molecules ATP or ADP (Gendron et al., 2002). Several transgenic and biochemical studies in *Arabidopsis thaliana*, and the legumes *Lotus japonicus* and *Glycine max* showed that apyrases play a role in developmental processes like sexual reproduction and nodulation, supporting a role of this enzyme rather in signaling than metabolism (Day et al., 2000; Steinebrunner et al., 2003; McAlvin and Stacey, 2005). Although it is widely accepted that plants posses plasma membrane localized apyrases facing the apoplast with their active sites, only two localization studies supported this view for the legume apyrases *Db-LNP* and *GS52*. Whilst *AtAPY2* was discussed as a membrane localized apyrase (Steinebrunner et al., 2000; Steinebrunner et al., 2003), Dunkley et al. (2004) suggested a Golgi localization of this enzyme on the basis of proteomic data.

In potato, the enzyme has been extensively characterized on the biochemical level (Kalckar, 1944; Molnar and Lorand, 1961; Kettlun et al., 1982) and has been cloned and sequenced in 1996 (Handa and Guidotti, 1996). Sequence comparison showed that this potato apyrase was relatively different to the plant apyrases cloned so far from Arabidopsis or other plant

species (Roberts et al., 1999). This was not surprising, since the potato apyrase is soluble in contrast to the membrane bound apyrases from other plant species. Although no information about the localization of this apyrase is available, indirect information suggest that it could also be localized in the apoplast. Firstly, it contains a putative signal peptide for the secretory pathway (Handa and Guidotti, 1996), and secondly, it has a pH optimum at around pH 6 (Kettlun et al., 1982), a value very close to the acidic pH in the apoplast. Bearing in mind the influence of apyrases on developmental processes found in previous studies, it could be possible that the high activity in potato tubers – compared to other plants tissues - is essential for apoplastic signaling events that may have an influence on the development of this storage organ. Independent of its potential apoplastic localization, the high activity of potato apyrase might have a significant influence on ATP (or ADP) turnover in potato tubers, being of particular importance for metabolic performance of this crop plant.

Aim of this work

We used a transgenic approach to specifically alter the ratios between the pools of ATP, ADP and AMP in different subcellular compartments of growing potato tuber tissue.
To perturb the cellular adenylate pools, we generated transgenic potato plants that were overexpressing an *E. coli* apyrase in the potato tuber amyloplast. Using this approach, we were asking the following questions: What are the processes that are specifically activated as a response to manipulations in the energy charge? What are the short and long term effects of a reduced energy charge in potato tubers? Can we dissect the response to a reduced energy charge from responses to hypoxic/anoxic treatments of potato tubers? This in particular will help to assign some of the complex responses to anoxic/hypoxic treatments to either oxygen- or energy-triggered mechanisms.
Localization studies were performed to clarify, whether the endogenous apyrase is in fact localized in the apoplast. By transgenic up and down regulation of its activity, the adenylate status in the compartment apyrase is localized in should be perturbed. This should give information on the function of this enzyme in potato and clarify, whether its activity has a significant influence on overall adenylate (and carbon) metabolism or plant development. Furthermore, the fate of extracellular ATP should be investigated to unravel how this metabolite is salvaged and imported into the cell.

8. Publication 1 (P1)

Metabolic and developmental adaptations of growing potato tubers in response to specific manipulations of the adenylate energy status

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Published in Plant Physiology **146**: 1579-1598 (2008).

Authors' contributions

The research was conceived and planned by Peter Geigenberger, Alisdair Fernie and David Riewe. The experimental work was done by David Riewe. Lukasz Grosman, Henrik Zauber and Cornelia Wucke assisted as student workers supervised by David Riewe.

8.1 Abstract

Heterotrophic carbon metabolism has been demonstrated to be limited by oxygen availability in a variety of plant tissues, which in turn inevitably affects the adenylate status. To study the effect of altering adenylate energy metabolism, without changing the oxygen supply, we expressed a plastidially targeted ATP/ADP hydrolyzing phosphatase (apyrase) in tubers of growing potato plants under the control of either inducible or constitutive promoters. Inducible apyrase expression in potato tubers, for a period of 24 hours, resulted in a decrease in the ATP-content and the ATP/ADP ratio in the tubers. As revealed by metabolic profiling, this was accompanied by a decrease in the intermediates of sucrose to starch conversion and several plastidially synthesized amino acids, indicating a general depression of tuber metabolism. Constitutive tuber-specific apyrase expression did not lead to a reduction of ATP, but rather a decrease in ADP and an increase in AMP levels. Starch accumulation was strongly inhibited and shifted to the production of amylopectin instead amylose in these tubers. Furthermore, the levels of almost all amino acids were decreased, although soluble sugars and hexose phosphates were highly abundant. Respiration was elevated in the constitutively expressing lines indicating a compensation for the dramatic increase in ATP hydrolysis. The increase in respiration did not affect the internal oxygen tensions in the tubers. However, the tubers developed a ginger-like phenotype having an elevated surface/volume ratio and a reduced mass per tuber. Decreased post-translational redox-activation of ADP-glucose pyrophosphorylase and a shift in the ratio of soluble starch synthase activity to granule bound starch synthase activity were found to be partially responsible for the alterations in starch structure and abundance. The activity of alcohol dehydrogenase was decreased and pyruvate decarboxylase was induced, but this was neither reflected by an increase in fermentation products nor in the cellular redox state, indicating that fermentation was not yet induced in the transgenic lines. When taken together the combined results of these studies allow the identification of both short- and long-term adaptation of plant metabolism and development to direct changes in the adenylate status.

8.2 Introduction

Photoassimilates are generated during photosynthesis in leaves and subsequently distributed to a variety of heterotrophic tissues, which utilize the incoming carbon for growth or store it for later use. Much recent research attention has focused on the regulation of carbon metabolism in heterotrophic tubers and seeds due to their major importance as regenerative organs in plants and their biotechnological importance for food and industrial uses. Potato tubers have been used as a model system to study the structure and regulation of the key-pathways of heterotrophic metabolism, specifically concentrating on the unloading of incoming sucrose and its subsequent metabolism within starch biosynthetic (Viola et al., 2001; Geigenberger, 2003; Biemelt and Sonnewald, 2006; Geigenberger and Fernie, 2006) and respiratory pathways (Fernie et al., 2004). Various transgenic plants have been created with alterations in the expression levels of enzymes and membrane transport proteins that constitute these pathways (Geigenberger et al., 2004; Davies et al., 2005) and their analysis has contributed greatly to the understanding of the control of starch synthesis and respiration in growing tubers (Fernie et al., 2002; Geigenberger et al., 2004; Lytovchenko et al., 2007). However, whilst most approaches were directly targeted to pathway enzymes and their regulatory properties, far less attention has been paid to the levels of cofactors and effectors.

Adenine and uridine nucleotides are important cofactors in a variety of heterotrophic pathways, affecting enzymatic activity *in vivo* by acting as substrates, allosteric effectors or signaling compounds which lead to translational or postranslational regulation (Berg et al., 2002). Uridine nucleotides are involved as substrates in the pathway of sucrose degradation via sucrose synthase and UDP-glucose pyrophosphorylase (Geigenberger and Stitt, 1993), while adenine nucleotides are involved in the conversion of hexoses to hexose-phosphates by hexokinase and their subsequent use, by ADP-glucose pyrophosphorylase (AGPase), to generate ADP-glucose, the ultimate precursor of starch synthesis in the plastid (Ghosh and Preiss, 1966). There have been a number of physiological and genetic approaches published in the past, showing that the prevailing levels of adenine (Tjaden et al., 1998; Loef et al., 2001; Regierer et al., 2002; Oliver et al., in press) and uridine nucleotides (Loef et al., 1999; Geigenberger et al., 2005) are co-limiting for respiration and starch synthesis in growing potato tubers.

While these studies mainly focused on the alteration of the overall pool levels of nucleotides by manipulating their biosynthetic pathways, interconversion or transportation capacities, less attention has been paid to the manipulation of the ratios between different adenylate pools or the adenylate energy state (Pradet and Raymond, 1983). In heterotrophic tissues, ADP is regenerated to ATP by glycolysis and mitochondrial oxidative phosphorylation, which requires the provision of oxygen to the tissue. In growing tubers and developing seeds,

internal oxygen concentrations can fall to relatively low levels, leading to adaptive responses such as decreased respiration, decreased adenylate energy state and inhibition of various biosynthetic processes (Geigenberger, 2003). Previous work to analyze the role of adenylates in this context focused mainly on incubation of plants at low external oxygen concentrations (Drew, 1997; Geigenberger et al., 2000). Whilst these studies provided useful correlative information, they did not allow specific manipulation of the adenylate status, due to a number of pleiotropic effects. Particularly problematic is the fact that incubation at low oxygen leads to a multitude of morphological and biochemical alterations, while it remains unclear whether these are due to direct oxygen signaling or to indirect signaling effects via changes in the adenylate energy status (Geigenberger, 2003; Bailey-Serres and Chang, 2005).

In this study, we used a transgenic approach to specifically alter the ratios between the pools of ATP, ADP and AMP in growing potato tubers. For this purpose, we generated transgenic potato plants overexpressing an *E. coli* apyrase in their amyloplast under the control of a tuber specific promoter. Apyrases specifically hydrolyze nucleoside tri- and diphosphates to produce the monophosphate. The apyrase was N-terminally fused to a transit peptide to direct it into the amyloplast. Subcellular metabolite analyses revealed high concentrations of adenylates in amyloplasts, while uridine nucleotides were mainly compartmented in the cytosol (Farre et al., 2001), the adenylate pools in cytosol and plastid being interlinked by an ATP/ADP translocator in the plastid membrane (Tjaden et al., 1998). Changes in the adenylate energy state in the plastid will therefore also affect total cellular adenylate energy states. To identify short time responses, we used a construct that allowed inducible apyrase expression in the plastid via the ethanol-inducible *alc*-system under the tuber specific B33-patatin promoter. This construct was designed to manipulate the adenylate energy state in a well defined temporal and spatial manner. To investigate the long term adaptation of potato tubers to reduced adenylate energy state, we also transformed plants with a construct expressing plastidial apyrase constitutively under the control of the B33-promoter leading to expression of apyrase throughout tuber development. The induction of apyrase for 24 hours led to decreased ATP-contents and specific alteration in metabolite profiles of growing tubers, indicating a general depression of sucrose metabolism as an adaptive response to save energy. Using correlation analysis, we identified several sites of metabolic regulation that can be attributed to a short-term decrease in ATP pool levels. In contrast, constitutive apyrase expression led to a long-term decrease in the adenylate energy state during tuber development which was accompanied by adaptive responses, such as a transition in carbon partitioning to decrease starch and increase respiration and alterations in the participating enzyme activities. These metabolic adaptations were accompanied by changes in tuber morphology to increase surface/volume ratios and excessive generation of side-tubers,

without changing internal tuber oxygen concentrations. These changes in tuber morphology and respiratory metabolism could not be reverted by increasing external oxygen supply. The results are discussed in terms of both short- and long-term strategies that plant heterotrophic tissues adopt to counter the effects of perturbation of the balance of the various adenylate pool sizes.

8.3 Results

Generation of transgenic potato plants expressing apyrase in growing tubers under the control of an inducible promoter

An apyrase gene from *E. coli* strain *HN280* was amplified from the virulence plasmid pINV (GenBank accession AJ315184, Santapaola et al., 2002) omitting the first 70 nt which encode the endogenous N-terminal periplasma targeting sequence. This fragment was fused in-frame N-terminally with the transitpeptide of the spinach ferredoxin-$NADP^+$-reductase (FNR, (Jansen et al., 1988) by cloning it into pART33-FNR, which possesses the tuber specific B33-promotor (Liu et al., 1990). For the production of the plasmid B33-*Alc-Apy*, which codes for the inducible tuber specific apyrase, the coding sequence of the chimeric gene was then cloned into p35S:*alcr* (Caddick et al., 1998) to link it to the alc-promotor. The region from the alc-promotor to the terminator was cloned into pB33-*alc* (Junker et al., 2003) to combine it with the *alc*-regulator under the tuber specific B33-promoter (for vector maps, see supplementary Fig. S1). After *Agrobacterium tumefaciens* mediated transformation and regeneration, plant material was propagated in tissue culture and explants were grown under controlled conditions to obtain plant material for biochemical analysis and phenotypic characterization.

Application of acetaldehyde leads to inducible expression of *E. coli* apyrase and decreased ATP levels in growing tubers

For induction of apyrase expression, acetaldehyde was chosen instead of ethanol, since it has been reported previously to have less influence on metabolism and to lead to a faster transgene expression (Junker et al., 2003). In these previous studies, transgene expression was induced by drenching whole pots with acetaldehyde solution (Junker et al., 2003; Junker et al., 2004). However, this procedure led to a patchy induction pattern of the transgene mainly in the basal and peripheral parts of the tuber (Junker et al., 2003). We therefore used a different approach involving the preparation of a fine bore-hole (1 mm diameter) through a

growing tuber attached to the plant using a coaxial biopsy needle and subsequently flushing this duct with 0.2% acetaldehyde solution. Within 24h, this procedure led to reproducible induction of transgene expression in a concentric region of 2-3 mm around the duct (Junker et al., 2004) which was then sampled for metabolic analysis. Untreated tubers or tubers flushed with water instead of acetaldehyde served as controls.

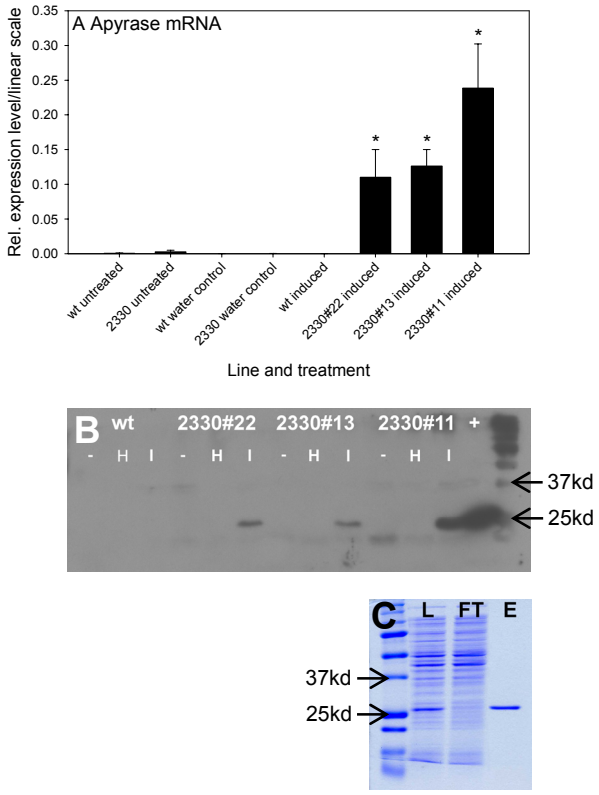

Fig. 1. Effect of plastidial apyrase induction for 24 hours on apyrase mRNA and protein levels in potato tuber tissue. Potato tuber tissue was either treated with acetaldehyde or water or not treated at all and analyzed regarding **(A)** apyrase mRNA using Real-Time-PCR and **(B)** apyrase protein on a Western Blot (- = untreated, H = water control, I = Induced with 0.2% acetaldehyde, + = positive control consisting of tuber protein from the weakest constitutive apyrase expressor 1330#45). **(C)** Migration of mature His-tagged apyrase expressed in *E. coli* in PAGE (L = loading, FT = flow through, E = eluate).

Wild-type and three independently transformed potato lines were analyzed with respect to *E. coli* apyrase transcript abundance and protein levels after incubation of tuber tissue with 0.2% acetaldehyde solution. Untreated tubers or tubers that were pseudo-induced by injecting water instead of acetaldehyde were used as additional controls. Apyrase transcript analysis using Real-Time-PCR (Fig. 1A) and protein analysis by immunoblotting using polyclonal antibodies (Fig. 1B) clearly showed that the heterologous apyrase was expressed in transgenic tubers 24h after treatment with acetaldehyde, while no significant expression was observed in the wild-type. There was no substantial apyrase expression in untreated tubers or in tubers treated with water instead of acetaldehyde. This provides direct evidence that apyrase expression was specifically induced by acetaldehyde. It also demonstrates that gene expression was tight, since no leaky expression took place in tubers that were not treated.

The FNR-transitpeptide, which was fused to the apyrase, has been successfully used to direct enzymes into the plastid in previous studies (see Lloyd et al., 1999b; van Voorthuysen et al., 2000; Farre et al., 2006). To investigate whether the apyrase was correctly targeted to the amyloplast by the FNR transitpeptide, the size of the processed protein was determined using PAGE. The predicted size of the plastidial *E. coli* apyrase is 281 aa/31 kD for the unprocessed protein and 223 aa/25 kD for the mature protein after removal of the transit peptide. The size of the *E. coli* apyrase detected on the immunoblot was slightly below 25 kD, according to the migration of the protein standard, in a resolution that allows a safe distinction between a 25 and a 31 kD protein (Fig. 1B). Furthermore, C-terminal his-tagged *E. coli* apyrase with a molecular mass of 233 aa/26 kD after processing in the periplasma was expressed in *E. coli*. This recombinant protein migrated approx. the same distance when separated under identical conditions and compared to the same protein standard (Fig. 1C). These findings provide evidence that the FNR-transit peptide has been cleaved off from the remainder of the apyrase protein in the transgenic tubers, suggesting that it was correctly targeted to the amyloplast, where the specific protease for the FNR-transit peptide resides.

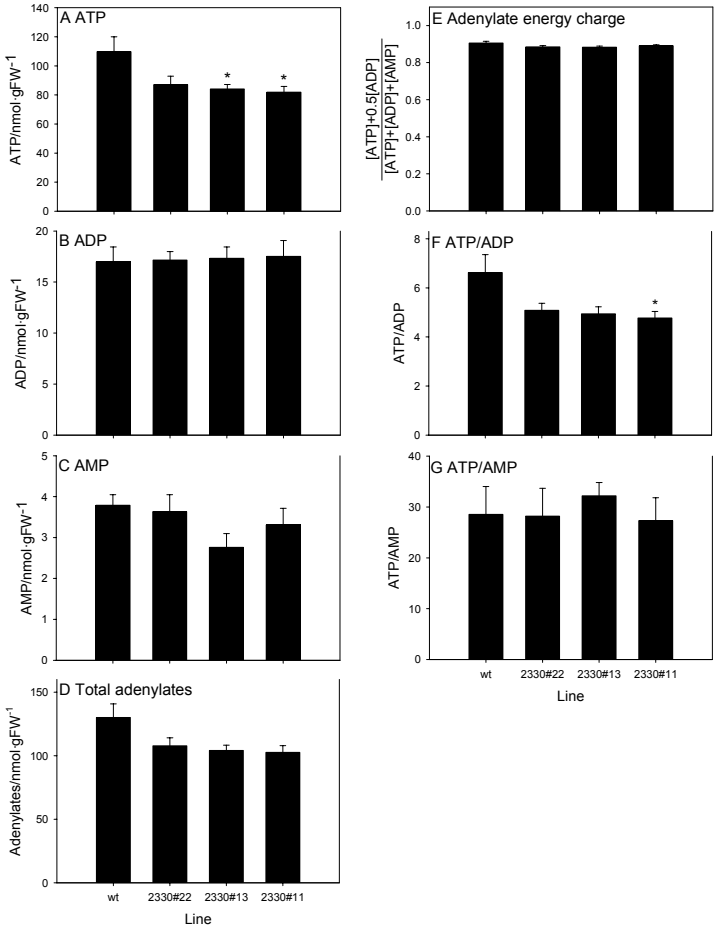

Fig. 2. Adenylate levels in potato tuber tissue after apyrase induction for 24 hours. (A) ATP, **(B)** ADP and **(C)** AMP. **(D)** Total adenylates and corresponding adenylate energy dependent parameter: **(E)** adenylate energy charge, **(F)** ATP/ADP ratio and **(G)** ATP/AMP ratio (*n* = 6-8). Significant differences to the wild-type according to the student's t-test are indicated with asterisks ($p < 0.05$).

To investigate the impact of induced expression of apyrase on the levels of individual adenylate pools, ATP, ADP and AMP were measured in growing tubers of wild-type and transgenic lines. The levels of ATP, ADP and AMP were not significantly different between wild-type and the transgenic lines, when non-treated tubers were compared (data not shown). This indicates that adenylates were not changed *per se* in the transformed lines as a result of the transformation procedure. In contrast to this, the levels of ATP were significantly decreased after acetaldehyde treatment in growing tubers of two of the three inducible lines, compared to wild-type (Fig. 2A). Induction of apyrase did not affect the concentration of ADP (Fig. 2B), but led to a slight reduction of AMP (Fig. 2C). Consequently, the total adenylate pool was decreased, indicating that either adenylate synthesis was decreased or breakdown was increased (Fig. 2D). In support of this, only the deduced ATP/ADP (Fig. 2F) ratio was reduced, significantly so in one line, whilst the adenylate energy charge and the ATP/AMP ratio were not altered (Fig. 2E and 2G).

Effect of inducible expression of *E. coli* apyrase on metabolite profiles in growing tubers

As indicated above, induction of apyrase led to alterations in tuber energy metabolism within 24h, including a decrease in the level of ATP and the ATP/ADP ratio. To study the effects of the reduced ATP availability on metabolism in more detail and to assess possible regulation sites, metabolite profiles were analyzed using GC-MS and enzymatic assays. The data are summarized in Fig. 3, showing the changes in metabolite levels in 24h-induced tubers of transgenic lines compared to wild-type tubers identically treated as control. In Table 1, individual metabolites were correlated to ATP using the Spearman algorithm. Results of this correlation analysis are also indicated in the color code of Fig. 3.

Induction of apyrase led to a consistent but non-significant decrease of sucrose, hexoses and UDP-glucose and a significant decrease of glucose-1-phosphate in all transgenic lines, indicating inhibition of sucrose transport or faster metabolization. Labeling studies using ^{14}C-sucrose, which was injected into the fine borehole of the tubers 24 hours after induction showed that sucrose metabolization was minorly, albeit, insignificantly inhibited to rates between 77 and 97% of the wild-type level in the transgenic lines (data not shown).

While the levels of glucose and fructose did not correlate with ATP using the Spearman test (see Table 1), the strong positive correlation between UDP-glucose and ATP indicates that sucrose degradation via sucrose synthase rather than invertase has been inhibited, which is in agreement with a more profound role of sucrose synthase compared to invertase in growing tubers (Zrenner et al., 1995). These findings indicate that the availability of ATP regulates sucrose degradation processes in growing tubers.

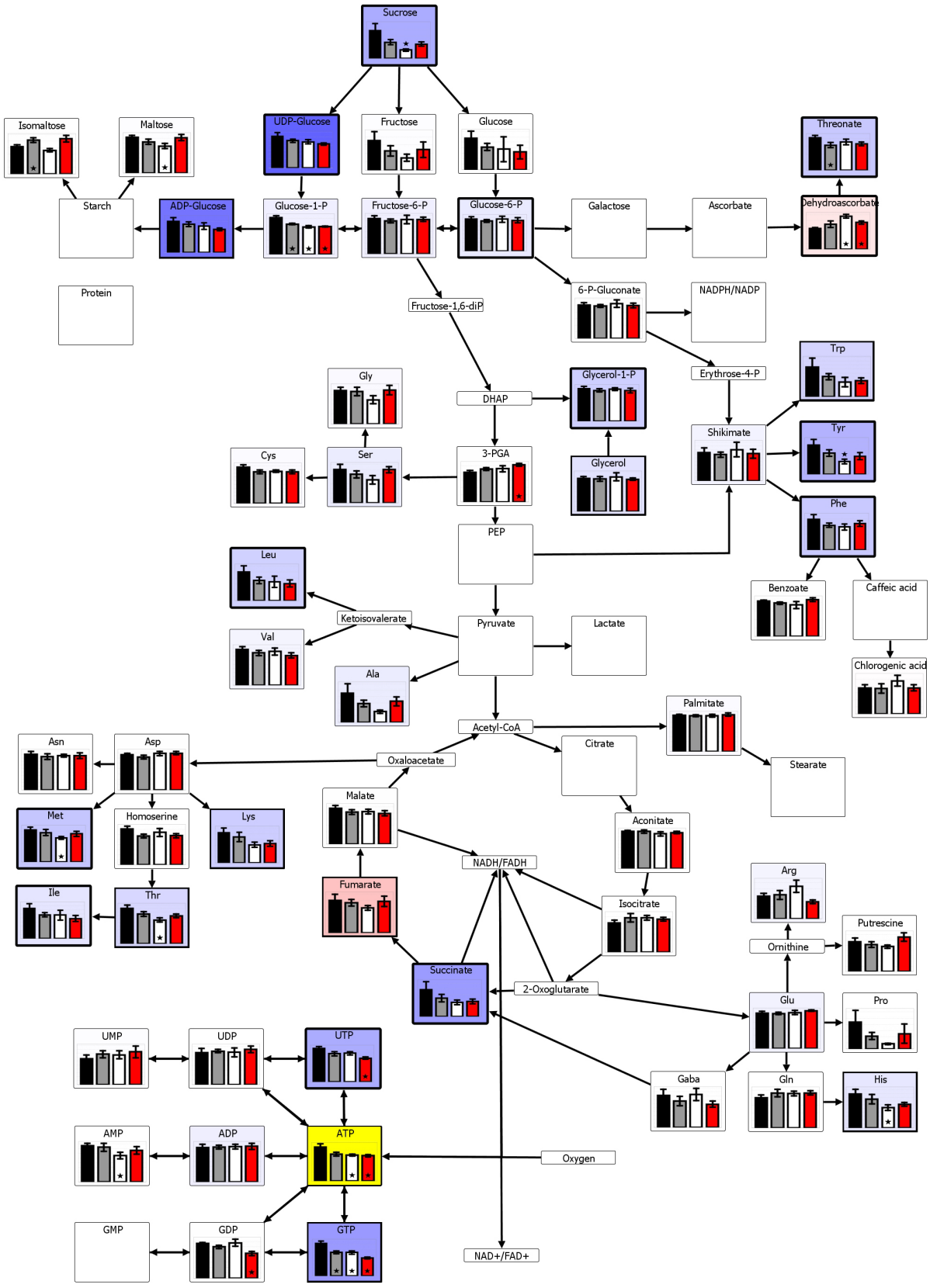

Fig. 3. (*Legend appears on following page*)

Fig. 3. Metabolite profile of tuber tissue after apyrase induction for 24 hours. Metabolite concentrations were measured using GC-MS and spectrophotometric techniques and visualized in their adequate pathways using VANTED (Junker et al., 2006). The bars represent the following lines: wt = black, 2330#22 = grey, 2330#13 = white and 2330#11 = red (*n* = 6-8). Significant differences in the mean values to the wild type according to the student's t-test are indicated with asterisks ($p < 0.05$). Positive correlations of individual metabolites to ATP (yellow) are visualized with blue background, negative correlations with red background depending on the intensity of the correlation (*n* = 35-39). Significant Spearman correlations corrected for multiple testing according to Benjamini and Hochberg (1995) are indicated with a frame around the diagram (fdr $p < 0.05$).

Table 1. Metabolites with significant Spearman correlation to ATP after apyrase induction for 24 hours. Negative correlations are displayed with a negative correlation coefficient. r = correlation coefficient, p = p-value, fdr p = false discovery rate corrected p-value according to Benjamini and Hochberg (1995), *n* = independent biological samples derived from wt (max. 7) and transgenic lines (max. 21).

Metabolite	r	p	fdr p	*n*
UDP-glucose	0.850	<0.000001	<0.00005	27
ADP-glucose	0.816	<0.000001	<0.00005	27
GTP	0.737	0.000008	0.00015	28
Succinate	0.734	0.000013	0.00018	27
Ribonic acid	0.687	0.000054	0.00060	28
Sucrose	0.690	0.000069	0.00061	27
UTP	0.687	0.000076	0.00061	27
Tyrosine	0.670	0.000179	0.00125	26
Fumarate	-0.622	0.000406	0.00253	28
Methionine	0.606	0.000803	0.00450	27
Threonate	0.599	0.000965	0.00491	27
Phenylalanine	0.595	0.001056	0.00493	27
Glycerol-1-p	0.572	0.001473	0.00635	28
Lysine	0.580	0.001890	0.00756	26
Leucine	0.564	0.002666	0.00994	26
Tryptophane	0.552	0.002841	0.00994	27
Threonine	0.537	0.003856	0.01270	27
Glycerol	0.485	0.008909	0.02772	28
Dehydroascorbate	-0.459	0.013961	0.03973	28
Isoleucine	0.466	0.014191	0.03973	27
Histidine	0.452	0.018000	0.04800	27
Glucose-6-p	0.440	0.019273	0.04906	28

Apyrase expression also led to a consistent but non-significant decrease in the level of ADP-glucose, the ultimate precursor of starch synthesis in the plastid, supporting the idea that AGPase and starch synthesis have been inhibited in response to decreased ATP levels. This hypothesis is further supported by the strong Spearman correlation between ADP-glucose and ATP shown in Table 1.

Induction of apyrase also led to changes in metabolic intermediates involved in glycolysis and TCA-cycle. There was an increase in the levels of 3-PGA, significantly in one out of three lines, which may be due to a shift in the glycolytic reaction of glycerate kinase, converting 1,3-bisphosphoglycerate and ADP to 3-PGA and ATP, potentially caused by the increased ATP/ADP ratio. The levels of aconitate and isocitrate remained either unchanged, (but appeared to increase marginally), whilst the levels of metabolites further downstream of the TCA cycle such as succinate, fumarate and malate if anything decreased, marginally (although non-significantly). Given the potential that the observed decrease in ATP may have led to an increased oxidation of succinate, resulting in the shift in TCA cycle intermediates observed we decided to look for further existence of a link between ATP and succinate. This was found in the strong positive correlation between these two metabolites in the analysis presented in Table 1. The reaction product of succinate oxidation, fumarate, showed a high negative correlation to ATP (Table 1). The correlation of ATP with the succinate/fumarate ratio was even slightly higher ($r = 0.81$, $p = 0.000001$, $n = 26$) than the correlation with succinate alone ($r = 0.73$, $p = 0.000013$, $n = 27$). Taken together, these data suggest that ATP availability has an influence on succinate oxidation in mitochondria.

Induction of apyrase led to decreases in the levels of many of the amino acids (however most of these changes were not statistically significant). This trend was especially marked with respect to amino acids deriving from the shikimate pathway, or belonging to the aspartate, pyruvate or serine family (Fig. 3). There was a relatively strong correlation between ATP and the levels of many amino acids (Table 1). These correlations were highest for amino acids that are mainly synthesized in the plastid rather than the cytosol, such as tryptophan, tyrosine, phenylalanine, methionine, lysine, threonine and isoleucine. Only very minor influence of ATP was found on the levels of aspartate, asparagine, glutamate, glutamine, glycine, valine, arginine, and proline. Given that aspartate, asparagine, glutamate and glutamine are imported from the shoot rather than produced *de novo* in tubers (Karley et al., 2002; Koch et al., 2003), only the production of arginine, histidine and proline demands further ATP consumption for their synthesis starting from intermediates of glycolysis and the TCA-cycle. In contrast to this, eight out of the twelve plastidial amino acids require further ATP for their production. The data therefore indicate that plastidial apyrase expression affected mainly the production of amino acids, whose synthesis requires ATP, and in particular also those, which are synthesized within the plastid

The changes in amino acids observed here are almost the direct opposite to those observed by Regierer et al. (2002), who altered the plastidial adenylate equilibrium by antisense inhibition of the plastidial adenylate kinase, leading to higher ATP levels and significantly increased levels of histidine, isoleucine, leucine, methionine phenylalanine, lysine, tryptophan and tyrosine. Interestingly, in the previous study the levels of the amino acids belonging to

the shikimate family were also more strongly affected than the others. In the present study the levels of benzoate and chlorogenic acid were not significantly changed, despite the significant reduction of their aromatic amino acid precursors (Fig. 3). Furthermore, in contrast to the general trend in metabolite levels, induction of apyrase led to a significant increase in the level of dehydroascorbate in two out of three lines (Fig. 3).

Generation of transgenic potato plants expressing *E. coli* apyrase under the control of a constitutive tuber-specific promoter

In the previous section, short term responses to decreased ATP levels were investigated using an inducible apyrase. In the following section we investigated long-term changes by constitutive expression of the *E. coli* apyrase under the tuber specific B33-promoter. The protein sequence is otherwise identical to the sequence used in the inducible apyrase-construct described above, comprising the FNR-transit-peptide fused to the *E. coli* apyrase without periplasmatic targeting sequence.

The plasmid B33-*apy* was produced by cloning the region from the B33-promoter to the terminator from the plasmid pART33-FNR-*apy* (see above) into pART27 (Gleave, 1992); for vector map, see supplementary Fig. S1B). After *Agrobacterium* mediated transformation and regeneration, plant material was propagated in tissue culture and explants were grown under controlled conditions to obtain plant material for biochemical analysis and phenotypic characterization.

Apyrase activity was determined in enzyme extracts from growing tubers of four independent transgenic lines (Fig. 4A). Compared to wild-type, apyrase activity increased significantly in all four transgenic lines, the increase being stronger in lines 1330#1 and 1330#8 than in lines 1330#45 and 1330#32 (Fig. 4A). Surprisingly, there was no change in the ATP-content of tubers from the transgenic plants with exception of the strongest line 1330#8, which even had a significantly higher ATP-content than the wild-type (Fig. 4B). In contrast to this, the level of ADP was significantly decreased in all lines (Fig. 4C), while the AMP level was substantially increased in all transgenic lines, compared to wild-type, the increase in AMP being significantly different in three of the lines (Fig. 4D). The total amount of adenylates remained unchanged compared to the wild-type with exception of line 1330#8, which contained significantly more adenylates (Fig. 4E). Moreover, both adenylate energy charge (Fig. 4F) and ATP/AMP ratio (Fig. 4H) were significantly decreased in three out of four of the transgenic lines, and the ATP/ADP ratio was significantly increased in all lines (Fig. 4G).

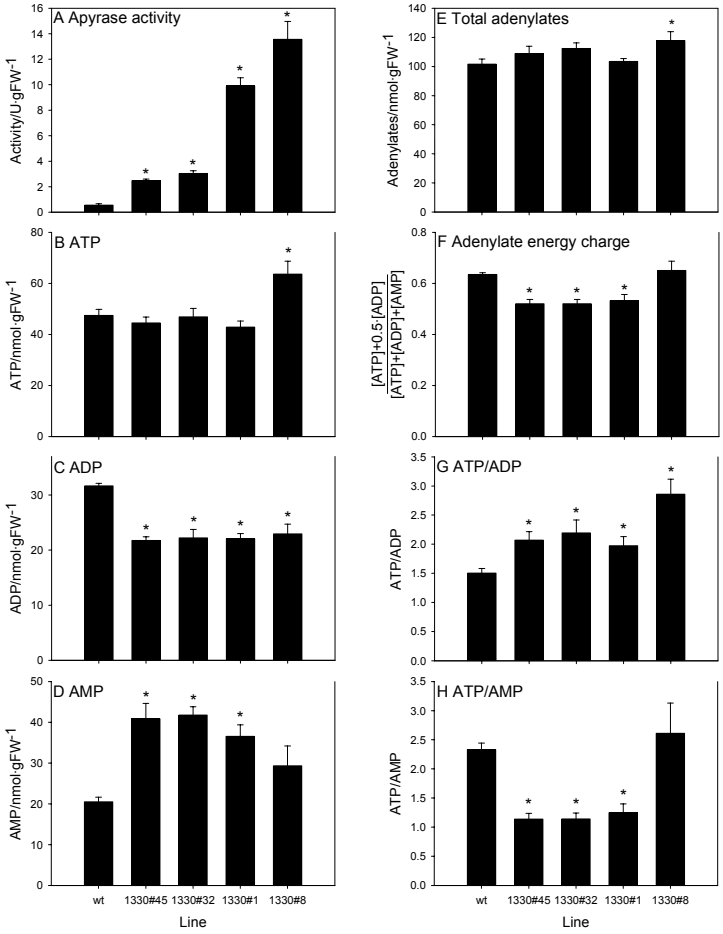

Fig. 4. Effect of constitutive plastidial apyrase expression on apyrase activity and adenylate levels in potato tubers. (A) Apyrase activity in potato tubers was measured using ATP as substrate. Concentrations of **(B)** ATP, **(C)** ADP and **(D)** AMP and resulting **(E)** total adenylates. Energy-related parameters were calculated: **(F)** adenylate energy charge, **(G)** ATP/ADP ratio and **(H)** ATP/AMP ratio (n = 7 or 8). Significant differences to the wild-type according to the student's t-test are indicated with asterisks ($p < 0.05$).

Constitutive expression of *E. coli* apyrase affects yield, tuber morphology, starch content and composition

Growing potato tubers of the lines expressing plastidial apyrase constitutively showed strong morphological alterations in response to apyrase expression (Fig. 5A). Expression of apyrase led to tubers with longitudinal shape, characterized by increased production of side tubers. These alterations were progressively more severe with increasing apyrase activity, resulting in excessive side-tuberization in the two strongest lines. With increased apyrase expression, stolons were getting thicker and the number of tubers per stolon was increased (Fig. 5C). A Lugol-stain of the tubers clearly showed that starch content progressively decreased with increasing apyrase activity (Fig. 5B). The number of individual tubers per plant was significantly increased in two of the transgenic lines, compared to wild-type, being almost three times higher in the strongest line, even when the high degree of side-tuberization was not taken into account (Fig. 6A). Although there was an increase in tuber number, total tuber mass per plant was not significantly altered (Fig. 6B) due to a dramatic decrease of the mass of the individual tubers, being significant for three out of the four lines (Fig. 6C). For better quantification, starch content was also measured by enzymatic assays. The data are

summarized in Fig. 6D and confirm the results from the Lugol-stain. Increased expression of apyrase led to a progressive and significant decrease in tuber starch content in all lines. Combining this with the tuber mass per plant, the starch production per plant was significantly reduced in three out of for lines to a level below 5% of the wild-type in the strongest line (Fig. 6E). Consistently, starch levels were highly negatively correlated to apyrase activity ($r = -0.85$, $p < 0.000001$, $n = 39$).

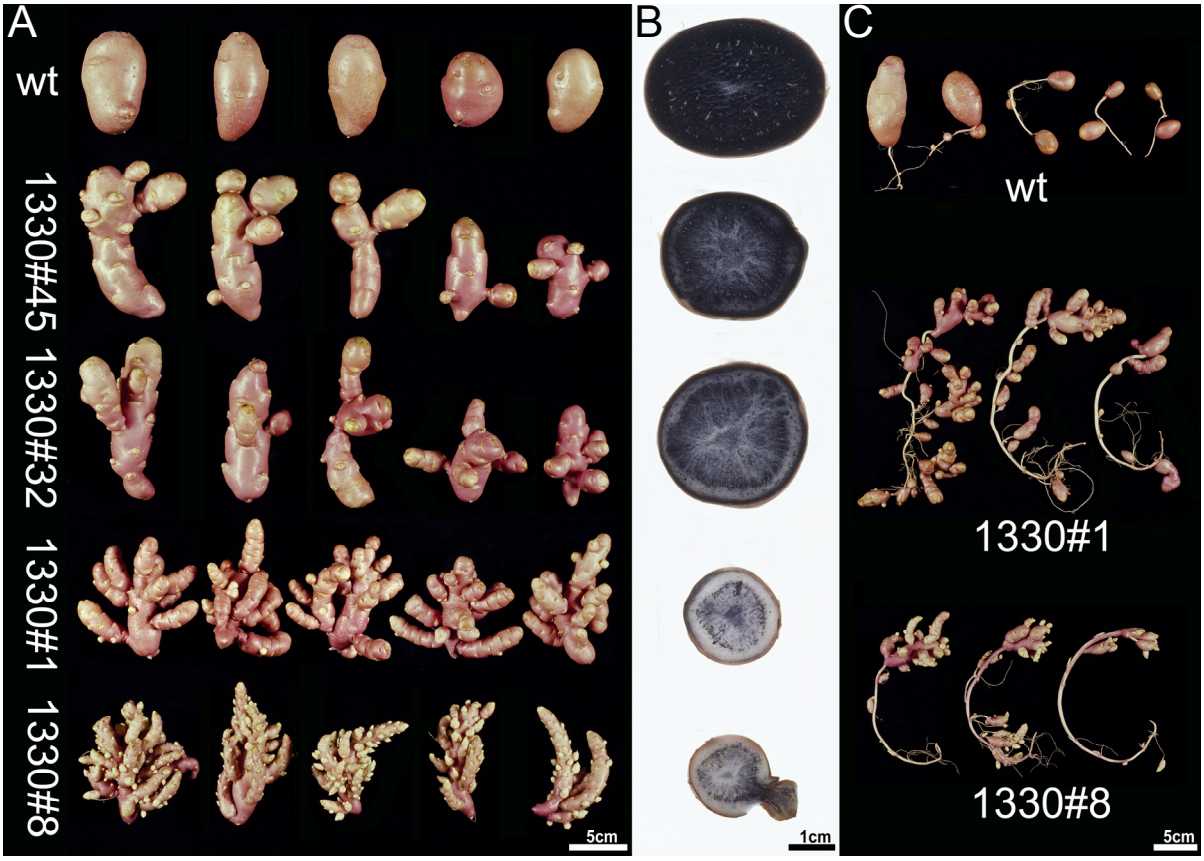

Fig. 5. Phenotype of fully mature potato tubers constitutively expressing plastidial apyrase. **(A)** With increasing apyrase activity, the tubers showed an increased surface/volume ratio resulting in excessive side-tuberization for the stronger lines. **(B)** Starch content as indicated by Lugol-stain. **(C)** The stolons of the two strongest lines were thicker and showed a markedly increased rate of tuber initiation per stolon. The plants were grown to maturity for 13 weeks in an uncontrolled greenhouse from May to July. Figures show representative examples.

Potato starch is composed of two different macromolecules: amylose, which is an alpha-1,4-linked linear glucose chain, and amylopectin, which also consists of alpha-1,6-branched glucan residues. The amylose/amylopectin ratio of potato tuber starch has been shown to be approx. 20% under normal conditions (Lloyd et al., 1999). To investigate whether apyrase expression affects the amylose/amylopectin ratio, tuber starch was analysed using a quantitative Lugol-based assay. The data show that the content of amylose, which is the

compound stained by the Lugol-solution, is significantly decreased in starch from all transgenic lines compared to wild-type. Also in this case, increased expression of apyrase led to a progressive decrease in the starch amylose content. The ratio of amylose to amylopectin is an important parameter for starch quality (Lloyd et al., 1999).

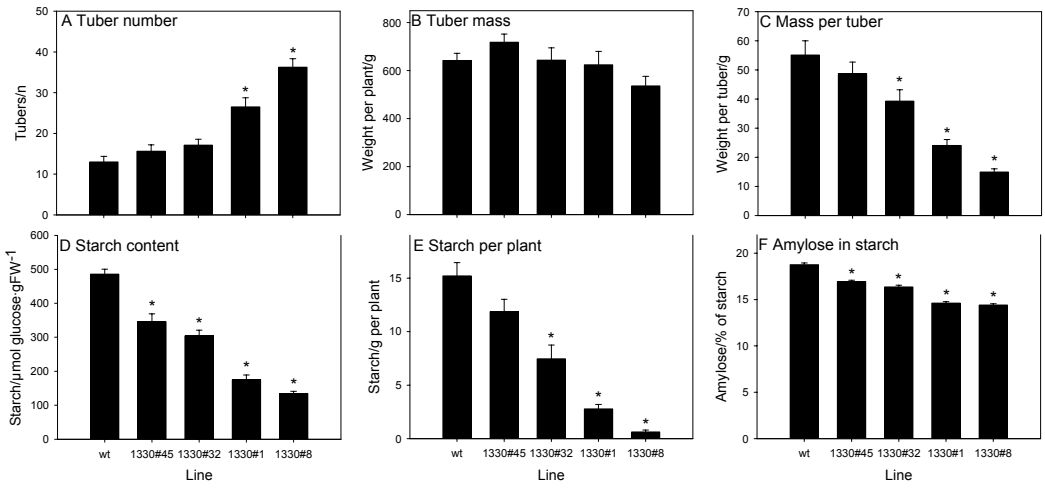

Fig. 6. Properties of tubers constitutively expressing plastidial apyrase. (A) Tuber number, **(B)** tuber mass and **(C)** resulting mass per tuber from plants grown to maturity for 13 weeks in an uncontrolled greenhouse from May to July (*n* = 8-15). **(D)** Starch content as determined enzymatically, resulting **(E)** starch yield per plant and **(F)** starch amylose content in tubers grown in a conditioned greenhouse (*n* = 8). Significant differences to the wild-type according to the student's t-test are indicated with asterisks ($p < 0.05$).

Constitutive expression of *E. coli* apyrase leads a compensatory increase in respiratory activity

Introduction of apyrase led to an additional sink for ATP and ADP, but our data did not reveal a decrease in the ATP-content of the tubers. This may be due to a stimulation of ATP-generating pathways, such as glycolysis and respiration. To determine overall respiration rates, oxygen consumption was measured in freshly-cut tuber slices using an oxygen electrode. Increased expression of apyrase led to a progressive induction of respiration rates, which were significantly elevated in the three strongest lines, compared to wild-type (Fig. 7A). This suggests that increased ATP-consumption due to introduction of apyrase is compensated for by stimulating ATP-production *via* increased flux through glycolysis and the TCA-cycle and elevated oxidative phosphorylation.

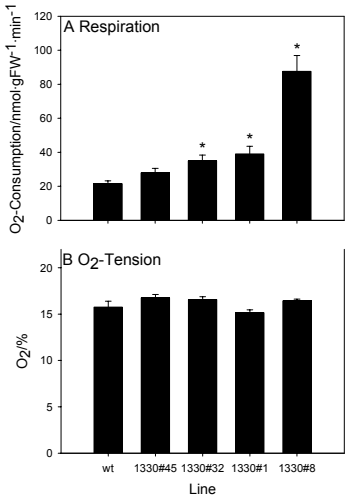

Fig. 7. Respiration rates and oxygen tensions in tubers constitutively expressing plastidial apyrase. (A) Respiration rates were determined from freshly prepared tuber slices in oxygen saturated buffer solution using a clark electrode (*n* = 6-8). **(B)** Oxygen tensions were determined 4 mm below the skin of growing tubers using an optical needle-type oxygen sensor with a tip diameter < 30 µm (*n* = 15-30). Significant differences to the wild-type according to the student's t-test are indicated with asterisks ($p < 0.05$).

Effect of constitutive expression of *E. coli* apyrase on internal oxygen concentrations in growing tubers

Previous studies showed that changes in respiration rates can lead to altered internal oxygen concentrations in growing tubers (Bologa et al., 2003). We therefore measured internal oxygen concentrations 4 mm below the tuber skin by inserting an oxygen micro sensor (Fig. 7B). Internal oxygen concentrations in wild-type tubers were found to be around 15% (v/v), which resembles the concentrations found within the tuber periphery in previous studies (Geigenberger et al., 2000; Bologa et al., 2003). Compared to wild-type, there was no significant change in the oxygen concentration measured within growing tubers expressing apyrase, indicating that apyrase did not affect internal oxygen concentrations.

Effect of constitutive expression of *E. coli* apyrase on metabolite profiles in growing tubers

To investigate metabolic adaptation to long term reduction of the energy charge in more detail and to determine further regulation sites, a comprehensive analysis of metabolite levels and enzyme activities was conducted in growing tubers. Metabolite profiles analyzed by GC-MS and enzymatic methods are depicted in Fig. 8, showing the changes in metabolite levels or ratios in the transgenic lines compared to wild-type.

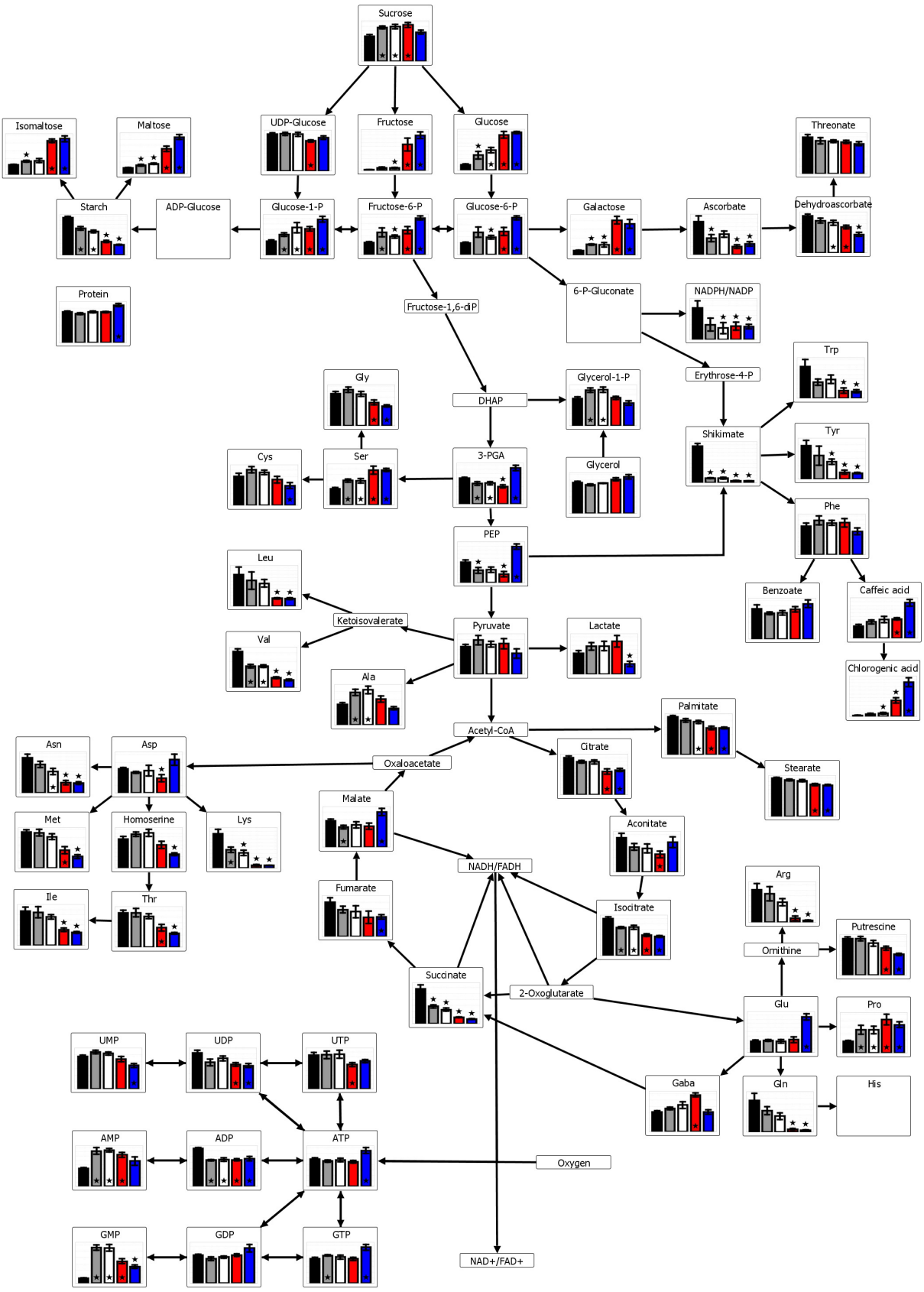

Fig. 8. (*Legend appears on following page*)

Fig. 8. Metabolite profile of tuber tissue with constitutive plastidial apyrase. Metabolite concentrations were measured using GC-MS and spectrophotometric techniques and displayed in their adequate pathways using VANTED (Junker et al., 2006). The bars represent the following lines: wild-type = black, 1330#45 = grey, 1330#32 = white 1330#1 = red and 1330#8 = blue (*n* = 7-8). Significant differences to the wild-type according to the student's t-test are indicated with asterisks ($p < 0.05$).

Sucrose was only slightly, but significantly increased in three out of four lines, while the levels of its degradation products such as glucose, fructose and hexose-phosphates increased significantly in at least three of the four lines with increasing apyrase activity (Fig. 8). In contrast, the level of UDP-glucose was not significantly changed.

Compared to wild-type, metabolite levels at the end of glycolysis such as 3-PGA and PEP were significantly decreased in all transgenic lines, with exception of the strongest line 1330#8, which had concentrations of these metabolites significantly above the wild-type level (Fig. 8). The decrease in PEP in lines 1330#45, 1330#32 and 1330#1 was accompanied by a minor yet non-significant increase in pyruvate levels and a consequent significant decrease in the deduced PEP/pyruvate ratio in three out of the four lines (not shown). The decrease in PEP/pyruvate ratio indicates pyruvate kinase as the primary regulatory step where glycolysis has been stimulated. This, however, did not apply to the strongest line 1330#8. Interestingly, a high correlation between the PEP/pyruvate and the ATP/AMP ratios ($r = 0.52$, $p = 0.0021$, $n = 33$) suggests a regulatory link between PEP to pyruvate conversion and adenylate energy status. By contrast, the PEP carboxylase reaction does not appear to be under strong regulation, since malate and aspartate showed an almost identical pattern of change as PEP.

Constitutive apyrase expression led to a general decrease in the levels of organic acids of the TCA-cycle, such as citrate, aconitate, isocitrate, succinate and fumarate. Of these metabolites, succinate showed the strongest decrease (which was significant in all lines and down to 12% of wild-type level in the most severe case; Fig. 8). The general reduction of TCA-cycle compounds is most likely due to the increased demand of NADH to support the elevated respiration rates (see Fig. 7A). The data also indicate that the primary regulation site leading to increased respiration is located in the respiratory chain. This is also confirmed by the strong decrease in the NADPH/NADP ratio upon apyrase expression, significantly for three out of four lines, indicating that induction of apyrase led to a strong decrease in the overall cellular redox-state (Fig. 8). Fermentation products such as lactate, alanine or glycerol-1-phosphate displayed few changes that were conserved across the transgenic lines, indicating that fermentative metabolism was not induced in the transgenic tubers.

Expression of apyrase led to a marked decrease in the amino acid content of the tubers (Fig. 8). There was a strong decrease in asparagine and glutamine, significant in three and two lines, respectively. These are the predominant amino acids in potato tubers which account for approx. 40% of total tuber amino acid content (Koch et al., 2003; Stiller et al., 2006). The

contents of most amino acids deriving from glycolytic intermediates were decreased (although the statistical significance of these decrease varies from metabolite to metabolite), with the exception of alanine and serine which were significantly increased (in at least two of the transgenic lines). In addition, two of the aromatic amino acids, tryptophan and tyrosine, were significantly decreased (in at least two of the transgenic lines), which may also be due to the strong decrease in shikimate levels upon apyrase expression. Interestingly, phenylalanine remained unchanged and there was a significant increase in the levels of phenylpropanoids such as caffeic acid (in two lines) and chlorogenic acid (in three out of the four lines). The levels of caffeic and chlorogenic acid showed no correlation to energy-related parameters, but did strongly correlate with the levels of individual sugars like glucose, fructose, arabinose or ribose for example, the correlation coefficient between chlorogenic acid and glucose was found to be 0.76 ($n = 38$, $P < 0.00001$).

There was a general decrease in the amino acids belonging to the aspartate family, with the exception of aspartate itself. The decrease was especially strong (significant in at least three lines), in the levels of the N-rich amino acids asparagine and lysine, leading to a strong decrease in the deduced aparagine to aspartate ratio (data not shown). A similar pattern can be observed for the amino acids belonging to the glutamate family. Whereas the level of glutamate remained unchanged (with exception of the strongest line, which contained significantly more glutamate), the N-rich amino acids glutamine and arginine tended to be reduced (significantly so in two of the four transgenic lines). There was also a significant increase in the proline level (all lines). Despite the reduced contents of most amino acids, the overall protein content was not substantially changed in the transgenic tubers apart from the strongest line, which had a significantly higher protein content compared to wild-type (Fig. 8).

Expression of apyrase led to a decrease in both ascorbate and dehydroascorbate levels (significant in three of the lines; Fig. 8). Ascorbate functions as antioxidant by reducing hydrogen peroxide formed by superoxide anions during mitochondrial electron transfer reactions (Buchanan et al., 2000). The deduced ascorbate/dehydroascorbate ratio was additionally significantly decreased in two of the lines (data not shown), most probably due to the decreased NADP redox-state (see above).

Effect of constitutive expression of *E. coli* apyrase on enzyme activities in growing tubers

Apyrase induced respiration was not due to changes in the overall activities of glycolytic enzymes. There was a slight, but for two lines significant decrease in pyruvate kinase activity (Fig. 9A), while PEP phosphatase activity significantly increased in two lines (Fig. 9B). No significant changes were observed in the activities of UDP-glucose pyrophosphorylase and

hexokinase (data not shown). This pinpoints the importance of post-translational mechanisms in regulating glycolysis in response to changes in the energy state.

To investigate whether introduction of apyrase led to long-term changes in the activities of key-enzymes involved in fermentation, we analysed the activities of lactate dehydrogenase (LDH), pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in growing tubers. The data show that apyrase expression led to no significant changes in LDH activity (Fig. 9C) but a strong increase in PDC (Fig. 9D), which was significant in the two strongest lines. In contrast, apyrase expression led to a significant decrease in the activity of ADH in three of the four transgenic lines (Fig. 9E).

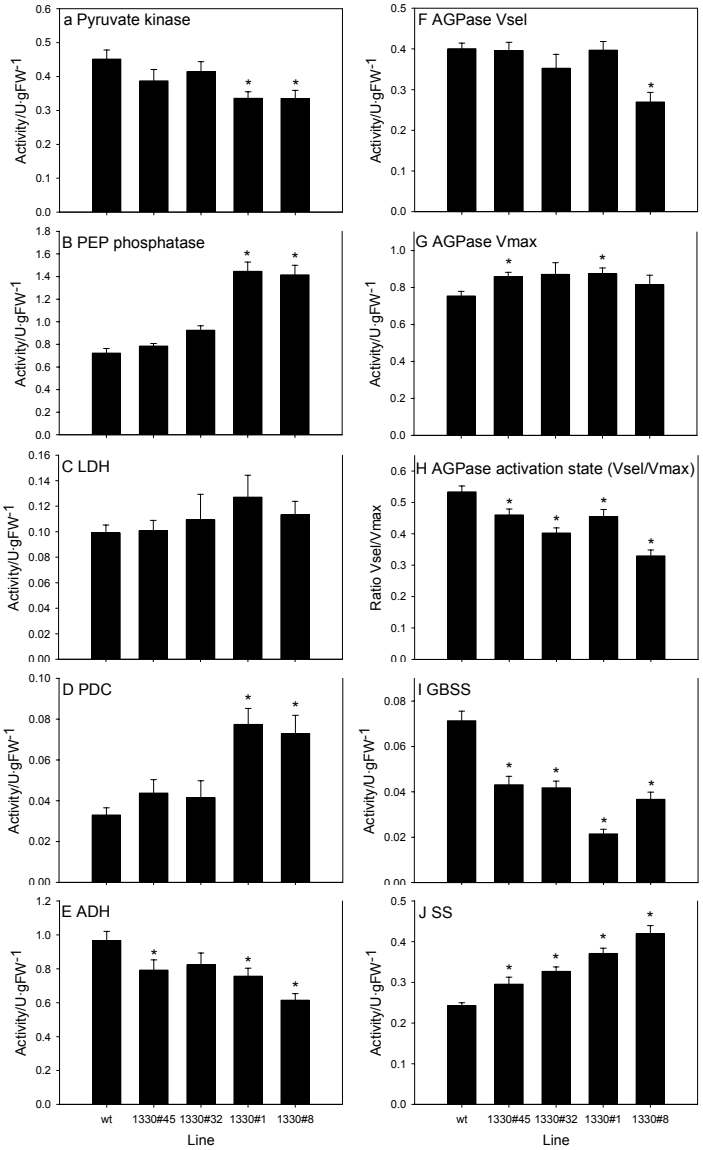

Fig. 9. Enzyme activities associated with glycolysis, fermentation and starch synthesis in tubers constitutively expressing apyrase. **(A)** Pyruvate kinase, **(B)** PEP phosphatase, **(C)** lactate dehydrogenase (LDH), **(D)** pyruvate decarboxylase (PDC), **(E)** alcohol dehydrogenase (ADH), **(F)** ADP-glucose pyrophosphorylase (AGPase) Vsel (= non-reductive assay), **(G)** AGPase Vmax (= reductive assay), **(H)** AGPase redox-activation state Vsel/Vmax, **(I)** granule bound starch synthase (GBSS), **(J)** soluble starch synthase (SSS) (*n* = 7-8). Significant differences to the wild-type according to the student's t-test are indicated with asterisks ($p < 0.05$).

In addition to the strong negative correlation between apyrase and starch (see above), which is very likely caused by ATP limitation of starch synthesis in the plastid, the inhibition of starch synthesis can also be partially explained by changes in the activities of plastidial enzymes involved in the pathway of starch synthesis. There was a significant decrease in the post-translational redox-activation of ADP-glucose pyrophosphorylase (AGPase), the near rate-limiting enzyme in the pathway of starch synthesis in potato tubers (Geigenberger et al., 2004), in all transgenic lines. The decrease in redox-activation was indicated by a decrease in the ratio between the activities measured under limiting (without reductant DTT, Fig. 9F) and optimal assay conditions (including 5 mM DTT; Fig. 9G), and is shown in Fig. 9H. Redox-activation of AGPase has been found to be a crucial mechanism in the regulation of starch synthesis in tubers and leaves (Tiessen et al., 2002; Hendriks et al., 2003). The strong positive correlation between AGPase redox-activation state and starch content ($r = 0.66$, $p = 0.00001$, $n = 37$) and the high negative correlation between AGPase redox-state and glucose-1-phosphate ($r = -0.61$, $p = 0.0001$, $n = 34$) is in support of an important role of this redox-based post-translational mechanism in the regulation of starch synthesis in response to apyrase expression in tubers.

Apyrase also led to a progressive and significant decrease in granule-bound starch synthase (GBSS) in all transgenic lines, while soluble starch synthase (SSS) activity progressively increased significantly in all lines (Fig. 9I and 9J). This provides a possible explanation for the decrease in starch amylose content in response to apyrase expression (see Fig. 6F). Previous studies with transgenic tubers showed that GBSS is crucial for the synthesis of amylose, while SSS is mainly involved in amylopectin synthesis (Visser et al., 1991; Lloyd et al., 1999).

8.4 Discussion

It is known from textbooks that cells need sufficient energy to fuel important processes such as maintenance, growth and biosynthetic activities. However, energy parameters can fall to low levels under conditions that lead to increased demand for ATP or that impair ATP generating processes, such as starvation or decreased oxygen concentrations. Under these conditions, adaptive mechanisms are required to balance ATP supply and demand. In plants, the knowledge about these mechanisms is very limited. Overall, there is surprisingly little experimental data available concerning the direct consequences of a specific change in energy parameters *in planta*. In the present paper we overexpressed an *E. coli* apyrase in the plastid of growing potato tubers to specifically decrease the levels of ATP and the adenylate energy state in the cells. Western-blotting revealed that the plastidial transitpeptide was cleaved off from the apyrase (Fig. 1). This can only happen within the plastid and assures that apyrase was properly targeted into this organelle. As no further band was detectable on the blot, we do not believe that considerable amounts of the exogenous apyrase are present in the cytosol or any other compartment. As supportive, albeit circumstantial evidence for the correct targeting of this enzyme is the fact that the metabolic consequences of the transformation are consistent with a plastidial localization of the enzyme (see Tjaden et al. 1998; Regierer et al. 2001) and inconsistent with patterns obtained when the expression of apyrase is altered in non-plastidial compartments (Riewe, Grosman, Fernie, Wucke, Geigenberger, unpublished data).

Short-term expression of apyrase using an inducible system led to a decrease in the ATP level and in the ATP/ADP ratio in growing tubers within 24h (Fig. 2A and F). It must be noted that the reported adenine nucleotide levels are overall levels and may not reflect the concentrations in the plastid. However, because expression of apyrase in the transgenic plants was targeted to the plastid, this should lead to changes in the plastidial levels of adenine nucleotides which are subsequently transformed to the cytosol and the mitochondria via ATP/ADP translocators located in the plastidial and mitochondrial membranes, respectively (Heldt, 1969; Vignais, 1976). The importance of the plastidial adenylate transporter to exchange ATP and ADP between cytosol and plastid has been elegantly demonstrated in transgenic studies in potato tubers (Tjaden et al. 1998) and Arabidopis leaves (Reinhold et al., 2007). Direct measurements of subcellular metabolite levels will, however, ultimately be needed to confirm our interpretation.

The decrease in ATP level and ATP/ADP ratio resulted in a general inhibition of sucrose degradation and metabolism, indicated by a decrease in the levels of sucrose, sucrose degradation products, organic acids involved in TCA cycle and most amino acid deriving from these pathways (Fig. 3). As the reduction in sucrose was apparently not caused by an

increased metabolism, it is highly likely that sucrose unloading was inhibited upon apyrase expression. This, however, remains to be verified by more direct approaches. Spearman correlation analysis confirmed our interpretation of a general inhibition of metabolism (Table 1), since most of the metabolites that were found to be significantly correlated to ATP showed positive correlations, while only 2 out of 22 showed negative correlation coefficients (fumarate and dehydroascorbate). Positive correlations to ATP were found for (i) UTP and GTP, indicating that adenine, uridine and guanidine nucleotide-pools are interlinked via nucleoside-diphosphokinase, (ii) sucrose, UDP-glucose and glucose-6-phosphate, indicating that sucrose degradation is linked to ATP, (iii) ADP-glucose, indicating a link between ATP and AGPase in the plastid, (iv) organic acids such as succinate and fumarate, indicating a link between ATP and succinate dehydrogenase in the TCA-cycle, and (v) aromatic amino acids (tyrosine, tryptophan and phenylalanine), amino acids of the aspartate family (methionine, lysine, leucine, threonine and isoleucine) as well as histidine, indicating a link between ATP and the synthesis of these amino acids in the plastid. In confirmation with this, the above mentioned amino acids were increased in potato tubers with antisense repression of a plastidial adenylate kinase having increased levels of ATP. In these lines, increased ATP levels also led to an increase in ADP-glucose and starch levels (Regierer et al., 2002).

Overall, these results show that a short-term decrease in ATP leads to a general suppression of tuber metabolism rather than a stimulation. These changes resemble the general metabolic depression in response to a decrease in internal oxygen concentrations that has been found in previous studies using potato tubers (Geigenberger et al., 2000; Geigenberger, 2003), wheat and rape seeds (Vigeolas et al., 2003; van Dongen et al., 2004) and rice plants (Fukao et al., 2006; Xu et al., 2006). This suggests that the low-oxygen induced inhibition of metabolism is at least partly due to the decrease in ATP levels, rather than a direct consequence of oxygen signalling. In this context, it remains to be determined whether ATP is acting as substrate, allosteric effector or a signal in its own right.

While a general inhibition of ATP-consuming processes such as sucrose degradation and the biosynthesis of starch and amino acids may have been expected as an adaptive response to save ATP, it is interesting that there was no stimulation of respiration within this time-frame to compensate for the fall in ATP. Correlative evidence links the reduction of ATP to a higher conversion of succinate to fumarate, a mitochondrial reaction yielding electrons for respiration. Although not clear at the moment, this process could lead to a higher chemiosmotic gradient across the inner mitochondrial membrane, enabling more efficient oxidative phosphorylation or faster respiration at a later stage. The resilience of the respiration rate to a short-term decrease in ATP levels will be beneficial in the context of the low internal oxygen concentrations that are prevailing in growing potato tubers (Geigenberger 2003b).

The short-term decrease in ATP did not lead to changes in ADP and a slight reduction of AMP instead of an accumulation. This must be a result of net degradation of adenylates. The opposite adenylate phenotype, an increase in ATP but no changes in ADP and AMP has been found after inhibition of the enzyme AMP deaminase with the herbicide carbocyclic coformycin (Dancer et al., 1997), and very recent results identified the deregulation of adenylates as cause of the toxicity of this herbicide (Sabina et al., 2007). It has been proposed that, if ATP decreases transiently, AMP deaminase stabilizes the adenylate energy charge by removal of AMP from the total pool to enable biochemical reactions dependent on the energy charge (Chapman and Atkinson, 1973; Dancer et al., 1997). Our results are in accordance with this hypothesis and suggest a participation of AMP deaminase in the process to compensate for low ATP in the short term.

To investigate long-term adaptations to a decrease in the adenylate energy state, apyrase was expressed and targeted into the tuber amyloplast under control of the constitutive B33 patatine promoter. In contrast to short-term expression, apyrase led to a decrease in tuber energy state mainly by decreasing the ratio between ATP and AMP or the adenylate energy charge (Fig. 4). The levels of AMP increased significantly under these conditions and total adenylates were not reduced, indicating that AMP deaminase was not able to compensate for the increase in AMP. There was no significant decrease in the overall ATP levels, which was mainly due to a large set of compensatory mechanisms including an increase in respiration rates that were more apparent in the lines with constitutively altered energy charge than in those with short-term reduction of ATP. Specifically the stimulation of respiration will lead to increased conversion of ADP to ATP in the mitochondria. It is therefore highly likely that increased production of ATP in the mitochondria compensated for the apyrase-induced conversion of ATP to ADP and AMP in the plastid. Line 1330#8 with the strongest constitutive expression of apyrase in the plastid revealed the strongest increase in respiration rates and the largest degree in compensation. The results are in agreement with an effective exchange of adenine nucleotides between the different subcelluar compartments.

The ATP-dependent short term changes in carbon metabolism in the inducible lines, such as the slight, but highly ATP-correlated decreases in intermediates of starch synthesis and amino acids of the shikimate and the aspartate family, were manifested under long term reduction of the energy charge (Fig. 8). While sugars accumulated, a strong inhibition of starch synthesis and a strong reduction in the levels of the above mentioned amino acids occurred in the constitutive lines. Also, succinate was much more decreased than any other TCA-cycle intermediate, again supporting that it may have an important role in energy dependent mitochondrial processes.

Unlike the inducible lines, long-term reduction of the energy charge led to an activation of respiration and changes in tuber morphology and growth resulting in an increased surface-to-volume ratio, which will improve oxygen access to the tuber tissue. Both features can be regarded as processes leading to a higher production of ATP. In the batch grown for eight weeks in the conditioned greenhouse used for biochemical analysis, the strongest line showed much more distinctive morphological adaptation than the other transgenic lines when compared to the same plants grown in an uncontrolled greenhouse to maturity for phenotypic analysis. This may explain why it contained more ATP and differed from the other lines in respect to intermediates of glycolysis. Similar changes in starch content and tuber morphology have been observed in transgenic plants with decreased plastidial ATP supply due to antisense inhibition of the plastidial ATP/ADP transporter (Tjaden et al., 1998).

The changes in carbon partitioning and tuber morphology were not accompanied by alterations in the internal oxygen concentrations of the tubers (Fig. 7). Moreover, the changes in morphology were not affected by alterations in external oxygen supply during long-term exposure of growing tubers to sub- or super-ambient oxygen concentrations (see supplemental Fig. S2). This provides evidence that these metabolic and morphological changes are linked to alterations in the energy state rather than being a direct oxygen effect.

The inhibition of starch synthesis was highly correlated to apyrase expression, suggesting that its synthesis was limited by ATP within the plastid. Additionally, the post-translational redox-activation state of the key-regulatory enzyme of starch synthesis, AGPase, was decreased upon apyrase expression, indicating that it may be linked to the adenylate energy state. In confirmation to this, redox-activation of AGPase and starch synthesis has been found to be increased in response to an increase in the energy state due to adenine-feeding to wild-type tubers or antisense expression of plastidial adenylate kinase (Oliver et al., in press). Previous studies showed that the redox-state of AGPase is altered in response to sugars, with decreased sugar levels leading to inactivation of AGPase (Tiessen et al., 2002). However, sugars increased or remained unchanged in response to apyrase expression, making it unlikely to be the reason for decreased AGPase activation under these conditions (Fig. 8). The decrease in the NADPH/NADP reduction state (see above) provides an alternative explanation for the redox-inactivation of AGPase. There is evidence that the NADPH/NADP system is involved in redox-transfer to AGPase in the plastid, and a decrease in the NADP reduction state may therefore lead directly to a decrease in AGPase redox-activation (data not shown).

In addition to the decrease in starch content, there was also a substantial decrease in the amylose/amylopectin ratio (Fig 6F.). This was mainly due to a decrease in GBSS activity, while SSS activity increased upon apyrase expression. Indeed, the amylose/amylopectin ratio strongly correlates with the GBSS/SS ratio ($r = 0.85$, $p < 0.000001$, $n = 36$). In addition

to this, the decrease in AGPase activation will lead to decreased ADP-glucose concentrations in the plastid, which will inhibit GBSS much stronger than SSS due to the differences in their K_m's for ADP-glucose (Baba et al., 1990; Lloyd et al., 1999). Recent studies in wheat also indicate that enzymes involved in starch synthesis in the plastid such as starch branching enzymes or starch synthases are regulated by reversible protein phosphorylation and protein complex formation (Tetlow et al., 2004). It will be interesting to determine whether changes in the protein phosphorylation pattern of these enzymes are linked to the ATP/AMP ratio in the plastid.

There were only marginal changes in the concentrations of fermentation products like lactate, alanine or glycerol-1-phosphate in response to apyrase expression, indicating that fermentation is rather a feature of hypoxia, but is not activated in a low energy state. Also the activities of ADH and LDH were either reduced or unaltered. Only the activity of PDC was significantly increased in the transgenic lines. PDC alone would not lead to an oxidation of NADH and due to its low affinity for pyruvate in comparison to pyruvate kinase it may not be active unless the concentration of pyruvate becomes very high (Tadege et al., 1999). Furthermore, constitutive expression of PDC in tobacco leaves does not alter production of acetaldehyde or ethanol under normoxic conditions, but under low oxygen the production of both metabolites accumulated 8-35 fold compared to the wild-type, showing that PDC limits ethanolic fermentation (Bucher et al., 1994). Its expression under conditions where energy is low, therefore may prepare metabolism to be ready for ethanolic fermentation in case it will be required.

In conclusion, the results of this study show that tuber metabolism, morphology and growth are severely altered as an adaptive response to a decrease in tuber energy parameters. While the short-term response mainly comprises a general depression of sucrose metabolism to avoid ATP-consuming processes, and maintenance of the energy charge, long-term responses also involve changes in carbon partitioning in favour of respiration, large changes in tuber morphology and growth resulting in an increased surface to volume ratio.

8.5 Materials and methods

DNA manipulation

Constitutive tuber specific plastidial apyrase (B33-apy): The DNA sequence corresponding to the mature protein sequence of the *Escherischia. coli* apyrase *apy* (GenBank accession No. AJ315184) ranging from nt 70-741 (without periplasma targeting sequence) was amplified from the virulence plasmid pINV (kindly provided by Mauro Nicoletti) isolated from *E.coli* strain *HN280* (Santapaola et al., 2002) using *pfu*-polymerase and the primers eapyHolof- (5'-CTG AAG GCA GAA GGT TTT CTC AC-3') and ecoliapyr (5'-CTG TGG ATC CTT ATG GGG TCA GTT CAT TGG TAG G-3'), the latter being flanked by a *Bam*HI restriction site. The pART7 derivative pART33-FNR-PHAG (kindly provided by Dr. Volker Mittendorf), was digested with *Nco*I and blunt-ended by incubation with Klenow enzyme and dNTPs. After digestion of both the PCR-product and the linearized plasmid with *Bam*HI, the PCR-product was ligated in frame to form a chimeric ORF consisting of 165 nt coding for the *Spinacia oleracea* Ferredoxin-$NADP^+$-Reductase transitpeptide (Jansen et al., 1988) at the 5'-end, the partial *apy* gene coding for the mature *E. coli* apyrase at the 3'-end and a linker consisting of the nucleotide sequence 5'-GGG GCC ATG-3'. Accuracy of the coding sequence was confirmed by sequencing. The cassette coding for the patatin B33-Promoter, the chimeric ORF and the octopine synthase terminator was subcloned into part27 using the *Not*I restriction sites

Ethanol-inducible tuber specific plastidial apyrase (B33-alc-apy): The sequence of the *E. coli* apyrase fused to the transitpeptide of the spinach ferredoxin-$NADP^+$-reductase was amplified from the plasmid B33-*apy* using the primers fnrapybamf (5'-ACT CGG ATC CAT GAC CAC CGC TGT CAC CGC C-3') and ecoliapyr (see above), both flanked with the restriction site for *Bam*HI. The PCR-product was digested with *Bam*HI and ligated into the *Bam*HI digested plasmid pUC19-*alcA* (obtained by Dr. Björn Junker). Accuracy of the coding sequence was confirmed by sequencing. The obtained plasmid was digested with *Hind*III to obtain the cassette containing the *alc*-promotor, the chimeric ORF and the 35S-terminator. This cassette was cloned into B33-*alc-GUS*, which was digested with *Hind*III to excise the region coding for the *alc*-promotor, GUS and NOS-terminator.

Lactose/IPTG-inducible His-tagged apyrase (apy-His): The complete sequence without stop codon of the *E. coli apy* gene (see above) was amplified with *pfu*-polymerase using the primers eapy+3f (5'-AAA ACC AAA AAC TTT CTT CTT TTT TG-3') and eapy+3r (5'-TCC AGG ATC CTG GGG TCA GTT CAT TGG TAG GAG TA-3'), the latter being flanked by a *Bam*HI restriction site. The plasmid pQE60 (Quiagen, Hilden, Germany) was digested with *Nco*I and blunted with Klenow enzyme. After digestion of the PCR-product and linearized

plasmid with *Bam*HI, the PCR-product was ligated into pQE60 in frame with a Hexa-His-tag and a subsequent stop codon.

Generation of transgenic potato plants

The plasmids B33-*apy* and B33-*alc-apy* were transformed into *Agrobacterium tumefaciens* strain *pGV2260* using a gene pulser electroporator (Biorad, Munich, Germany) as described by Mattanovich et al. (Mattanovich et al., 1989). The recombinant *Agrobacteria* were used to transform sterile, freshly injured leaves of *Solanum tuberosum L. cv. Desiree* by the method established by Rocha-Sosa et al. (1989).

Plant growth conditions

Potato plants *cv. Desiree* were cultivated in tissue culture for long term storage and propagation. Cuttings were transferred to soil and grown in a phytotron (soil, 60/75% humidity d/n, 22/16°C d/n, 150 $\mu mol \cdot s^{-1} \cdot m^2$ light intensity, 16/18h d/n) for 2 weeks. Then the plants were transferred to pots with a diameter of 18 cm and grown in a conditioned glasshouse (soil, 60% humidity 22/16°C d/n, 350 $\mu mol \cdot s^{-1} \cdot m^2$ light intensity, 16/18h d/n) for eight or ten weeks for the biochemical analysis, or for thirteen weeks in an uncontrolled greenhouse for phenotypical characterization.

Induction of apyrase in tubers *in planta*

Tubers attached to plants grown for eight weeks were pierced with a coaxial needle (diameter 0.9 mm, HS Hospital Service, Aprilia – Latina, Italy). The duct was filled with 50 µl 0.2% acetaldehyde solution and the pipet tip remained sticking in the duct filled with 150 µl acetaldehyde solution. This allowed further supply with acetaldehyde and identification of the duct after the tuber was covered again with soil. After 24 hours, only the part surrounding the duct was sampled with a corkborer (6 mm diameter) and immediately frozen in liquid nitrogen.

Incubation of tubers with different oxygen concentration in planta

After two weeks of growth in a phytotron and one week of growth in the conditioned greenhouse, the pots were transferred into boxes (Allibert-Buckhorn Limited, Bromsgrove, UK, five plants per box) with water supply and grown for two weeks to allow the shoot to grow through a 6 cm high duct consisting of a round tissue culture vessel (6.5 cm diameter,

Greiner Bio-One, Solingen, Germany) with sawed off bottom placed on holes with 6.5 cm diameter in the lid of the boxes. Then the duct and the lid of the boxes were sealed with cotton and tape. After another two weeks the boxes were aerated constantly for another five weeks with gas with different oxygen concentration and (4, 21 and 40% oxygen, 350 ppm carbon dioxide, rest nitrogen) mixed by a flow controller (Brooks Instruments, Westphal Mess und Regeltechnik Vertriebs-GmbH, Hahn, Germany). After this period, the tubers were harvested together with tubers from plants grown next to the boxes as control plants.

Expression of His-tagged apyrase

The plasmid *apy*-His was heat-shock transformed into *E. coli* strain *M15*. An isolated clone with elevated apyrase expression was selected to produce metal affinity purified apyrase protein using TALON affinity resin (Clontech, Mountain View, CA, USA) following the manufacturer's instructions.

Real-Time-PCR

RNA was extracted from 20 mg FW using the RNeasy Plant Mini Kit (Quiagen, Hilden Germany) and DNA was digested on column as suggested by the supplier. 200 ng RNA were used to produce cDNA in a final volume of 130 µl. For cDNA quantification, 2 µl were used as template in a Real-Time-PCR-reaction mixed with 10 µl Power SYBR-Green (Applied Bioscience, Warrington, UK) and 10 µl primer mix (forward and reverse primer, each 0.5 µM). The following primer mixes were used for quantification: Eapy (forward: 5'-GAG GCT GGG CGT TTA ATG GGA G-3', reverse: 5'- GGG GTC AGT TCA TTG GTA GGA GT -3'), StGAPDH5' (forward: 5'-AAG GAC AAG GCT GCT GCT CAC-3', reverse: 5'-AAC TCT GGC TTG TAT TCA TTC TCG-3') and StGAPDH3' (forward: 5'-TTC AAC ATC ATC CCT AGC AGC ACT-3', reverse: 5'-TAA GGT CGA CAA CAG AAA CAT CAG-3'). The amount of cDNA of the induced apyrase was related to the amount of GAPDH set as one. Only samples with equal threshold cycles (±1) for the GAPDH cDNA 5'- and 3'-ends were considered for analysis.

Polyacrylamide electrophoresis (PAGE) and Western blotting

Non-reducing PAGE, coomassie stain and Western blotting were performed according to the standard protocol described by Sambrook and Russel (2001) using a 12.5% acrylamide gel. After semi-dry transfer on a PVDF membrane (Roche, Mannheim, Germany), the membrane was probed with a serum directed against *E. coli* apyrase (kindly provided by Francesca

Berlutti). Binding was detected enzymatically using a secondary antibody conjugated to horseradish peroxidase (Biorad, Munich, Germany).

Metabolite analysis

Glucose, fructose sucrose, hexose-phosphates, UDP-glucose, 3-PGA, PEP, pyruvate and *starch* were extracted or precipitated with trichloroacetic acid (TCA) extracts as described by Jelitto et al. (1992) and analyzed using enzyme based assays as described by Geigenberger et al. (1998). *Amylose* content of starch was determined using a Lugol-based colorimetric assay (Hovenkamp-Hermelink et al., 1988). Nucleotides were quantified from TCA-extracts using a Kontron HPLC (Bio-Tek Instruments, Bad Friedrichshall) equipped with a Partisil-SAX 10 anion-exchange column and a UV-photometer detecting at 254 nm (Geigenberger et al., 1998) with exception of AMP in tubers expressing inducible apyrase, which was measured using a photometric assay (Bergmeyer, 1985). Protein content was quantified from alkalized TCA precipitations using the dye based Bradford assay (Bradford, 1976). $NADP^+$ and NADPH were quantified from freshly prepared ethanol extracts using a cycling assay (Kolbe et al., 2005). ADP-glucose was measured from TCA extracts using a cycling assay (Gibon et al., 2002). GCMS analysis and preceding extraction and derivatization was performed as described by Roessner at al. (Roessner et al., 2001) using either a GCMS system consisting of a GC 8000 gas chromatograph and a Voyager quadrupole mass spectrometer (ThermoQuest, Manchester, GB) for the analysis of the plants expressing plastidial apyrase constitutively (1330) or as described by Lisec et al. (2006) using a HP 5980 gas chromatograph (Agilent, Waldbronn, Germany) coupled to a Pegasus II Time-of-Flight mass spectrometer (Leco Corporation, St. Joseph, MI, USA) for the analysis of the plants expressing inducible plastidial apyrase (2330).

Enzyme analysis

Enzymes were extracted as described previously by Geigenberger and Stitt (1993) with pefabloc and PVPP instead of PMSF in the extraction buffer and omitting BSA. Enzymes were extracted from 30-100 mg frozen homogenized tissue using 1500µl 50 mM HEPES/KOH pH 7.4 containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X100, 10% glycerol, 2 mM benzamidine, 2 mM γ-aminocaproic acid, 0.5 mM pefabloc, 5 mM DTT, 0.1% PVPP. Extracts were kept on 4°C prior measurement. For the measurement of AGPase DTT was omitted in the extraction buffer and for the extraction of starch synthases triton X100 was omitted.

Apyrase: Apyrase was measured via P_i release from ATP as described by Ames (1966) using a buffer optimized for the *E. coli* apyrase (50 mM HEPES/KOH pH7.4, 1 mM EDTA, 1 mM EGTA). One Unit is defined as liberation of 1 µmol P_i per minute.

ADP-glucose-pyrophosphorylase (AGPase): AGPase was assayed in absence or presence of DTT as described by Tiessen et al. (2002). Oxygen in extraction and assay buffer was displaced by N_2 before use. Extraction and subsequent measurement did not exceed 30 minutes.

UDP-glucose-pyrophosphorylase (UGPase): UGPase was measured as described by Zrenner et al. (1993).

PEP-Phosphatase and *pyruvate-kinase*: PEP-dephosphorylating activities were measured as described by Ireland et al. (1980) by sequential addition of PEP and ADP to the reaction mix.

Lactate-dehydrogenase (LDH): LDH was quantified as previously described by Bergmeyer (1985).

Pyruvat-decarboxylase (PDC): PDC activity was assayed as performed by Bouny and Saglio (1996) in the presence of the cofactor thiamin-pyrophosphate and the LDH-inhibitor oxamate.

Alcohol-dehydrogenase (ADH): ADH activity was determined as described by Bergmeyer (1985).

Soluble starch synthase (SSS): SSS was extracted from 30 mg FW without triton X100 and was assayed by determination of incorporation of [U-^{14}C]ADP-glucose into amylopectin as described by Abel et al. (1996).

Granule bound starch synthase (GBSS): The insoluble pellet from the extract prepared for SSS-determination (see above) was washed three times with 50 mM Tricine/NaOH pH 8.5, 25 mM potassium acetate, 2 mM EDTA, 2 mM DTT. The pellet was then suspended in 500 µl buffer and the reaction was started by addition of [U-^{14}C]ADP-glucose in a final concentration of 1 mM and a specific activity of 3.5 kBq/µmol. After several time points aliquots of 100 µl were subtracted and incorporation of [U-^{14}C]ADP-glucose into amylose was determined as described for SSS.

Analysis of respiration rates

Repiration rates of two freshly prepared tuber discs (diameter 8 mm, thickness 2mm) were analyzed in an oxygen electrode (Hansatech, Norfolk, UK) filled with 1 ml 20 mM MES/KOH pH 6.0 as described previously (Loef et al., 2001).

Analysis of internal oxygen concentration

Tubers were harvested, freed from soil and immediately fixed in a micromanipulator (Saur Laborbedarf, Reutlingen, Germany) without previous washing with water. Without delay a MikroTX2 oxygen microsensor with a tip diameter of 30 µm (Presens, Regensburg, Germany; see also van Dongen et al. (2003) was phased in to a depth of 4 mm under the tuber skin and the oxygen concentration was recorded until it was stable.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AJ315184.

8.6 Acknowledgements

We are grateful to Volker Mittendorf (BASF Plant Science, Durham, NC, USA) for the plasmid pART33-FNR-PHAG, Mauro Nicolleti (Università G. D'Annunzio, Chieti, Italy) for the plasmid pINV, Francesca Berlutti (Università La Sapienza, Rome, Italy) for the serum raised against *E. coli* apyrase, and Ilse Balbo and Aenne Eckardt (MPIMP, Potsdam, Germany) for help with GC-MS analysis.

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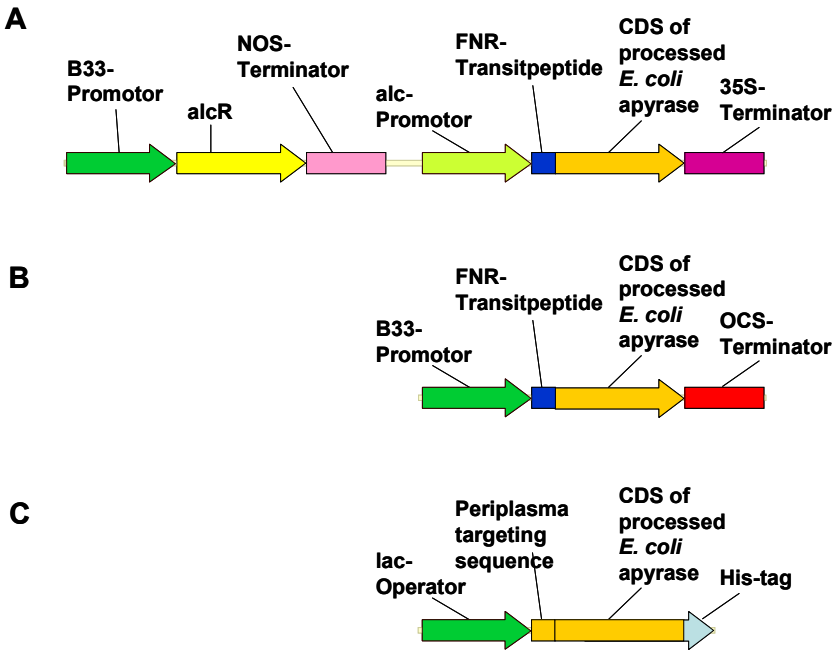
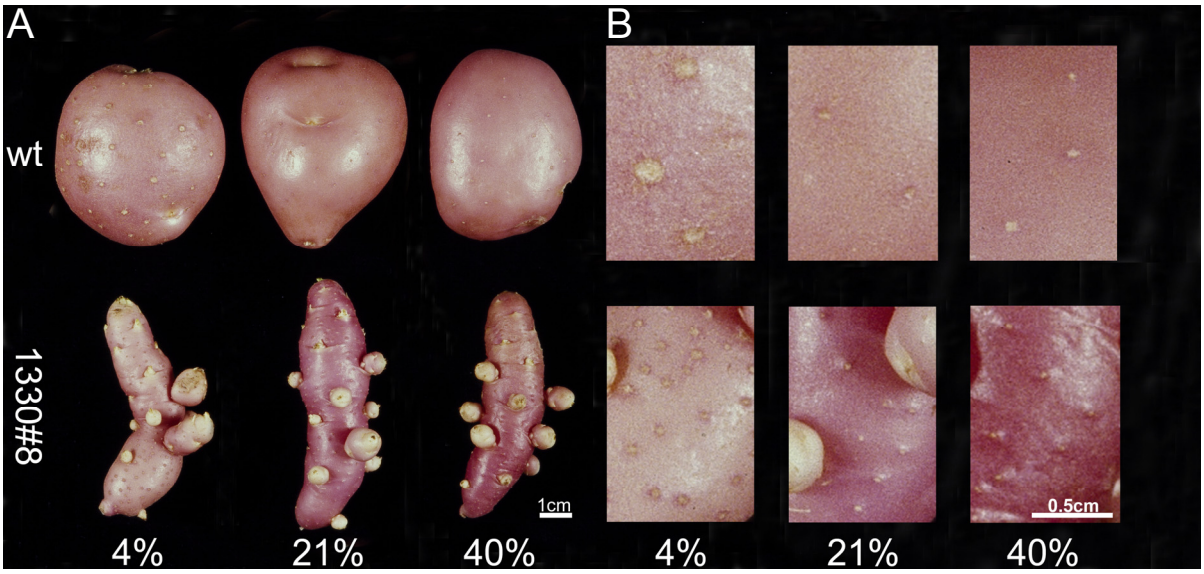
8.8 Supplemental data



Supplemental Fig. S1. Plasmids used for expression of apyrase in *S. tuberosum* and *E. coli*. **(A)** Inducible tuber specific plastidial apyrase (B33-*alc-apy*), **(B)** constitutive tuber specific plastidial apyrase (B33-*apy*) and **(C)** lactose/IPTG inducible His-tagged apyrase (*apy*-His).



Supplemental Fig. S2. Growth performance of tubers exposed to different oxygen concentrations. The pots of potato plants from wild-type and line 1330#8 were aerated with 4, 21 and 40 % (v/v) oxygen from the fifth to the tenth week of growth, before representative pictures of **(A)** tuber morphology and close-ups showing **(B)** lenticel size were taken.

9. Publication 2 (P2)

The potato-specific apyrase is apoplastically localized and has influence on gene expression, growth and development

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In press in Plant Physiology, PubMed Unique Identifier: 18480378. The published version has been revised.

Authors' contributions

The research was conceived and planned by Peter Geigenberger, Alisdair Fernie and David Riewe. The experimental work was done by David Riewe. Lukasz Grosman and Cornelia Wucke assisted as student workers supervised by David Riewe.

9.1 Abstract

Apyrases hydrolyze nucleoside triphosphates and diphosphates and are found in all eukaryotes and a few prokaryotes. Although their enzymatic properties have been well characterized, relatively little is known regarding their subcellular localization and physiological function in plants. In the present study we used reverse genetic and biochemical approaches to investigate the role of potato-specific apyrase. Silencing of the apyrase gene family with RNAi-constructs under the control of the constitutive 35S-promoter led to a strong decrease in apyrase activity to below 10% of the wild-type level. This decreased activity led to phenotypic changes in the transgenic lines including growth retardation, delayed flowering and changes in flower morphology. These changes were accompanied by an increase in tuber number per plant and differences in tuber morphology. In contrast, apyrase overexpressing plants, showing an up to 10-times higher apyrase activity, flowered earlier than wild-type and showed alterations in flower morphology which were opposite to the changes reported for RNAi plants. Silencing of apyrase under control of a tuber-specific promoter led to similar changes in tuber morphology, compared to constitutive suppression, as well as to increased starch content. DNA micro-arrays revealed that decreased expression of apyrase leads to increased levels of transcripts coding for cell wall proteins involved in growth and genes involved in energy transfer and starch synthesis. To place these results in context, we determined the subcellular localization of the potato-specific apyrase. Using a combination of approaches we were able to demonstrate that this enzyme is localized to the apoplast. We describe the evidence underlying this fact and that potato-specific apyrase has a crucial role in regulating plant growth and development through changes in the expression of nuclear genes involved in cell-wall extension, energy metabolism and starch synthesis.

8.2 Introduction

ATP-diphosphohydrolases, or apyrases, are enzymes that hydrolyze both the γ- and ß-phosphates of ATP and ADP. They are distinct from other phosphohydrolases with respect to their specific activity, nucleotide substrate specificity, divalent cation requirement and sensitivity to inhibitors (Plesner, 1995; Handa and Guidotti, 1996). Apyrases are ubiquitously expressed in eukaryotes and have additionally been found in some prokaryotes, indicating a general role for these enzymes across species. Furthermore, apyrase function has been intensively studied in mammalian systems, where the role of apyrase is to hydrolyze extracellular adenosine nucleoside tri- and diphosphates, which is important both in the inactivation of synaptic ATP as a neurotransmitter following nerve stimulation (Todorov et al., 1997) and the inhibition of ADP-induced platelet aggregation to prevent thrombosis (Marcus et al., 1997). Adenosine deriving from the concerted action of apyrase and ecto 5'-nucleotidase is subsequently transported into mammalian cells via a sodium/adenosine co-transporter (Che et al., 1992).

In contrast to the situation described above, relatively little is known on the function of apyrase in plants. The enzyme has been characterized at the biochemical level in potato (Kalckar, 1944; Molnar and Lorand, 1961; Kettlun et al., 1982) and various legumes such as *Glycine max*, *Medicago truncatula* and *Pisum sativum* (Day et al., 2000; Hsieh et al., 2000; Cohn et al., 2001). Potato apyrase has additionally been speculated to be involved in starch synthesis (Fanta et al., 1988). The reasoning behind this hypothesis was the fact that many steps of the starch biosynthetic pathway are regulated by the levels of ATP, ADP or P_i and that potato tuber, which accumulates vast quantities of starch (Geigenberger and Fernie, 2006), contains a specific apyrase distinct from those found in other plants (Handa and Guidotti, 1996; Roberts et al., 1999). A special feature of potato specific apyrase is that it is soluble and does not appear to be strongly bound to membranes as it is the case for all other plant apyrases characterized to date. On the basis of phylogenetic analysis and a DNA hybridization experiment reported by Handa and Guidotti (1996), it has been postulated that potato additionally contains apyrases which more closely resemble general plant apyrases (Roberts et al. 1999). The deduced amino acid sequence of the potato specific cDNA contains a putative N-terminal signal peptide for the secretory pathway (see Supplemental Fig. S1). In *Medicago truncatula*, four apyrase cDNAs were isolated and two of them showed increased transcript levels in response to nodulation (Cohn et al., 2001). Nod(ulation promoting)-factors were also shown to bind to a lectin domain of an apyrase found in roots of *Dolichos biflorus*, indicating a role of this enzyme during nodulation (Etzler et al., 1999). However, this lectin domain appears to be conserved also being present in the potato apyrase and apyrases *AtAPY1* and *2* from *Arabidopsis thaliana* (Steinebrunner et al., 2000).

In addition to sequence analysis, some information is available regarding subcellular localization of these proteins with the legume specific apyrase *Db-LNP* from *Dolichos biflorus* demonstrated to have a cell membrane localization (Etzler et al., 1999). Plasma membrane localization was also found for the *Glycine max* apyrase *GS52* (Day et al., 2000; Kalsi and Etzler, 2000), and a second, structurally similar apyrase has been documented as being localized to the Golgi apparatus (Day et al., 2000). While *GS52*-specific antibodies blocked nodulation in *Glycine max* (Day et al., 2000), overexpression of *GS52* in *Lotus japonicus* enhanced nodulation (McAlvin and Stacey, 2005). These studies point to a specific function of apyrase in nodulation processes of legumes. Furthermore, studies of apyrase function in non-legumes have been performed in Arabidopsis. These studies include the analysis of transgenic plants overexpressing an apyrase from *Pisum sativum* and T-DNA knock-out mutants of two apyrase genes (*AtAPY1* and *AtAPY2*) identified in Arabidopsis. Interestingly, expression of the heterologous apyrase led to increased scavenging of extracellular phosphate (Thomas et al., 1999) and to higher resistance to xenobiotics (Thomas et al., 2000) and herbicides (Windsor et al., 2003). In contrast, single knock-out mutants of *AtAPY1* or *AtAPY2* lack a discernable phenotype, whilst the corresponding double mutant exhibited a complete inhibition of pollen germination in Arabidopsis, indicating a role of apyrase in sexual reproduction of plants (Steinebrunner et al., 2003).

Although *AtAPY1* and *2* are thought to be ecto-apyrases, only indirect evidence supporting an apoplastic localization for these enzymes has been presented to date (Wu et al., 2007). Apyrase activity, occasionally termed ”latent UDPase” has additionally been identified in Golgi vesicles from *Pisum sativum* wherein it appears to use UDP as substrate (Orellana et al., 1997; Neckelmann and Orellana, 1998). It is believed that this activity hydrolyzes UDP to UMP, which is subsequently translocated into the symplast in exchange for a nucleotide sugar. The sugar-moiety of this nucleotide sugar is subsequently transferred to a nascent cell wall polysaccharide or protein glycosyl residue yielding UDP. It seems likely that the elimination of UDP is required for continued operation of the cycle since it is an inhibitor of the glucoysltransferase catalysed reactions that constitute a portion of the cycle (Neckelmann and Orellana, 1998). On the basis of their proteomic analyses of Golgi preparations, Dunkley et al. (2004) suggest a Golgi localization of *AtAPY2* Whilst these previous studies revealed a number of possible functions of apyrase, the general importance of this enzyme in plants has, as yet, not been fully resolved. Moreover, the subcellular localization of plant apyrases in general, and the potato-specific apyrase in particular have not been unambiguously demonstrated. In the following study, we cloned two new potato specific apyrases closely related to the one identified by Handa and Guidotti (1998). Using database searches, we were able to find tentative consensus sequences of potato apyrases that in fact more closely resemble general plant apyrases, supporting the assumption that

potato contains additional apyrases with special function in addition to general plant apyrases (Roberts et al., 1999). We used a reverse genetic approach to investigate the importance and role of potato-specific apyrase. Transgenic analyses were performed to study the effect of altered apyrase expression on growth, development, metabolism and gene expression. In addition, the subcellular localization of the potato apyrase was investigated using non-aqueous fractionation, GFP-fusion constructs, apoplastic washings and tuber slice incubation experiments. The combined results of these studies indicated that potato apyrase is an apoplastic enzyme of major importance for plant growth and development.

9.3 Results

Potato contains at least three different potato-specific apyrases with high expression in sink tissues

Using primers based on the 5'- and 3'-UTR sequences of the previously cloned potato apyrase (Handa and Guidotti, 1996), two apyrases with very high identity to this apyrase were cloned. These novel clones were thus termed *StAPY2* and *StAPY3* (with *StAPY1* being the apyrase cloned previously). *StAPY3* showed 98.7% identity to *StAPY1*, and *StAPY2* still showed 92.6% identity to *StAPY1* on the protein level (alignment included in Supplemental, Fig. S1 and Table S1). A comprehensive database search revealed tentative consensus (TC) sequences generated from expressed sequence tags (ESTs) for all three apyrases in the *Solanum tuberosum* gene indices database provided by the Computational Biology and Functional Genomics Laboratory (Supplemental Table S1). *StAPY2* was assigned to TC134863 and both *StAPY1* and *StAPY3* were assigned to TC137054 alone, but the SNP-report for this TC clearly verifies the existence of both very similar isoforms. In addition to this, TCs putatively coding for other apyrases were identified in this database (TC142822 and TC144101, see Table S1). Complete or partial protein sequences were derived from these TC-sequences and analysed regarding identity towards *StAPY1-3* and *AtAPY1* and *AtAPY2*. Whilst comparison at the nucleotide level was relatively unclear, the protein sequences of these TCs were markedly more similar to the protein sequences of the Arabidopsis apyrases (59-66%) than to *StAPY1-3* (49-52%). This comparison thus provides support for the assumption by Roberts et al. (1999) that in addition to potato specific apyrase additional apyrases with high similarity to general plant apyrases, such as *AtAPY1* and *AtAPY2* , exist in the potato genome.

The cDNA of *StAPY2* has a comparable length to *StAPY1* and *3*. However, in addition to the TAA stop codon found for the latter apyrases at position 1363-1365, it has an additional stop codon at position 850-852. The protein sequence of both isoforms contain all four apyrase conserved regions identified by Handa and Guidotti (1996) (see Supplemental Fig. S1). The anterior stop codon, however, was not found in several ESTs from *StAPY2* cDNA cloned from the cultivar *Shepody* and it is hence possible that this cDNA is not translated into a functional apyrase protein in the cultivar *Desiree*. Due to the high identity between the different potato-specific apyrases (>92%, see Supplemental Table S1) and their lower identity with other detected apyrases at the nucleotide level, it was possible to determine the combined transcript levels of all three potato-specific isoforms in different tissues using a probe generated from the cDNA sequence of *StAPY3*. Expression was found to occur to a considerable extent in sink tissues such as root, tuber and flower and to a much lesser extent in stolon tissue (Fig. 1A). In contrast, virtually no transcript was detectable in source leaves, senescent leaves or stem tissue.

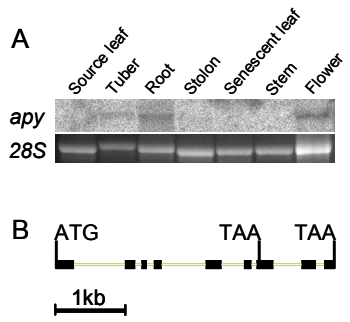

Fig. 1. Gene expression pattern of potato apyrase and gene structure of the potato apyrase 2. (A) 50 µg RNA of different tissues were separated and transcript levels of *StAPY1-3* quantified with a probe consisting of *StAPY3*. The detected band had a size of 1.5 kb. As loading control, the band of the 28S-ribosomal RNA (*28S*) in the agarose gel is also shown. **(B)** The complete gene of the truncated potato apyrase *StAPY2* consisting of nine exons (black blocks) and eight introns is shown including start codon and the stop codon leading to truncation in exon 7 as well as the stop codon found in *StAPY1-3* in the last exon.

Isolation of a genomic clone of *StAPY2* confirmed that the truncation found in the cDNA was not an artifact of the cloning procedure. The 4 Kb sequence obtained contained the 3909 bp long DNA sequence from the start to the posterior stop codon identified in the cDNA (Fig. 1B). In total agreement with the anterior stop codon found in the cDNA of *StAPY2*, the genomic sequence contained the corresponding stop codon in exon 7 at position 2855-2857. The gene consists of nine exons and eight introns, exactly like the Arabidopsis apyrases *AtAPY1* and *AtAPY2*, and some of the splicing sites are conserved between the potato and Arabidopsis apyrases. It thus seems likely that the general plant apyrases and the potato-specific apyrases originated from a common ancestral protein.

Generation of transgenic potato plants with altered expression of apyrase

Potato plants with reduced/elevated apyrase expression were generated by Agrobacterium-mediated transformation of potato plants, *cv. Desiree*, as described by Rocha-Sosa et al. (1989). The full length sequence of *StAPY3* was cloned into pART7/pART27 (Gleave, 1992) to create a plasmid overexpressing *StAPY3* under the ubiquitous CaMV 35S-promoter. For the production of the 35S-RNAi plasmid, the first 500 nt of *StAPY3* were cloned in sense-intron-antisense orientation into pHANNIBAL/pART27 (Wesley et al., 2001; for full cloning details see Supplemental Fig. S2A-C). Due to the high identity of *StAPY1-3* at the RNA level it seems probable that these RNAi constructs would affect the transcript levels of all three potato-specific apyrases, but not of those with higher identity to *AtAPY1* and *AtAPY2*. Furthermore, a database search in the potato gene indices of the Computational Biology and functional Genomics Laboratory using the 500 bp RNAi-fragment as query, suggested that it was highly unlikely that this construct would affect any other unintended target. A third plasmid for tuber-specific reduction of apyrase was produced by replacement of the 35S-promoter with the B33-promoter. Following regeneration, transformed plant material was clonally propagated, cuttings were cultivated in tissue culture and explants were grown under controlled conditions in order to generate plant material for biochemical analysis and phenotypic characterization.

Lines with altered expression of apyrase under the control of the constitutive 35S-promoter showed marked changes in apyrase mRNA and activity

To investigate expression of apyrase in the transgenic lines, both apyrase-mRNA and activity were determined. The primers used for the Real-Time-PCR were specific for isoforms *StApy1* and *StApy3*, but would not allow amplification of *StApy2*. Differentiation between *StApy1* and *StApy3* was virtually impossible due to the high similarity on the nucleotide level downstream of the sequence used for RNAi-induction. Apyrase activity was measured in the soluble fraction of enzyme extracts using ATP as substrate and in the absence of phosphatase-inhibitors, since potato apyrase is the predominant ATP-hydrolyzing phosphatase in enzyme extracts of potato tubers (see below) As demonstrated in Fig. 2A and B, both the apyrase mRNA and activity of all 35S-RNAi-lines were significantly reduced, whereas the apyrase mRNA and activity of the 35S-overexpressors were significantly higher than those in wild type tubers. The only exception to this statement was the overexpressing line 35S:143 which displayed insignificantly decreased levels of transcript and activity – a phenomena most likely attributable to co-supression effects. Whereas line 35S:17 was clearly most strongly reduced in *StAPY1/3* transcript levels (0.13% of the wt) compared to

lines 35S:35 (5.3%) and 35S:23 (18%), apyrase activity was similarly reduced in all transgenic lines. Line 35S:23 appeared to be the most affected followed by line 35S:17 and 35S:35 at the level of activity. However these lines were not very different from one another, with activities ranging from 6.0-7.1% of the total activity. From this we conclude that neither transcript levels nor phosphatase activities allow unimpeachable conclusions on the degree of reduction of apyrase in these lines but clearly show that apyrase is reduced in all of them.

Using specific primers for *StAPY2*, we found that this isoform is expressed to a lower extent when compared to *StAPY1* and *StAPY3* and that its expression is also reduced in the 35S-RNAi-lines (data not shown). As observed for the wild type, apyrase activities of the transgenics were similar when UTP, ADP and UDP were provided as substrate (see Fig. 8H). However, it should be noted that the turnover of the monophosphates was markedly lower, suggesting that ATP was hydrolyzed by apyrase, not by any phosphatase. The relative turnover of the tri- and diphosphates tested was invarient in the overexpressing lines.

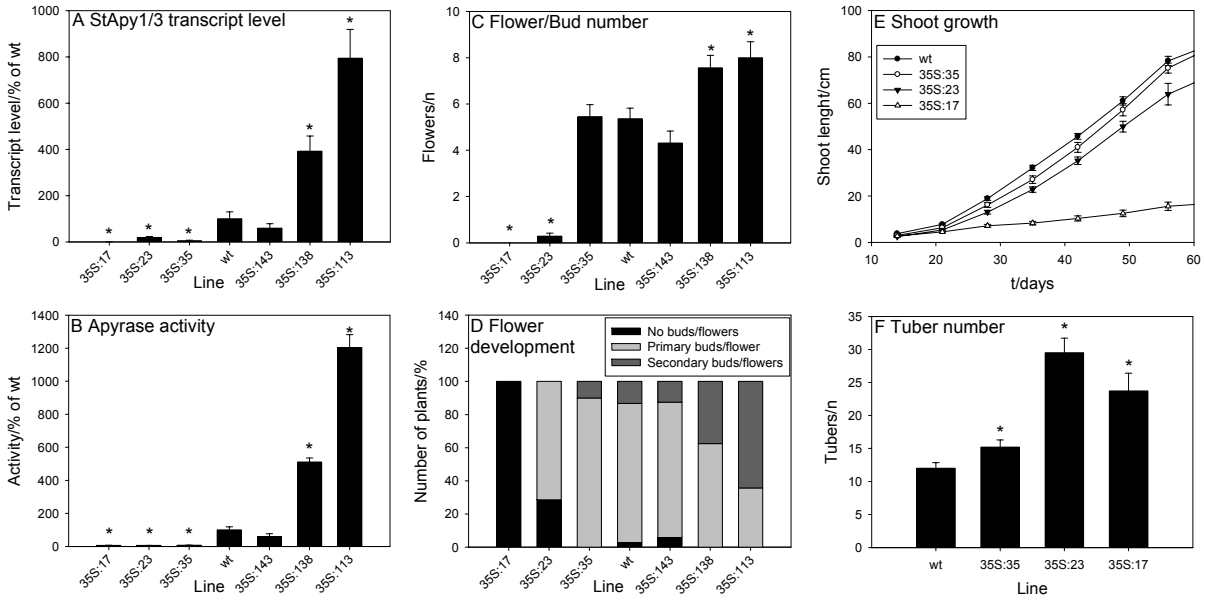

Fig. 2. Altered apyrase transcript and activity under the control of the 35S-promoter affects plant development. (A) Combined transcript levels of *StApy1* and *StApy3* in tuber tissue of RNAi-lines (2-digit code) and overexpressors (3-digit code) were determined from cDNA by Real-Time-PCR and expressed as % of the wild type. **(B)** Apyrase activity was analysed using an assay containing 1 mM ATP as substrate, and the activities expressed as % of the wild type. **(C)** Flower number and **(D)** flower development was analyzed in 49 and 56 day old plants, respectively. **(E)** Shoot growth was weekly monitored for 70 days and **(F)** tuber number was determined in in matured plants. Results are means ± SE, *n* = 6-8 for **(A and B)** and 10-13 for **(C-F)**. Significant differences to the wild-type according to a student's t-test are indicated with asterisks ($p < 0.05$). Shoot growth was significantly lower for all transgenic lines compared to the wild-type from day 14 to 35, for lines 35S:23 and 35S:17 from day 42 to 56 ($p < 0.05$).

All lines exhibit a rest ATP-hydrolyzing activity of approximately 6% of that found in the wild type. It seems unlikely that all of this activity corresponds to residual potato-specific apyrase since apyrase protein was not detectable on a Western blot in the strongest RNAi line and consistently increased in the strongest overexpressing line, (see Supplemental Fig. S3). This result suggests that the remaining ATP-hydrolysis activity is either catalyzed by apyrase isoforms that do not cross-react with the antibody or by less specific phosphatases. Irrespective of what is responsible for this residual activity the data presented here clearly demonstrate that potato-specific apyrase isoforms contribute to the vast majority of apyrase activity measured in potato tubers.

Lines with decreased expression of apyrase under the control of the constitutive 35S-promoter showed dramatic changes in growth and development

Potato plants with decreased expression of apyrase under the control of the constitutive 35S-promoter showed various developmental alterations compared to wild-type when grown in the greenhouse. Shoot growth was clearly retarded in two lines (Fig. 2E and 3A) and the line with the strongest decrease in StApy1/3 mRNA had problems to reach maturity in an uncontrolled greenhouse but was, however, fit enough to produce flowers in a fully conditioned greenhouse and tubers in a phytotron (data not shown). The 35S-RNAi lines produced a larger number of tubers but these were considerably smaller in size (Fig 2F and 3C) and displayed a more longitudinal shape than tubers of the wild type (Fig 3C). Flowering was clearly delayed in two of the transformants (Fig. 2C), with a detailed scoring of flower development at day 56 revealing that there were almost no secondary flowers/buds on plants of these lines at this time point (Fig. 2D). In addition to the alterations in flowering time, flower morphology was also severely altered (Fig. 3B, red arrow). Here a relative reduction of style length was observed with the stigma being unable to leave the anther in the case of the RNAi line exhibiting the strongest inhibition. This was due to curling of the style (Fig. 3B, yellow arrow) which additionally forced contact between the stigma and the anthers which would inevitable lead to self fertilization of the flower.

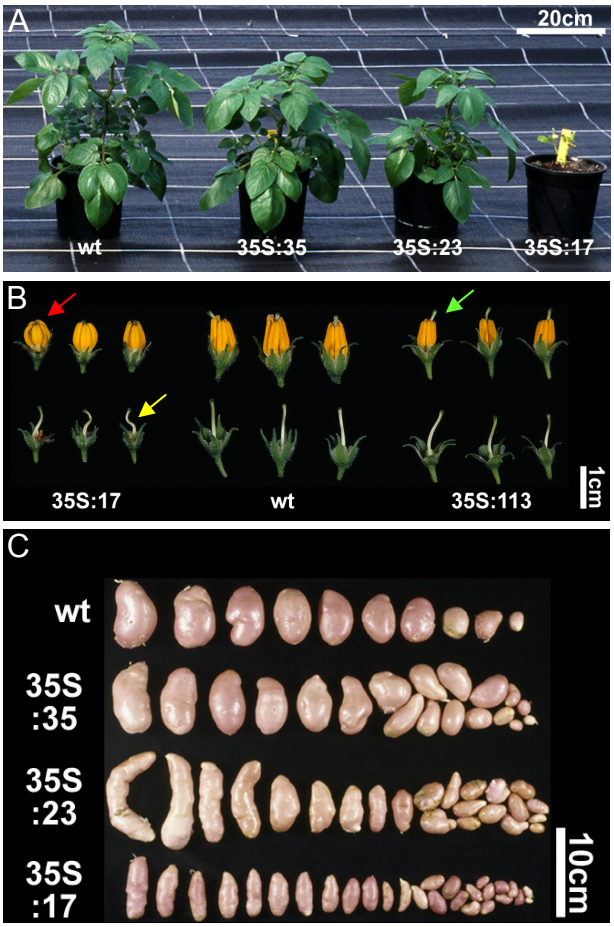

Fig. 3. Visible phenotype of lines with altered apyrase activity under the control of the 35S-promoter. (A) Significantly retarded shoot growth documented in seven-week old plants with decreased expression of apyrase. **(B)** Morphological analysis of flowers in ten-week old plants: reduced apyrase by RNAi led to decrease style length (red arrow) due to curling (yellow arrow), while increased apyrase by overexpression led to exerted stigmas (green arrow). **(C)** Lines with decreased apyrase expression grown for ten weeks in a phytotron produced a higher number of smaller tubers showing a rod like shape. The figure panels show typical examples. For coding of the lines see Fig. 2.

Lines with increased expression of apyrase under the control of the constitutive 35S-promoter showed changes in flower development that are opposite to the changes achieved in lines with decreased expression of apyrase

Plants with increased expression of apyrase under the control of the constitutive 35S-promoter exhibited no significant changes in overall plant growth or tuber development, compared to wild type. However, increased expression of apyrase led to changes in flower development that were opposite to the changes achieved in response to decreased expression of apyrase. In addition, flowering was significantly accelerated, with more flowers/buds emerging after 49 days of plant growth (Fig 2C.), and the formation of secondary flowers/buds was significantly advanced at day 56 (Fig. 2D). Also the stigmata were remarkable exerted to grow to a more distal position from the anthers (Fig. 3B. green arrow). Consequently, the stigmata present little chance of self-pollination, but a much higher probability of getting cross-pollinated by insects. These changes clearly correlated with apyrase activity even in line 35S:143, which had consequently less flowers/buds after 49 days, underlining that changes in flower development are a direct feature of altered apyrase expression.

Lines with decreased expression of apyrase under the control of the tuber specific B33-promoter showed increased longitudinal tuber growth and starch content

To investigate, whether tuber development was specifically affected by a decrease in apyrase activity in the growing tuber, as opposed to pleiotropic changes as a consequence of its reduced expression in other plant tissues, further transgenic lines were generated that expressed the apyrase-RNAi chimera under the control of the tuber-specific B33 patatin promoter. Following screening of the resultant transformants, four lines were selected that displayed a strong decrease in both tuber apyrase mRNA and activity with respect to the wild type (see Fig. 4A and B). As would perhaps be expected, there was no substantial decrease in shoot growth when apyrase expression was decreased using the B33-promoter (data not shown), since this promoter has been demonstrated to confer tuber-specific expression (Liu et al., 1990). However, the total tuber number per plant was slightly increased in the transformants (Fig. 4C). Furthermore, tuber morphoplogy was altered in the tissue-specific lines in a similar manner to that described above for the lines exhibiting constitutive repression of apyrase expression (compare Fig. 4E with Fig. 3C). When taken together, these data provide strong evidence that the changes in tuber morphology are directly linked to alterations in the tuber apyrase activity rather than consequences of pleiotropic effects of the modulation of apyrase activity in other parts of the plant. The data also indicate that the influence of apyrase on tuber number may depend on the timing of apyrase silencing.

There was also an increase in the starch content of the tubers reaching 115-117% of the wild-type starch content in the strongest line in two independent trials (Fig 4D). This increase was also significant for line B33:10 in the second trial. Due to an increase in tuber mass per plant, the total starch content per plant was also increased significantly to a level of 123-142% in these two lines in both trials (data not shown). In contrast to starch, the reduction of apyrase activity did not substantially affect the relative contents of total protein (data not shown) or of cell-wall components such as cellulose, and uronic acids (see Supplemental Fig. S4A and B). Also the composition of cell-wall matrix polysaccharides was unchanged in the transgenic lines, providing evidence that cell-wall matrix architecture was not affected by the change in apyrase activity (see Supplemental Fig. S4C). Since uronic acids and cell wall matrix polysaccharides are produced in the Golgi, the lack of changes in these components argues against the possibility that potato specific apyrases act as latent UDPases functionally linked to the production of such cell wall structures in the Golgi as has been proposed for a *Pisum sativum* apyrase (Orellana et al., 1997; Neckelmann and Orellana, 1998).

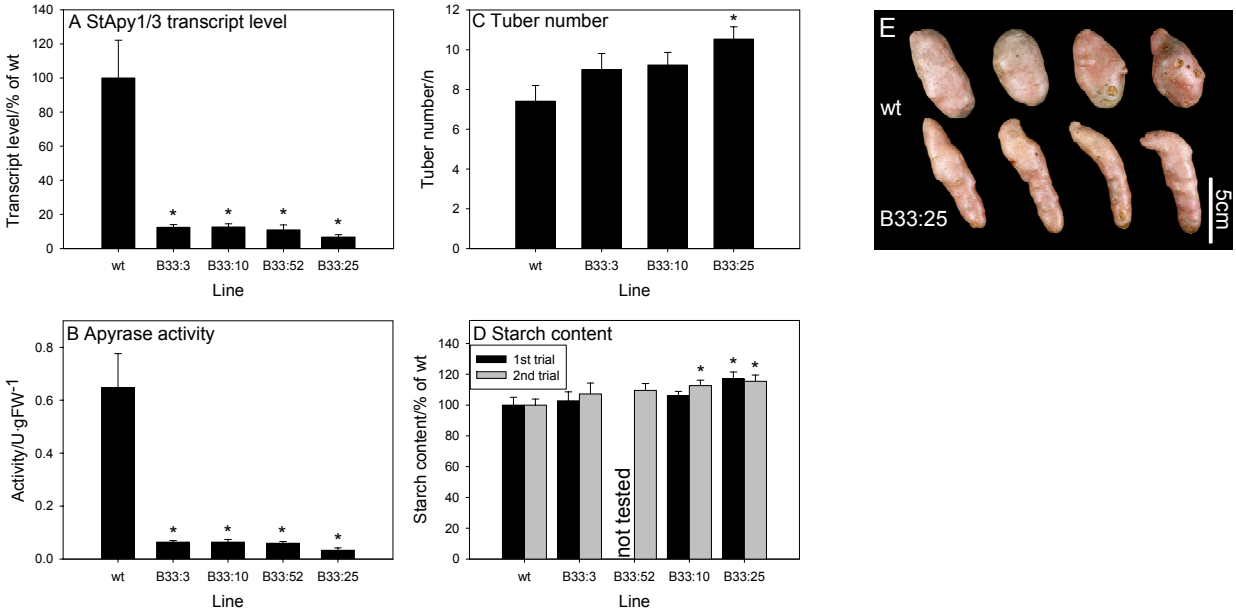

Fig. 4. Altered apyrase transcript and activity under the control of the tuber-specific B33-promoter affects tuber phenotype and starch content. (A) Combined transcript levels of *StApy1* and *StApy3* in tubers in tubers of eight week old plants were determined from cDNA by Real-Time-PCR and expressed as % of the wild type. **(B)** Apyrase activity was analysed using an assay containing 1 mM ATP as substrate and displayed in units per g freshweight. **(C)** Tuber number and **(D)** starch content in two independent trials were determined in tubers from 13 week old plants. There were characteristic changes in **(E)** tuber morphology, which were similar to those observed in lines where apyrase was decreased using the constitutive 35S-promoter (see Fig. 3). Results are means ± SE, *n* = 8 for **(A and B)** and 12-15 for **(C and D)**. Panel **(E)** shows a typical example. Significant differences to the wild-type according to the student's t-test are indicated with asterisks ($p < 0.05$).

Decreased expression of apyrase under the control of the tuber-specific B33-promoter did not lead to substantial alterations in metabolite levels

To investigate whether the changes in tuber development and starch content in the transformants are attributable to changes in metabolite levels, the metabolite profiles were analyzed in growing tubers using HPLC and GC-MS. There were no significant changes in the levels of adenylate pools or adenylate-related parameters for cellular energy charge in the different genotypes, with exception of the concentration of AMP in line B33:3 (see Supplemental Fig. S5). The other uridylates and guanylates (UTP/UDP/UMP/GTP/GDP/GMP) measured by HPLC were also unaltered in comparison to the wild type (data not shown).

Although steady-state levels of nucleotides were unchanged, we postulated that reduction of apyrase may have lead to a decline in the turnover of nucleotides. Such a scenario would be anticipated to result in lowered respiration rates, since it would entail lower demand for respiratory ATP production. For this reason oxygen consumption of tubers was determined

by transferring freshly cut tuber-slices into a Clarke-type electrode. However, this parameter was largely unaltered in the transformants (see Supplemental Fig. S6), suggesting that modification of apyrase did not greatly affect overall cellular ATP turnover.

Systematic profiling of metabolites using a GC-MS-based method failed to reveal major changes in metabolite levels in the transformants (see Supplemental Table S2). Furthermore, even minor changes were not statistically significant following correction of the p-values returned using the false discovery rate method (Benjamini and Hochberg, 1995) implemented in the R software (n = 8). This observation has important implications since it suggests that the changes in growth caused by reduction of apyrase are not primarily due to an effect of altered apyrase activity upon metabolism.

Decreased expression of apyrase under the control of the tuber-specific B33 promoter leads to changes in transcript profiles

To investigate whether the changes in tuber development and starch content in response to the decreased apyrase activity are attributable to broader changes in gene expression, transcript profiles were analyzed in growing tubers using the TIGR 10k EST potato array. This array contains 12091 cDNAs in replicates and covers approx. 25% of the 46000 potato genes found so far (Canadian Potato Genome Project, 2007, http://www.cpgp.ca/). Four arrays were analyzed that were hybridized with two independent tuber samples from the line B33:10 versus wt samples and two independent tuber samples from line B33:25 versus wt samples. The p-values obtained after normalization were corrected for multiple testing using the FDR algorithm implemented in the R software (The FDR corrected p-values are subsequently referred to as q-values). In some cases several clones of one gene were spotted on the array. After elimination of the redundant clones, 11375 individual genes derived from unique tentative consensus sequences remained. Of these, 540 genes (= 4.7% of all genes) were differentially regulated. Only 58 of them were downregulated compared to 481 genes that were upregulated, showing that reduced apyrase activity leads to a preferential activation of genes. Of the upregulated genes, 48 showed a greater than two-fold induction, however no repression of corresponding magnitude was observed in the downregulated genes. Using a recently developed MapMan mapping file (Rotter et al., 2007), all genes were sorted into bins arranged according to functional ontologies. This also allowed visualization of the genes involved in general metabolism using the MapMan software (Thimm et al., 2004) (Fig. 5). In addition, all of the genes exhibiting a greater than two-fold change were manually explored for tentative functions using BLASTN (Altschul et al., 1997), in attempt to identify homologs. This facilitated the association of several previously unassigned genes to a functional ontology. However, 18 such genes remained unidentified

and are thus listed as coding for hypothetical/expressed proteins. Three genes had contradictory 5'- and 3'-EST sequences derived from the clone spotted on the array. The inconsistency between the 5' and the 3' end of a clone is probably caused by chimeras consisting of more than one cDNA in one plasmid. The remaining 27 genes are listed in Table 1.

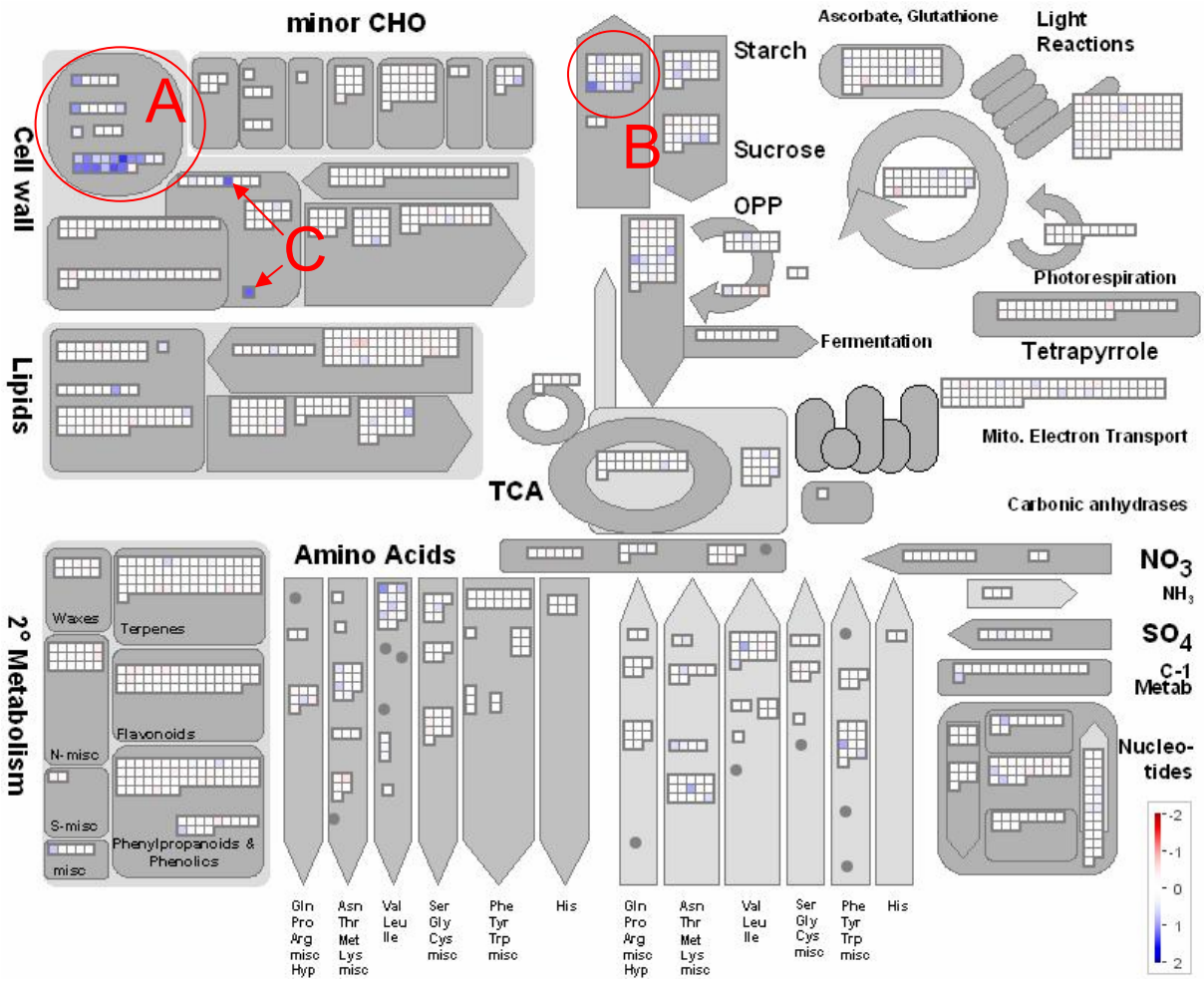

Fig. 5. Tubers with decreased apyrase under the control of the tuber-specific B33-promoter show specific changes in their transcript profiles compared to wild-type. RNA was extracted from growing tubers of separate plants from the wild-type and lines B33:10 and B33:25 and converted into dye-labeled second strand cDNA. The second strand DNA was hybridized together with complementary labeled wild-type second strand cDNA on the TIGR 10K potato array giving four hybridized arrays in total. Within the two replicate arrays for one of the two lines a dye-swap was performed. Data are shown a a snapshot of the metabolic overview slide of MapMan (Thimm et al., 2004), using a recently developed mapping file (Rotter et al., 2007). Changes in transcript levels coding for **(A)** extensins and related cell wall proteins, **(B)** genes involved in starch synthesis, and **(C)** glycosyl-transferases are highlighted.

Table 1. Summary of differentially regulated transcripts in tubers exhibiting decreased apyrase expression under the control of the tuber-specific B33-promoter, in comparison to wild-type. Only genes with tentative function that passed the t-test corrected for multiple comparisons using the false discovery rate (FDR) and which are at least two fold changed compared to wild-type are displayed. Genes were assigned into bins as suggested by the MapMan mapping file for the TIGR 10k potato array developed by Rotter et al. (2007). Transcript changes are given in $\log_2$ scale and the q-value corresponds to the p-value corrected for multiple comparison. Sequence information of the spotted clones can be retrieved using the identifier from GenBank or the TIGR Solanaceae Genomics Resource. If significant induction was observed on alternative spots on the array coding for the same gene, their identifier is given in brackets.

Bin	Identifier/Clone	log2	q-value	Description
amino acid metabolism.synthesis.branched chain group.common	STMHX23	1.06	0.0010	homologue to UP|Q9SM58_PEA (Q9SM58) Acetohydroxy acid isomeroreductase precursor , partial (36%)
cell wall.cell wall proteins	STMIJ81	1.23	0.0016	similar to UP|Q02021_LYCES (Q02021) Glycine-rich protein, partial (97%)
cell wall.cell wall proteins.AGPs	STMCJ47	1.02	0.0071	homologue to UP|Q52QJ6_LYCES (Q52QJ6) cell wall attached arabinogalactan/Proline rich protein, partial (98%)
cell wall.cell wall proteins.HRGP	STMJK30 (STMHU57)	1.02	0.0118	similar to TIGR_Ath1|At1g53645.1 68414.m06102 hydroxyproline-rich glycoprotein family protein , partial (47%)
cell wall.cell wall proteins.proline rich proteins	STMGX29	1.05	0.0063	similar to UP|Q40402 (Q40402) N.plumbaginifolia extensin (ext) precursor, partial (28%)
	STMIA95	1.08	0.0033	homologue to UP|Q40402 (Q40402) N.plumbaginifolia extensin (ext) precursor, partial (33%)
	STMHV77	1.17	0.0033	similar to UP|Q6QNA3 (Q6QNA3) Proline-rich protein 1 (Extensin), partial (92%)
	STMID50 (STMIK90)	1.21	0.0016	homologue to UP|Q41707 (Q41707) Extensin class 1 protein precursor, partial (56%)
	STMJI39	1.12	0.0029	homologue to UP|O82066 (O82066) Proline-rich protein (Extensin), partial (91%)
	STMJN80	1.21	0.0016	homologue to L.esculentum extensin (class I) gene (partial 76%)
	STMHZ32	1.45	0.0010	homologue to UP|Q09084 (Q09084) Extensin (Class II) precursor, partial (26%)
	STMIR05	1.33	0.0011	Solanum tuberosum mRNA for extensin
	STMIG94 (STMIH94)	1.25	0.0012	homologue to UP|Q43504 (Q43504) Extensin-like protein Dif10 precursor (Fragment), partial (24%)
	STMIK16	1.02	0.0026	similar to UP|Q43504 (Q43504) Extensin-like protein Dif10 precursor (Fragment), partial (21%)
cell wall.pectin synthesis	STMHQ60	1.26	0.0041	similar to TIGR_Ath1|At3g25140.1 68416.m03139 glycosyl transferase family 8 protein, partial (88%)
misc.UDP glucosyl and glucoronyl transferases	STMHU42 (STMJB73)	1.19	0.0062	similar to TIGR_Ath1|At5g60700.1 68418.m07617 glycosyltransferase family protein 2, partial (16%)
	STMHQ80	1.25	0.0101	similar to TIGR_Ath1|At1g18580.1 68414.m02317 glycosyltransferase family protein 8
major CHO metabolism.synthesis.starch.transporter/ transport.metabolite transporters at the mitochondrial membrane	STMDR46 (STMDI11) (STMGA51) (STMGJ88) (STMHK79)	1.20	0.0010	UP|ADT2_SOLTU (P27081) ADP,ATP carrier protein 2, mitochondrial precursor (ADP/ATP translocase 2) (Adenine nucleotide translocator 2) (ANT2) (Fragment), complete
redox.thioredoxin	STMHQ72	1.21	0.0010	homoloque to plastidial thioredoxin reductase (cNTR) AT2G41680
protein.degradation.ubiquitin.E3.RING	STMGI84	1.03	0.0122	similar to TIGR_Ath1|At1g68070.1 68414.m07776 zinc finger (C3HC4-type RING finger) family protein.
protein.synthesis.misc ribosomal protein	STMJN92 (STMDU75)	1.02	0.0016	homologue to UP|RS18_ARATH (P34788) 40S ribosomal protein S18, complete
RNA.processing.splicing	STMGI77	1.08	0.0190	similar to TIGR_Ath1|At5g51300.1 68418.m06359 splicing factor-related, partial (26%)
RNA.RNA binding	STMIY63	1.42	0.0071	similar to TIGR_Ath1|At3g13224.2 68416.m01658 RNA recognition motif -containing protein, partial (59%)
signalling.G-proteins	STMEF69	1.07	0.0067	similar to TIGR_Ath1|At5g27540.1 68418.m03297 GTP-binding protein-related, partial (39%)
signalling.unspecified	STMIM39	1.27	0.0024	TIP41-like family protein, contains Pfam PF04176: TIP41-like family; identical to cDNA putative cytoskeletal protein mRNA, partial cds GI:5031529
stress.abiotic.cold	STMIJ15	1.10	0.0016	similar to UP|GRP2_NICSY (P27484) Glycine-rich protein 2, partial (95%)
stress.abiotic.heat	STMJJ69	1.22	0.0067	similar to TIGR_Ath1|At1g74250.1 68414.m08599 DNAJ heat shock N-terminal domain-containing protein, partial (97%)

Almost half of the genes displaying a two-fold increase in transcript levels code for apoplastic proteins belonging to the class of extensins or related proteins (see Table 1 and Fig. 5A). Extensins are hydroxyproline-rich proteins which are highly *O*-glycosylated at the hydroxyproline residues. They are structural components of the cell wall with signaling

function and have been implicated in many developmental processes and various biotic and abiotic stress responses (Baumberger et al., 2001; Hall and Cannon, 2002; Merkouropoulos and Shirsat, 2003). Extensins are also involved in reproductive processes. They are enriched in the pistils and are postulated to interact with the male gametophyte during pollen tube growth. These interactions are not restricted to physical contact, but are believed to mediate cell-to-cell communication that ensures the correct growth of the pollen tube (Wu et al., 2001).

Decreased apyrase led to increased levels of transcripts involved in the provision of carbon and energy for starch synthesis in the amyloplast (see Fig. 5B). Sucrose-synthase activity is known to be crucial for starch synthesis and is induced at an early stage of tuber development to efficiently convert sucrose to UDP-glucose and fructose (Geigenberger, 2003). Both Sucrose-synthase genes 2 (STMDV33, $\log_2 = 0.55$, $q = 0.0279$) and 4 (STMHF04, $\log_2 = 0.85$, $q = 0.0018$) were induced in response to the decreased expression of apyrase. Also the genes coding for the mitochondrial ATP/ADP translocator 2 (ANT2, see Table 1) and ANT1 were significantly upregulated (STMDW96, $\log_2 = 0.76$, $q = 0.0010$) as well as the plastidic ATP/ADP translocator 1 (AANT1, STMGG40, $\log_2 = 0.60$, $q = 0.0399$) as were the glucose-6-phosphate translocator 1 (STMJF75, $\log_2 = 0.66$, $q = 0.0066$) and 2 (STMEC08, $\log_2 = 0.59$, $q = 0.0429$). These changes are in agreement with previous studies showing that the activity of the plastidic ATP/ADP-translocator and the plastidic glucose-6-phosphate transporter positively correlates with starch content in potato tubers (Tjaden et al., 1998); (Zhang et al., 2006). The nuclear encoded subunit of the key enzyme of starch synthesis, ADP-glucose-pyrophosphorylase (AGPase), which has a high control coefficient over starch synthesis in potato tubers (Sweetlove et al., 1999; Geigenberger et al., 2004), is significantly induced (STMDW17, $\log_2 = 0.71$, $q = 0.0422$). There was also a strong increase in transcripts coding for a plastidial thioredoxin reductase (cNTR, Table 1) in response to reduced apyrase. Recently, the post-translational redox regulation of AGPase has been found to be a powerful mechanism in the regulation of starch synthesis in the plastid (Tiessen et al., 2002); Kolbe et al., 2005).

Although no influence on the composition and amount of cell wall matrix sugars or uronic acids was found (see Supplemental Figs. S4B and C), two genes coding for glycosyltransferases were strongly upregulated in response to a decrease in apyrase (see Table 1 and Fig. 5C). A third, potentially upregulated, galactosyltransferase was rejected from the list because the spotted clone had contradictory 5'- and 3'-sequences (see above).

Investigation of the subcellular localization of potato apyrase

The lack of significant changes in nucleotide pool levels upon reduction of apyrase indicate that the potato apyrase resides in a compartment that is physically separated from the major nucleotide pools of the cell – those found in the cytosol, mitochondria and plastid. Unfortunately, the subcellular localization of apyrases in plants is still a matter of debate. The apyrases *GS52* from *Glycine max* and *Db-LNP* from the legume *Dolichos biflorus* were found to be localized to the cell membrane (Day et al., 2000; Kalsi and Etzler, 2000), while evidence has been provided that another, structural similar, apyrase termed *GS50* is localized in the Golgi apparatus of *Glycine max* (Day et al., 2000). In Arabidopsis, apyrases have been identified by mass spectrometry, only in membrane fractions containing Golgi proteins (Neckelmann and Orellana, 1998; Dunkley et al., 2004). Controversially, Dunkley et al. have suggested that *ATAPY2* is Golgi localized, whilst Roux and co-workers suggested it to be localized in the cell membrane with the active site facing the apoplast (Steinebrunner et al., 2003). In contrast to the apyrases from other plant species, potato specific apyrases are soluble when extracted under native conditions (Fig. 2B and 4B). This solubility is probably not an artefact of proteolytic cleavage, because the protein migrates at predicted size independently whether extracted under native conditions (Supplemental Fig. S3, lane A) or with Lämmli buffer containing high concentration of ionic detergent (Supplemental Fig. S3, lanes B-D). However, given the crucial importance of this question, we decided to investigate the subcellular localization of potato apyrase by following several different approaches in parallel.

Determination of apyrase in fractionated tuber tissue. Potato tuber tissue can be fractionated using a non-aqueous fractionation technique to analyze the subcellular localization of a specific protein or metabolite using marker enzymes (Farre et al., 2001; Tiessen et al., 2002). To investigate the distribution of apyrase, six gradients consisting of five different fractions each were analyzed, and the distribution of apyrase activity was compared with the distribution of the activities of several marker enzymes in these fractions such as UGPase (cytosol), AGPase (plastid), citrate synthase (CS, mitochondria) and mannosidase (vacuole + apoplast).

The pattern of apyrase activity was substantially different from the pattern of the cytosolic, plastidial or mitochondrial marker activities (Fig. 6A-E). Thus it is unlikely that apyrase is localized in the cytosol, plastids or mitochondria. The high recovery rate of apyrase activity (100 ± 20% for all gradients, data not shown) also excludes that ATP hydrolysis was carried out by another phosphatase than apyrase. The vacuolar/apoplastic marker mannosidase shows a similar pattern like apyrase and correlated highly significantly with apyrase in its

distribution in the representative gradient 11a (Fig. 6A) and all five gradients tested additionally (R = 0.977, p < 0.0001, n = 30, see Fig. 6B). At the moment it is not possible to distinguish between vacuole and apoplast in the non-aqueous gradients due to cofractionation of apoplastic and vacuolar markers see (Fettke et al., 2005). It can therefore not be determined with this method, whether apyrase is localized in the apoplast or in the vacuole.

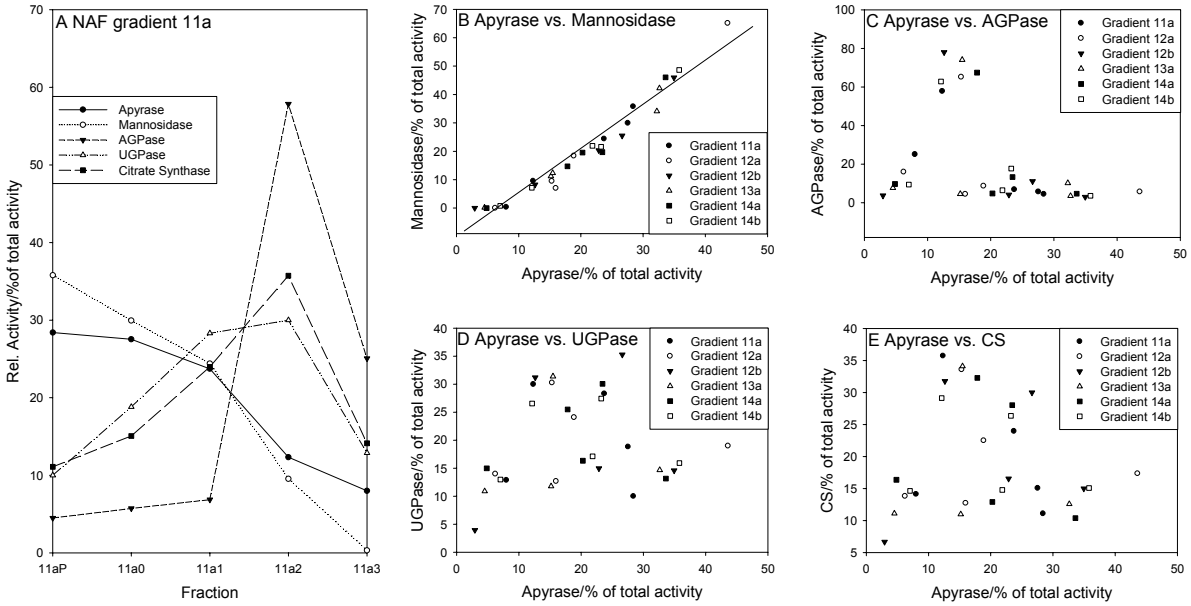

Fig. 6. Comparison of apyrase and marker enzymes in non-aqueous gradients of lyophilized wild-type tuber tissue. (A) The activities of subcellular marker enzymes and apyrase are shown along a typical gradient consisting of five fractions with increasing density from right to the left. Correlations between apyrase and the markers for vacuole and apoplast (mannosidase, **B**), plastid (AGPase, **C**), cytosol (UGPase, **D**) and mitochondria (citrate synthase, CS, **E**) were calculated from activities obtained from six individual gradients. The same non-aqueous gradients were used that were characterized previously (Tiessen et al., 2002).

Transient expression of apyrase-GFP in leaf epidermis cells. The data obtained from the NAF-gradients suggest vacuolar or apoplastic localization for apyrase. To distinguish between these possibilities, localization was further investigated by the use of an apyrase-GFP fusion protein. Potato apyrase *StAPY3* was cloned into pA7-GFP (Sohlenkamp et al., 2002); (Voelker et al., 2006); see also Supplemental Fig. S2D). The resultant plasmid was able to code for potato apyrase C-terminal fused in frame to enhanced GFP. A preparation of the plasmid coding for the apyrase-GFP fusion protein was loaded onto gold particles and shot into *Arabidopsis thaliana* leaves with a biolistic gun. After two days incubation at 25°C, the leaves were examined with a confocal microscope and images of cells temporarily expressing apyrase-GFP were recorded. As shown in Fig. 7A, C and D, GFP accumulates in a layer that is surrounding particular epidermis cells of the bombarded leaves. The signal is

diffusely spread in the apoplastic space, and can be clearly distinguished from the auto-fluorescence of the chloroplasts recorded at a higher wavelength (Fig. 7B). This shows that apyrase is localized in the apoplast rather the vacuole. Another control plasmid coding for the membrane-localized ammonium-transporter *AtAMT2* fused to GFP (Sohlenkamp et al., 2002) was also shot into leaves,however, the fluorescence pattern of this transporter was clearly different to the diffusive signal of StApy3-GFP and restricted to the plasma membrane of a single cell instead (Supplemental Fig. S7).

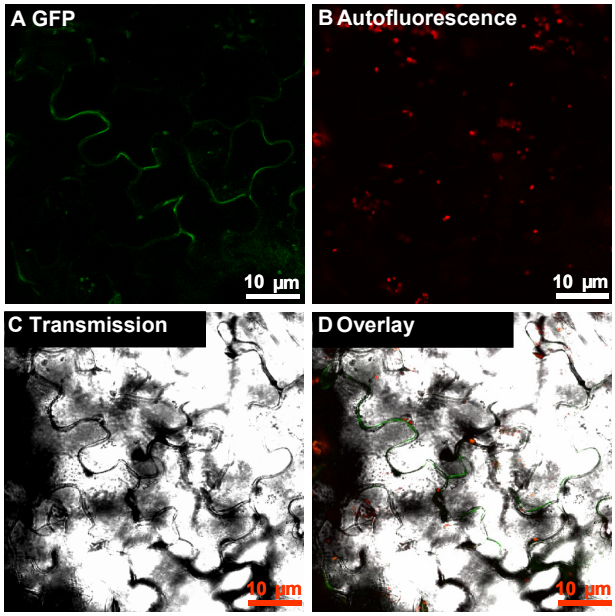

Fig. 7. Localization of a temporally expressed StAPY3-GFP fusion protein. Arabidopsis leaf epidermal cells were bombarded with a plasmid coding for *StAPY3* N-terminal fused with GFP. **(A)** Fluorescence of GFP recorded between 500-525 nm. **(B)** Auto-fluorescence of the chloroplasts recorded between 630-690 nm. **(C)** Bright field image of the section. **(D)** An overlay of all three images (given in A-C). Typical examples are shown.

Determination of apyrase activity in apoplastic washing fluid (AWF) from leaves of potato wild type plants. If apyrase is a soluble apoplastic protein or easily detachable from an apoplastic component, it should be possible to detect its activity in apoplastic washing fluid (AWF). This method has been already used not only for the determination of apoplastic metabolites, but also for the extraction of apoplastic proteins (Fecht-Christoffers et al., 2006); (Boudart et al., 2005). Pools of AWF of leaves from eight six-week old wt potato plants with reasonable low activity of UGPase - a soluble cytosolic enzyme with high activity and an almost identical molecular mass to that of apyrase - were prepared to analyze apyrase activity. In parallel, total cellular enzyme extracts from leaves of all eight plants were also prepared for comparison. The AWF contained less than 3% of specific UGPase-activity compared to that contained in whole leaf extracts (Fig. 8A), which is indicative of a relatively low level of contamination of the AWF with cytosolic proteins. In contrast, the AWF contained 13-times higher values of specific apyrase activity than the whole leaf extracts (Fig. 8B). These data give clear evidence for the existence of apoplastic apyrase in potato leaves. This apyrase

turns over ATP, UTP and ADP with the same speed, UDP is hydrolyzed almost twice as fast as the other nucleotides. Hydrolysis of AMP and UMP was almost undetectable (Fig. 8C). The highly specific turnover of tri- and diphosphates but not of the monophosphate in the AWF indicates that the phosphatase activity measured was carried out by an apyrase and not by an unspecific phosphatase. Apyrase measured in the whole leaf preparations displayed different kinetic properties than the enzyme in the AWF. UTP and ADP were hydrolyzed twice as fast as ATP, UDP even four times as fast (Fig. 8D). There was a substantial hydrolysis of AMP and UMP, indicating apyrase independent turnover of nucleotides by unspecific phosphatases in the whole leaf extract. When the turnover of unspecific nucleoside-monophosphate hydrolysis was subtracted from the hydrolysis of tri- and diphosphates to provide a more specific measure of apyrase activity, the differences between the turnover rates for the respective nucleoside tri- and diphosphates were even more pronounced.

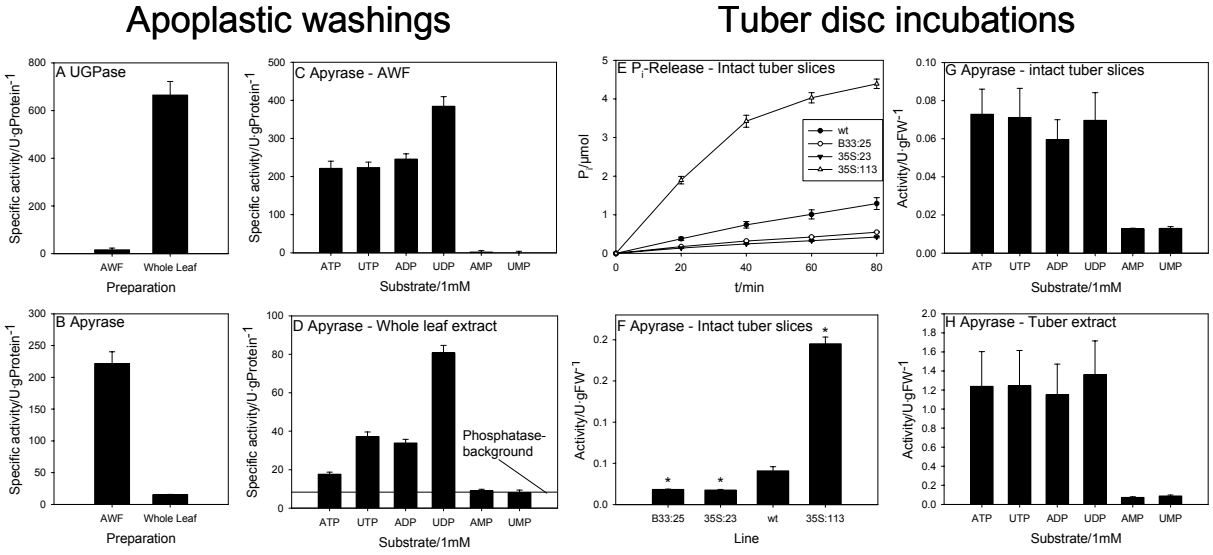

Fig. 8. Apyrase activity in apoplastic washings and in tuber slices incubation experiments. (A-D) To investigate the subcellular localization of apyrase, the specific activities of **(B)** apyrase (1 mM ATP as substrate) and of the cytosolic marker enzyme **(A)** UGPase were determined in apoplastic washing fluid (AWF) obtained by centrifugation of infiltrated wild-type potato leaves, and compared with the activities assayed in whole leaf extract obtained from the same plants. Substrate specificity of apyrase/5'-nucleotidase was assayed in both **(C)** apoplastic washing fluid and **(D)** whole leaf extract**,** using 1 mM ATP, UTP, ADP, UDP, AMP or UMP. The horizontal line indicates the level of non-specific phosphatase activity on the basis of nucleoside monophosphate hydrolysis. **(E-H)** Experiment with tuber slices: Freshly prepared and washed tuber slices of wild-type, B33-RNAi line B33:25, 35S-RNAi line 35S:23, and 35S-overexpressor line 35S:113 were incubated with 3 µmol ATP in 3 ml buffer in a final concentration of 1 mM to determine apoplastic apyrase activity as **(E)** release of Pi from ATP in a time course or **(F)** in absolute activities per gFW. **(G)** Substrate specificity of apyrase/5'-nucleotidase in intact tuber slices and **(H)** in total tuber enzyme extracts produced from wild-type plants using substrate concentrations of 1 mM are also shown. Results are means ± SE (*n* = 6-8). In Fig. F, significant differences to the wild-type according to the student's t-test are indicated with asterisks ($p < 0.05$).

Although it is possible that the enzyme might have been modified in one of the fractions or that the presence of effectors led to the differences in kinetic properties, these results suggest the existence of apyrases with different kinetic properties localized in different subcellular compartments in potato leaves. The leaf apoplastic apyrase possesses comparable relative turnover rates for the different purine and pyrimidine nucleotides tested. These kinetic properties also apply to the apyrases changed in activity in this study, and it is therefore highly likely that they are identical. A second leaf apyrase shows preference to the pyrimidine nucleotide UDP. From the experiment performed, no clear conclusions can be drawn concerning its localization with the exception of the fact that we can rule out that it is not a soluble apoplastic protein. Indeed, its substrate preference suggests it is more likely to be the Golgi localized latent UDPase, the apyrase potentially involved in the nucleotide sugar transfer (Orellana et al., 1997; Neckelmann and Orellana, 1998).

Determination of apyrase activity in intact tuber slices of potato wild type plants and lines with altered apyrase activity. Since apoplastic washings cannot be performed with potato tubers, we used a different experimental approach to measure apoplastic apyrase activity using tuber slices. Tuber slices consist of compactly packed cells without physical barriers such as cuticles which can be kept in buffer for several hours without loss of cell-membrane integrity. In contrast to tuber cells in culture, a freshly prepared tuber slice represents a much more physiological relevant state of this tissue since it harbours naturally organized cell walls. For this reason, tubers from eight-week old wild-type and transgenic plants were sliced into discs. The slices were washed five times with buffer to withdraw intracellular contaminations and finally incubated in buffer. To determine apoplastic apyrase activity, ATP was added to the slices. After different time-intervals, aliquots were collected from the external medium and analyzed for apyrase activity by determining the conversion of the external ATP to P_i. By this method, apyrase activity of tuber slices of the strongest B33-RNAi line, the second strongest 35S-RNAi line (the strongest was incapable of producing suitable tuber material for analysis) and the strongest 35S-overexpressor was determined. As shown in Fig. 8E, apyrase activity could be measured as P_i formation in the medium containing the wild type tuber slices after addition of ATP. This release is linear with time indicating an enzymatic turnover of ATP at saturating substrate concentration rather than a leakage of P_i from damaged cells. The absolute activity determined approximates to 0.05-8 U/gFW for intact wild-type slices (Fig. 8F and 8G), which corresponds to in the region of 10% of total cellular activity (see Fig. 4B and 8H). In comparison to this, UGPase activity measured in washed intact slices did not exceed 0.3% of the activity in total enzyme extracts (data not shown).

The apyrase activities of intact tuber slices from the strongest B33- and the second strongest 35S-RNAi line (B33:25 and 35S:23) were significantly reduced to a level of 45 and 44% of the wild type activity, respectively (Fig 8f). By contrast, the apyrase activity was strongly elevated in the strongest 35S-overexpressor (Fig. 8E). The rate of hydrolysis of adenosine tri- and diphospates is apparently much higher than the rate of hydrolysis of the monophosphate in wild-type tuber slices (Fig. 8G). This finding, when taken together with the fact that apyrase activity in the discs of the different lines was altered according to the respective apyrase expression level, provides clear evidence that hydrolysis was carried out by an apyrase rather than an unspecific phosphatase. Furthermore, the maximal activities for the substrates ATP, UTP, ADP and UDP were identical, as was the case for apyrase characterized in tuber extracts from wild type (Fig. 8H) and also in tuber extracts from the strongest *StAPY3*-overexpressor 35S:113 (data not shown), and similar to those determined in the AWF from wild type potato leaves (Fig. 8C).

9.4 Discussion

Potato-specific apyrases may comprise a small gene family

The cloning of three very similar potato specific apyrases, one of them truncated, may suggest that these apyrases comprise a small gene family. Whether these different isoforms are encoded on different loci or are merely different alleles of the same gene cannot currently be confidently stated due to the high degree of heterozygosity and the tetraploid genome of potato. The fact that *StAPY2* and *StAPY3* were cloned using primers that bind to the UTR, which generally exhibits low conservation between different genes would favor the one gene different allele scenario. However, the existence of all three isoforms in the cultivar *Bintje* and a southern blot analysis showing several bands when probed with *StAPY3* (data not shown) provide strong support for the assumption of a small gene family. Additionally, two more apyrases were identified from TC-sequences (Supplemental table S1) that resemble more closely general plant apyrases like *AtAPY 1* and *2*, but their total number is probably higher because Arabidopsis already contains seven predicted apyrases (The Arabidopsis Information Resource). Potato specific apyrases are expressed predominantly in sink tissues like root, flower, stolon and tuber, but to a much lower extent, if at all, in leaf or stem tissue (Fig. 1A). When expression of the potato-specific apyrase was altered in transgenic plants, strong morphological alterations were found in sink tissues such as flowers or tubers. The RNAi approach led to the reduction of nearly all the apyrase activity in growing tubers,

indicating that these potato-specific apyrases encode for most of the apyrase activity in this tissue (Fig. 2B and 4B).

Potato apyrase is confined to the apoplast

To date most plant apyrases have been demonstrate or postulated to be localized at the cell membrane (Thomas et al., 1999);(Etzler et al., 1999); (Day et al., 2000); (Wu et al., 2007). However, several independent lines of evidence are presented indicating that the potato-specific apyrases characterized in this study are rather localized in the apoplast without strong interaction to structural components of the cell. Furthermore, these studies revealed that the potato-specific apyrases account for the majority of apyrase activity in this compartment. First, apyrase activity correlated strongly with the distribution of a vacuolar/apoplastic marker in tuber fractions separated on non-aqueous gradients. When taken in the context of the high contribution of the potato specific apyrases to the total tuber activity, this suggests that, of the major cellular compartments, the potato apyrases may be either localized in the vacuole or apoplast, but not in the cytosol, mitochondria or plastids. Secondly, the *StAPY3-GFP* fusion-protein was targeted to the apoplast, when transiently expressed in *Arabidopsis thaliana* leaves. While we cannot fully exclude mis-targeting of apyrase within the secretory pathway leading to apoplastic accumulation of the *StAPY3-GFP* fusion protein, it is very unlikely that the fusion protein was entering this path due to C-terminal fusion of GFP to apyrase since targeting sequences for this path are located at the N-terminus and were predicted for all three potato apyrases. Moreover, the strong sequence identity makes it very likely that all three potato apyrase will have the same topogenesis. Thirdly, apyrase activity was detected in the apoplast of tuber slices; showing identical substrate specificity as *StAPY3*, and being altered in response to *StAPY3* or general potato specific apyrase expression in a corresponding manner. Fourthly, apyrase activity was also demonstrated in apoplastic leaf washings, having almost identical substrate specificity to the apyrase analyzed in the apoplast of tubers but displaying different substrate specificity to the apyrase activity found in the total tissue extracts of potato leaves. The latter is showing high activity when UDP is supplied as substrate, making it quite likely that it corresponds to a latent UDPase that has been shown to by localized to the Golgi (Orellana et al., 1997). In combination, these independent experiments provide evidence that the potato-specific apyrases are apoplastically localized and suggest that they have no strong association to membranes of the cell.

Potato apyrase is crucial for development affecting the reproduction strategy of the plant

Changes in the expression of apyrase under the control of the constitutive 35S-promoter revealed strong effects of apyrase on plant development. A strong ubiquitous reduction of expression led to a reduction in growth partially dependent on the growth condition, with the strongest effect leading to the death of the line with strongest reduction in apyrase-mRNA when grown in an uncontrolled greenhouse (Fig. 3A) but more modest differences from the wild type when grown in a carefully controlled phytotron (Figs. 3B and C).

Our data provide evidence that apyrase affects the reproductive strategy of the plant. Apyrase activity influenced both flowering time and style development (Figs. 2C and D and 3B). Plants with reduced apyrase activity clearly flowered later and produced flowers with inserted curled styles (that inevitably will lead to self-pollination of the flower), whereas plants overexpressing apyrase flowered earlier and produced flowers with exerted stigmata (that have a higher probability of getting cross-pollinated). Thus reduced apyrase activity would be predicted to enhance autogamic mating whilst higher apyrase activity could be anticipated to promote allogamic mating. Plants with reduced apyrase and exhibiting allogamic mating type also produced a higher tuber number per plant. This would lead to more clonally-identical progeny. This phenotypical alteration follows the allogamic strategy observed for the flowers and implies a reproduction strategy that conserves the plant's gene pool.

Potato apyrase affects starch accumulation without changing the metabolite profiles in tubers

Reduction of apyrase using a tuber-specific promoter resulted in an increased starch accumulation (Fig. 4D) . Similar changes were observed in two independent trials. Very recent results by Wolf et al. (2007) also report the influence of apyrase on starch accumulation, although this point was not commented on by the authors of this article. It was shown that a conditional double knockout of *AtAPY1* and *2* had larger starch granules in the chloroplasts of *Arabidopsis thaliana* leaves. The increase in starch accumulation in the tubers was not caused by a higher overall adenylate energy state or changes in respiratory activity as a result of the potato-specific apyrase silencing (Supplemental Figs. S5 and S6), providing circumstantial evidence that this activity is not localized in a compartment with high metabolic activity. The overall impact on metabolic intermediates was low, and no significant changes in the levels of metabolites were found by metabolic profiling using GC-MS techniques (Supplemental Table 2). In the context of a lack of changes in metabolite levels, the increase in starch levels suggests regulation of the biosynthesis of starch at the transcriptional rather

than the post-transcriptional level (see also below). The function of potato starch is to serve as carbohydrate source when a sprout develops to form a new plant. The higher tuber starch content is therefore consistent with the strategy to increase vegetative reproduction in tubers. Taken together, the increase in tuber number and starch accumulation in response to decreased expression of apyrase can thus be regarded as a strategy to strengthen clonal reproduction.

Interestingly, reduction of apyrase did not alter cell wall composition (Supplemental Fig. S4). Previous studies revealed that the Golgi apparatus contains an apyrase that is presumably involved in the Golgi sugar transfer system (Orellana et al., 1997; Neckelmann and Orellana, 1998). However, the localization studies performed here did not support a role for the potato-specific apyrase in this system. To test a more direct evidence on participation of potato apyrase on Golgi sugar transfer, an analysis of the cell wall with particular focus on structural components assembled in the Golgi was conducted. This analysis revealed neither differences in the absolute amounts of uronic acids nor in the composition and absolute amounts of cell wall matrix sugars. These structures are probably the biggest sink of sugars transferred via the Golgi sugar transfer system. It therefore follows that if apyrase was part of this pathway then reduction of its activity should result in alterations in the cell wall matrix. The fact that this was not the case suggests it unlikely that the potato-specific apyrase is involved in this pathway.

Potato apyrase leads to characteristic changes in the expression pattern of nuclear genes

Although changes in apyrase expression did not affect metabolite profiles, they led to characteristic changes in the expression pattern of nuclear genes (Fig. 5 and Table 1). By far the most striking change was the large induction of transcripts coding for cell wall proteins belonging to the class of extensins and related proteins, which made up 13 out of the 27 genes displaying a considerable upregulation at the level of the transcript. We can rule out that this strong upregulation is caused by one or a few dominant extensin-cDNAs hybridizing to the spots coding for the other extensin genes, since all of the differentially regulated extensins are fairly different in nucleotide sequence (data not shown). Extensins represent a multi-gene family coding for cell wall proteins with structural and signaling function in the apoplast. As already mentioned, there is good correlative evidence for extensins being involved in wounding, biotic and abiotic stress responses, as well as in developmental processes such as pollen recognition and fertilization, embryo development, cell division, differentiation, abscission and senescence (Roberts and Shirsat, 2006). Unfortunately, little insight into the function of extensins has been obtained from more direct approaches such as

the use of mutants or transgenic plants with altered extensin expression. Two studies using Arabidopsis null mutants or overexpressors of the extensins *LRX* and *Atext1* showed that these manipulations had only weak effects on inflorescence growth and root hair morphogenesis (Baumberger et al., 2001; Roberts and Shirsat, 2006). A third study showed that the knock out of an Arabidopsis extensin encoded by the *RSH* gene is embryo-lethal (Hall and Cannon, 2002). In *Nicotinia* species the, 120 kDa extensin is needed for intra- and interspecific pollen rejection, thus also having influence on reproduction strategy like apyrase (Hancock et al., 2005). Microscopic studies of Baumberger et al. (2001) and Hall and Cannon (2002) revealed that disruption of the extensins *LRX* and *RSH* led to abnormal development caused by defective cell expansion and cell shape in the tissue affected. In both studies this was linked to a function of extensins in polarized growth. The authors assume that this polar growth may be achieved by extensins connecting the cell membrane with the cell wall at specific sites of growth.

Reduction of apyrase expression led to increased expression of several extensin genes and to pronounced morphological changes, most predominantly in tubers and flowers. There is not much else known about extensins in potato, other than that they are induced in response to wounding, hypoxia and infection with *Erwinia carotovora* (Rumeau et al., 1990). However, there is considerable evidence that extensins have a predominant function in flower development (Wu et al., 2001). The general impact of extensins on plant development and their strong enrichment in reproductive tissues may explain why morphological changes appeared to be most accentuated in flowers and tubers.

The majority of transcripts encoding genes with function in central metabolism were not affected by reduction of apyrase, consistent with the lack of changes in metabolite levels of intermediates of these pathways. Interestingly, many transcripts encoding proteins related to starch metabolism were altered in a manner that would promote starch synthesis. This involved both proteins in the pathway of sucrose to starch conversion such as sucrose synthase, the plastidial glucose-6-P translocator and ADP-glucose pyrophosphorylase (Geigenberger, 2003), but also proteins esoteric to the pathway with important functions to promote starch synthesis such as adenylate transporters in mitochondria and plastids and plastidial adenylate kinase (Geigenberger et al., 2004; Oliver et al., 2008). Interestingly, a plastidial form of a NADP-thioredoxin-reductase containing a thioredoxin domain (Serrato et al., 2004) was also found to be upregulated, which could be important for post-translational redox-regulation of starch synthesis (Tiessen et al., 2002; Kolbe et al., 2005). Although it was not investigated whether these changes in transcript abundance do lead to subsequent changes in the protein levels or activities, the sum of these changes would be in favor of an increase in starch production by transcriptional regulation at several sites in the pathway.

They therefore provide a potential explanation for the finding that starch accumulation is increased without major changes in metabolite levels in tubers of apyrase silenced lines.

A possible role of apyrase in the apoplast

Our present study provides evidence that potato apyrases might be components of a signaling pathway that regulates the expression of a number of extensins and other genes. Apyrase was found to be localized in the apoplast and a decrease in its activity led to increased expression of nuclear genes that encode proteins such as extensins with important cell-wall and signaling functions. The specific effects of altered apoplastic apyrase activity on gene expression rather then metabolism fits into models linking this activity to apoplastic signaling processes, analogous to mammalian systems (Chivasa et al., 2005; Kim et al., 2006; Song et al., 2006; Wu et al., 2007). In the study by Chivasa et al., an increase of apoplastic ATP by adding external (e)ATP increased the viability of the cell, while removal of eATP by external application of apyrase led to opposite effects. Moreover, visualization studies *in planta* show that the level of eATP is highest in actively growing regions, providing evidence that it has a major role to support growth (Kim et al., 2006). Our data show that a decrease in the expression of apyrase via RNAi leads to stimulation of longitudinal growth in potato tubers, which is governed by increased expression of extensins and other proteins that are suggested to play a role in polar growth (Baumberger et al., 2001); (Hall and Cannon, 2002).

The effect of a decrease in apyrase on growth and development may vary depending on the tissue or developmental stage. Crucially, while decreased apyrase under the control of a tuber specific promoter increased longitudinal growth in natural growing tubers (Fig. 4E), it obviously led to an inhibition of growth of the shoot when decreased under the control of a constitutive promoter (Fig. 2E and 3A). This may be attributable to the different cellular organization of the tissues, which is relatively simple in tubers and very complex in apical meristems, flowers and leaves. When apyrase is reduced in these complex developing tissues, this may disturb coordinated polar growth of the different cell types and ultimatively lead to severe growth defects. Consistent with this, decreased apyrase due to incubation with antibodies has been reported to lead to decreased growth of pollen-tubes, despite an increase in eATP, and there have been reports showing an overall reduction of growth in the shoot when apyrase was reduced by different genetic manipulations in Arabidopsis (Wolf et al., 2007; Wu et al., 2007). Further evidence for an influence of eATP on polar growth was provided by earlier studies of Tang et al. (2003), who showed that application of eATP to growth medium did not inhibit growth, but led to curled root growth and a loss of gravitropic control.

Whilst this study provides strong support for potato apyrases having a role in apoplastic signaling events, several open questions remain. Amongst these is the role (and properties) of purinergic receptors or other elements of a signaling pathway. It seems likely that genetics-based approaches in *Arabidopsis thaliana* will be the most appropriate way to tackle such a question. A second open question is – what is the fate of the hydrolyzed nucleotides in the apoplastic space? In an attempt to address this we are currently investigating the enzyme complement of the potato apoplast that is capable of converting AMP to products that are known to be transported across the cell membrane. However, irrespective of the exact details of how this signal transduction is mediated, the results presented here reveal an important role for the apoplastically localized potato-specific apyrase in growth and development in this important crop species.

9.5 Materials and methods

Cloning work

cDNA of StAPY2 and StAPY3: The coding sequences of *StAPY2* and *StAPY3* were amplified by PCR from potato tuber cDNA using the oligonucleotides flaypyf (5'-T CTT GGA TCC GGG GCA AAA TGT TGA ACC AAA ATA G-3') and flapyr (5'-A TTG GAA TTC CAA CAC ATT AAG ATG ATG CAA CTC-3'), both of them flanked with restriction sites. After digestion with *Bam*H1 and *Eco*RI, the PCR products were ligated into pBluescript SK+ (Stratagene, La Jolla, CA). Each isoform was cloned three times in three independent PCRs out of two different cDNA preparations and sequenced (AGOWA, Berlin, Germany).

gDNA of StAPY2: The genomic sequence of *StAPY2* was amplified by PCR from potato genomic DNA using the oligonucleotides allapyf (5'-CTC TTC AAA TAG GGG CAA AAT GTT GAA CC-3') and apy2UTR (5'-CAA GTT TCC CAC CAA TAC AAG TAC AAG ATT T-3') and ligated into pCR2.1 (Invitrogen, Karlsruhe, Germany). The fragment was cloned only once since exons did not diverge from the sequence of the cDNA of *StAPY2* according to the sequencing results.

35S-overexpressor: The full length sequence of *StAPY3* in pBluescript was amplified using the oligonucleotides RNAis2f (5'- T CTT CTC GAG GGG GCA AAA TGT TGA ACC AAA ATA G-3') and flapyr (see above), both of them flanked with restriction sites. After digestion with *Eco*RI and *Xho*I, the fragment was ligated into pART7 (Gleave, 1992). The cassette

containing 35S-promoter, *StAPY3* full length and OCS-terminator was excised using *Not*I and ligated into pART27 (Gleave, 1992).

35S-RNAi: The partial sequence of *StAPY3* from nt -8 to nt 489 was amplified from *StAPY3* in pBluescript using the oligonucleotides RNAis2f (see above) and RNAis2r (5'-ATC AGG TAC CGT GAC CCA TTG ATC TTT GCT ATG G-3'), both flanked with restriction sites. After digestion with *Xho*I and *Kpn*I the fragment was ligated in sense orientation into pHannibal (Wesley et al., 2001). The identical partial sequence was also amplified using the oligonucleotides RNAiaf (5'-A ACA GGA TCC CTT AGG ATG TTA AAA GGG GAT GCA GC-3') und RNAia2r (5'-ATC AAT CGA TGT GAC CCA TTG ATC TTT GCT ATG G-3'), both flanked with restriction sites. After digestion with *Bam*HI and *Cla*I the fragment was ligated in antisense orientation into pART7 containing the sense fragment. The cassette containing the 35S-promoter, the RNAi-construct and the OCS-terminator was excised using *Not*I and ligated into pART27.

B33-RNAi: Using *Bam*HI und *Xho*I, the RNAi-construct consisting of the *StAPY3*-sense and the antisense fragment comprising the *pdk*-intron was exised from pHannibal (see above) and ligated into pART33, a derivative of pART7 with a B33-promoter instead the 35S-promoter. The cassette containing the B33-promoter, the RNAi-construct and the OCS-terminator was excised and ligated into pART27 using *Not*I.

StAPY3-GFP: The sequence of *StAPY3* was amplified from *StAPY3* in pBluescript using the oligonucleotides RNAis2f (see above) and allapy1r (5'-CAA CGT CGA CAG ATG ATG CAA CTC TAA TTT TG-3'), both flanked with restriction sites. After digestion with *Xho*I und *Sal*I the full length fragment without stop codon was ligated into dephosphorylated pA7-GFP. The coding sequence of *StAPY3* of this plasmid was sequenced to guarantee that it was accurate.

Sequence analysis

Alignments were produced using the ClustalW algorythm embedded in the MegAlign software (DNAstar, Madison, WI, USA). This software was also used for sequence identity calculations using identity weight matrix. For Homology searches, the GenBank database was explored using BLASTN (Altschul et al., 1997).

Generation of transgenic potato plants

The plasmids were transformed into *Agrobacterium tumefaciens* strain *pGV2260* using a gene pulser electroporator (Biorad, Munich, Germany) as described by Mattanovich et al. (Mattanovich et al., 1989). The recombinant *Agrobacteria* were used to transform sterile,

freshly injured leaves of *Solanum tuberosum L. cv. Desiree* by the method established by Rocha-Sosa et al. (1989).

Plant growth conditions

Potato plants *cv. Desiree* were cultivated in tissue culture for long term storage and propagation. Cuttings were transferred to soil and grown in a phytotron for 2 weeks. Then the plants were transferred to pots with a diameter of 18 cm and grown either in the phytotron (soil, 60/75% humidity d/n, 22/16°C d/n, 400 $\mu mol \cdot s^{-1} \cdot m^2$ light intensity, 16/18h d/n), a conditioned glasshouse (soil, 60% humidity 22/16°C d/n, 350 $\mu mol \cdot s^{-1} \cdot m^2$ light intensity, 16/18h d/n), or in an uncontrolled greenhouse.

Northern Blot analysis

After separation of 20 µg RNA derived from different tissues on a denaturing agarose gel and transfer on a membrane (Hybond-XL, APBiotech), the membrane was probed with the full length fragment of *StAPY3* labelled with ^{32}P. Hybridization was detected using a BAS-1800II phosphoimager (Fuji, Düsseldorf, Germany).

Western Blot analysis

After separation of 5 µg tuber protein using PAGE and transfer on a PVDF membrane (Roche, Mannheim, Germany), the membrane was probed with a serum directed against potato apyrase kindly provided by Dr. Pal Nyren (Royal Institute of Technology, Stockholm, Sweden). Binding was detected enzymatically using a secondary antibody conjugated to horseradish peroxidase (Biorad, Munich, Germany).

Real-Time-PCR

RNA was extracted from 60 mg FW using the RNeasy Plant Mini Kit (Quiagen, Hilden Germany) and DNA was digested as suggested by the supplier. 500 ng RNA were used to produce cDNA in a final volume of 100 µl using reverse transcriptase and RNase inhibitor provided by Invitrogen (Karlsruhe, Germany). For cDNA quantification, 2 µl were used as template in a Real-Time-PCR-reaction mixed with 10 µl Power SYBR-Green (Applied Bioscience, Warrington, UK) and 10 µl primer mix (forward and reverse primer, each 0.5 µM). The following primer mixes were used for quantification: *StAPY1* and *StAPY3* (forward: 5'-GCT TGT TGA TGG ATT TGG ACT AAA-3', reverse: 5'- GC CAT GCT GCT CCA ACT AGA

TAG -3'), *StAPY2* (forward: 5'-GCT TGT TGA TGG ATT TGG ACT AAA-3', reverse: 5'- GC CAT GCT GCT TTA ATT TGG TAA -3'), *StGAPDH5'* (forward: 5'-AAG GAC AAG GCT GCT GCT CAC-3', reverse: 5'-AAC TCT GGC TTG TAT TCA TTC TCG-3') and *StGAPDH3'* (forward: 5'-TTC AAC ATC ATC CCT AGC AGC ACT-3', reverse: 5'-TAA GGT CGA CAA CAG AAA CAT CAG-3'). *EF1-*α (forward: 5'- CAT TGC TTG CTT TCA CCC TTG GTG -3', reverse: 5'- CCT AGC CTT GGA GTA CTT GGG GG -3'). The amount of cDNA of the induced apyrase was related to the amount of *EF1-*α. Only samples with equal ct-values (±1) for the GAPDH cDNA 5'- and 3'-ends were considered for analysis.

Expression profiling using the TIGR 10k potato array

RNA extraction, cDNA synthesis, labeling, hybridization and scanning were performed as described previously (Degenkolbe et al., 2005). This method includes quality control of 1st strand cDNA synthesis by real time PCR using 3' and 5' primers for the GAPDH transcript (see above). 10K TIGR potato arrays (TIGR Solanaceae Genomics Resource) were hybridized and scanned with a Fuji MAS FLA-8000 micro array scanner (Düsseldorf, Germany). The GeneSpotter software (MicroDiscovery, Berlin, Germany) was used for the grid positioning and signal quantification. The resulting data were analyzed using the LIMMA package (Smyth et al., 2005) for the bioconductor software (Gentleman et al., 2004). Data were normalized using within-array print-tip loess and between-array quantile normalization. Statistical analysis was performed using a mixed model incorporating the duplicate spots for each transcript. P-values were corrected using a False discovery rate (FDR) correction (Benjamini and Hochberg, 1995). Transcripts were considered to be differentially expressed if they had a FDR p-value (= q value) < 0.05. Data were visualized with the MapMan software (Thimm et al., 2004) using a mapping file developed by Rotter et al. (2007).

Transient expression of GFP fusion proteins in *Arabidopsis thaliana*

50 µl gold particle suspension (1 µm in diameter, 6% w/v in 50% glycerol) were mixed with 5 µl plasmid DNA (0.5 µg/µl), 50 µl 2.5 M CaCl2 and 20 µl 0.1 M spermidine. After vortexing, the suspension was shortly centrifuged and washed 4 times with 100% EtOH. The suspension was then resuspended in 48 µl 100% EtOH. 10 µl gold particle suspension were transferred on a 1100 psi rupture disc (Biorad, München, Germany) and shot on freshly cut *Arabidopsis thaliana C24* leaves placed upside down in a distance of 9 cm in a BioRad PDS-1000 microprojectile bombardment system (München, Germany) as previously described (Sohlenkamp et al., 2002). The bombarded leaves were incubated for 24-48 hours at 25°C and then inspected under a Leica DM IRBE microscope equipped with a Leica TCS SPII

confocal scanner (Heidelberg, Germany). Excitation wavelength was 488 nm, emission window for the GFP-channel covered 500-525 nm, autofluorescence was recorded in a window between 630-690 nm.

Metabolite analysis

Nucleotides were quantified from TCA-extracts prepared as described by (Jelitto et al., 1992) using a Kontron HPLC (Bio-Tek Instruments, Bad Friedrichshall) equipped with a Partisil-SAX 10 anion-exchange column and a UV-photometer detecting at 254 nm (Geigenberger et al., 1998). Starch was measured as glucose obtained after three digestions of the TCA precipitate with amylase and amyloglucosidase by an enzyme linked assay described previously (Geigenberger et al., 1998). GCMS analysis and preceding extraction and derivatization was performed as described by (Roessner et al., 2001) using a HP 5980 gas chromatograph (Agilent, Waldbronn, Germany) coupled to a Pegasus II Time-of-Flight mass spectrometer (Leco Corporation, St. Joseph, MI, USA).

Cell wall analysis

Determination of matrix sugar composition: The pellet of a TCA-extract of 200 mg potato tissue was washed once with 1.5 ml MeOH/chloroform 1:1 and once with 1.5 ml H_2O. To dispose starch, the pellets were digested three times over night with 1.5 ml amylose/amyloglucosidase solution as described above (Geigenberger et al., 1998) and washed then twice again with 1.5 ml H_2O. The pellet was then mixed with 50 µg inositol and digested with 250 µl 2 M trifluoracetic acid for one hour at 121°C. 300 µl 2-propanol were added to the solution and the mix evaporated at 40°C with a N_2-stream. Evaporation was repeated 3 times in total. The pellet was extracted with 400 µl H_2O using a sonificator. The pellet was dried and used for the determination of crystalline cellulose. 50 µl of the watery extract was used for the determination of uronic acids. The remaining 350 µl were dried and used for the determination of matrix sugar composition according to Englyst and Cummings (1984). Analysis was done by GC-MS on a SP^{TM}-2380 fused silica capillary column (30 m x 0,25 mm, 0,2 µm film thickness, Supelco Bellefonte, PA) in an Agilent 6890N gas chromatograph coupled to an Agilent 5973 mass selective detector (Agilent Technologies, Palo Alto, CA). The temperature program started at 160ºC for two min, then increased to 200ºC at 20ºC/min, held for 5 min, then increased to 245ºC at 20ºC/min and held for 12 min. The system was calibrated for glucose, galactose, arabinose, xylose, rhamnose, fucose, mannose and inositol.

Determination of uronic acids: Uronic acids were determined in a 50 µl aliquot separated after TFA-hydrolysis as described above using the biphenyl assay as described by Filisetti-Cozzi and Carpita (1991) with galacturonic acid as standard.
Determination of crystalline cellulose: The preparation and hydrolysis of crystalline cellulose from the pellet left after TFA-hydrolysis above was done as described previously (Updegraff, 1969). The cellulose derived glucose was determined using the anthrone assay (Dische, 1962)..

Enzyme analysis

Enzyme extraction. Enzymes were extracted as described previously by Geigenberger and Stitt (1993) with pefabloc and PVPP instead of PMSF in the extraction buffer and omitting BSA. Enzymes were extracted from 30-100 mg frozen homogenized tissue using 1500µl 50 mM HEPES/KOH pH 7.4 containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X100, 10% glycerol, 2 mM benzamidine, 2 mM γ-aminocaproic acid, 0.5 mM pefabloc, 5 mM DTT, 0.1% PVPP. Extracts were kept on 4°C prior measurement.
Preparation of apoplastic washings. 32 leaves of a potato plant not older than six weeks were vacuum infiltrated with 20 mM MES pH 6.0 using a 60 ml syringe (Braun, Melsungen, Germany). The infiltrated leaves were quickly dried with tissue and rolled into an Eppendorf 5 ml pipet tip (Wesseling-Berzdorf, Germany) which was plugged with a 1.5 ml Eppendorf cup containing 2 µl 20 mM benzamidine, 20 mM gamma-aminocaproic acid,5 mM pefabloc, 50 mM DTT, 0.1% PVPP in MES buffer. The washings were then collected by centrifugation at 250 g for 4 minutes. 1 µl of each washing was tested for cytosolic UGPase activity to check for cytosolic contaminations as suggested by Farran et al. (2002). Only washings with reasonable low UGPase activity (<2.5% of overall leaf UGPase activity) were pooled for further analysis.
Apyrase/5'-Nucleotidase in soluble extracts or apoplastic washing fluid (AWF): 5 µl extract or AWF was incubated in 75µl of buffer containing 100mM MES/KOH pH 6.0, 5 mM $CaCl_2$. Enzymatic reaction was started by adding the substrate (ATP/UTP/ADP/UDP/AMP/UMP) to a final concentration of 1 mM. The reaction was terminated by adding 210 µl developer solution (10% ascorbic acid: 0.42% ammonium molybdate in 1 N H_2SO_4 1:6 mixed before use) as used by Ames (1966). After 20 minutes incubation at RT extinction at 820 nm was recorded and released P_i was determined using a P_i standard. The release of 1 µmol P_i per minute was defined as one Unit.
Apyrase/5'-Nucleotidase in intact tuber slices: Five tuber slices were washed five times with buffer containing 20 mM MES/KOH pH 6.0, 1 mM $CaCl_2$ and incubated in three ml of this buffer. The reaction was started by adding the substrate to a final concentration of 1 mM.

After certain time points, aliquots of 80 µl were taken to determine P_i-liberation as described for the soluble extracts.

UDP-glucose-pyrophosphorylase (UGPase) in soluble extracts or AWF: UGPase was assayed in the direction of glucose-1-phosphate as described by Zrenner et al. (1993). *In intact tuber slices:* Five tuber slices (8 mm diameter and 2 mm thick) were washed three times with 20 mM MES/KOH pH 6.0, 1 mM $CaCl_2$ twice with 100 mM Tris-HCL containing 2 mM $MgCl_2$. Then the slices were incubated with UGPase assay mix and the reaction was started with PP_i as described above. After several time points aliquots of 200 µl were subtracted and NADPH production was immediately measured in a photometer. There was no production of NADPH detectable in the absence of PP_i.

Analysis of respiration rates

Respiration rates of two freshly prepared tuber discs (diameter 8 mm, thickness 2mm) were analyzed in an oxygen electrode (Hansatech, Norfolk, UK) filled with 1 ml 20 mM MES/KOH pH 6.0 as described previously (Loef et al., 2001).

Accession numbers

Sequence data from this article can be found in the GenBank data libraries under accession numbers AF535135 (*StAPY2* mRNA), EU125183 (*StAPY3* mRNA) and EU125182 (*StAPY2* gDNA).

9.6 Acknowledgements

We wish to thank Ewa Urbanczyk-Wochniak (Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore) and Matthew Hannah (MPI Molecular Plant Physiology, Golm, Germany) for their support on the micro-array analysis. We are grateful to Ulrike Haensel (Bayer CropScience AG, Frankfurt, Germany) and Peter Immerzeel (Umea Plant Science Center, Umea, Sweden) for their support in the cell-wall analysis, to Axel Tiessen (CINVESTAV, Mexico) for providing non-aqueous fractions and marker activities, to Pal Nyren (Royal Institute of Technology, Stockholm, Sweden) for providing the anti apyrase serum and to Jens Kossman (Stellenbosch, South Africa) and Jeremy Clark (MPI Molecular Plant Physiology, Golm, Germany) for advice in the beginning of the experiments.

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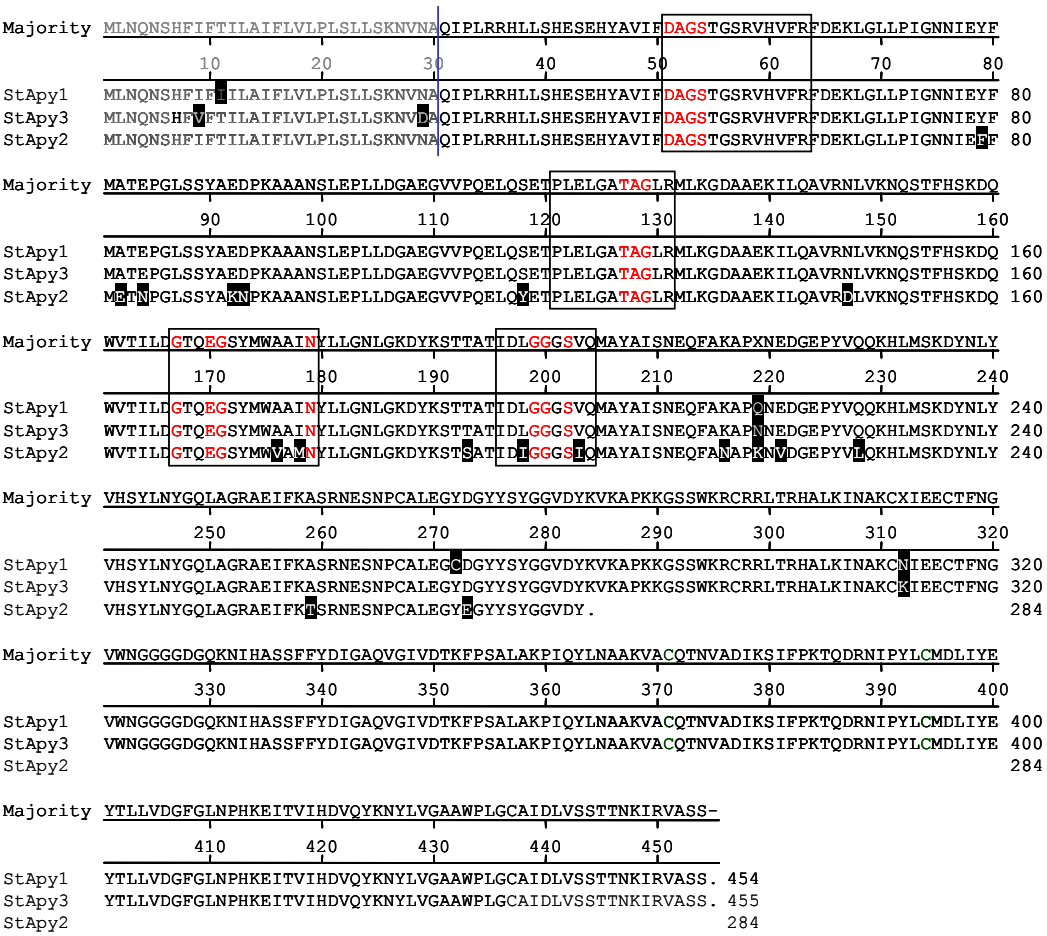
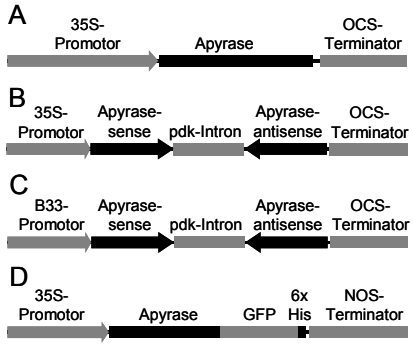
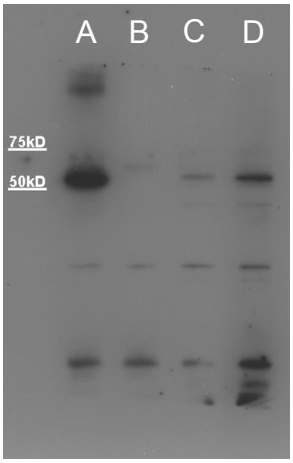
9.8 Supplemental data



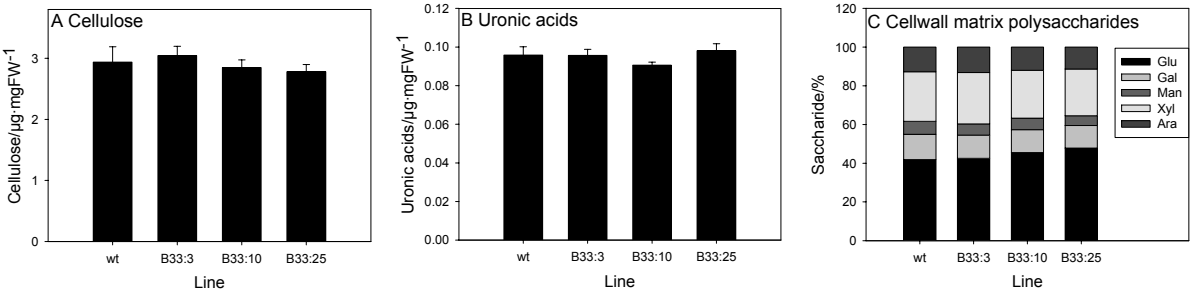
Supplemental Fig. S1. Alignment of potato specific apyrases cloned to date. Residues not matching the consensus sequence are shaded black, the apyrase conserved regions (ACRs) are enclosed by a black frame and highly conserved amino acids within the ACRs are highlighted in red. The amino acids of the putative secretory signal peptide from residue 1 to 30 are highlighted in grey and the putative processing site between amino acid 30 and 31 is marked with a blue line.



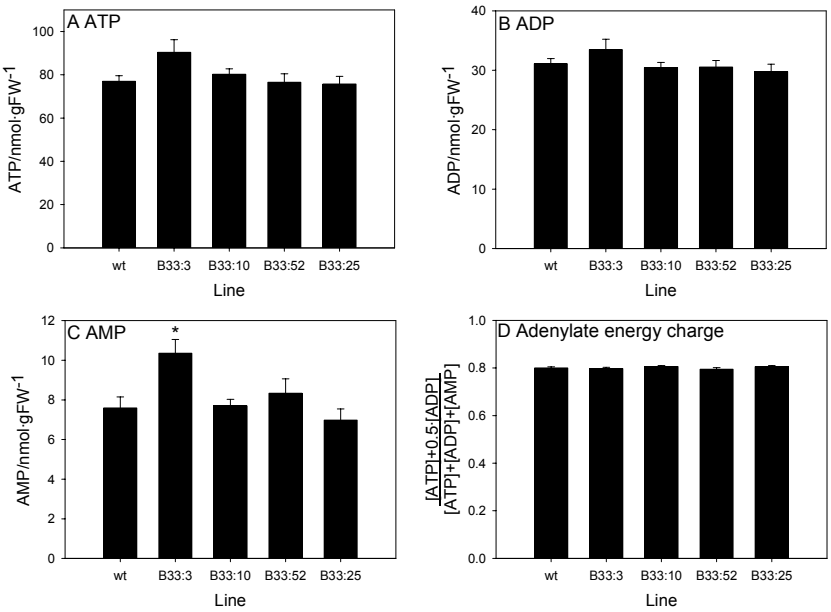
Supplemental Fig. S2. Plasmids used for manipulation and localization of apyrase. (A) 35S-overexpressor, **(B)** 35S-RNAi, **(C)** B33-RNAi and **(D)** 35S-apyrase-GFP.



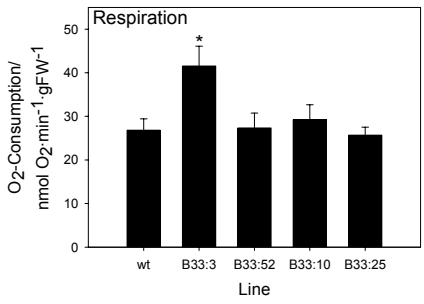
Supplemental Fig. S3. Immunodetection of potato apyrase. Five µg protein was analyzed using a serum raised against apyrase. **(A)** 35S:17 + 50 mU commercially available apyrase (SIGMA), **(B)** 35S:17 without additional apyrase, **(C)** wt, **(D)** 35S:113. The band appearing at appr. 52kD corresponds to potato apyrase.



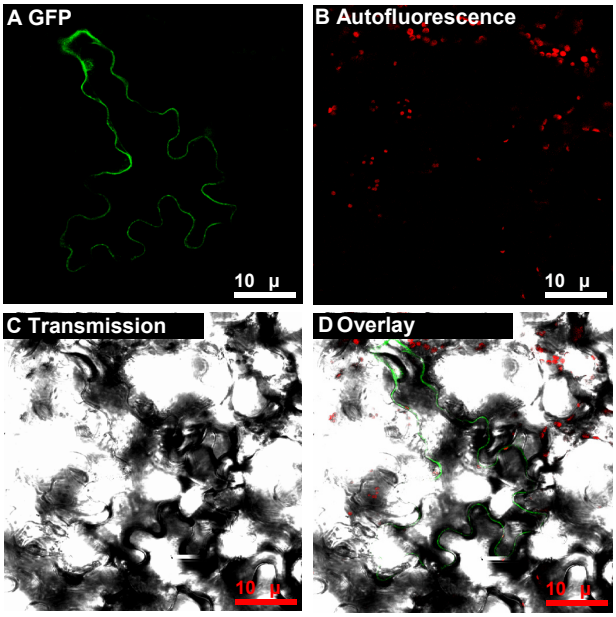
Supplemental Fig. S4 Cell wall composition of tubers from the B33-RNAi lines. (A) Cellulose, **(B)** uronic acids and **(C)** matrix polysaccharides (Glu = glucose, Gal = galactose, Man = mannose, Xyl = xylose, Ara = arabinose). Results are means ±SE (*n* = 8).



Supplemental Fig. S5. Overall adenylate levels in tubers with decreased apyrase under the control of the tuber-specific B33-promoter. Growing tubers from 8 week-old plants were harvested and material immediately shock-frozen in liquid nitrogen before extraction for the determination of overall adenine nucleotides: **(A)** ATP, **(B)** ADP, **(C)** AMP, and **(D)** adenylate energy charge calculated using the data from (A-C). Results are means ± SE (*n* = 7-8).



Supplemental Fig. S6. Respiration rates of tuber slices of the B33-RNAi lines. Results are means ±SE (*n* = 8). Significant differences to the wild-type according to the student's t-test are indicated with asterisks ($p < 0.05$).



Supplemental Fig. S7. Localization of a temporally expressed plasma membrane localized GFP fusion protein. Arabidopsis leaf epidermal cells were bombarded with a plasmid coding for *AtAMT2* fused to GFP (Sohlenkamp et al., 2002). **(A)** Fluorescence of GFP recorded between 500-525 nm. **(B)** Auto-fluorescence of the chloroplasts recorded between 630-690 nm. **(C)** Bright field image of the section. **(D)** An overlay of all three images given in (A-C). Typical examples are shown.

Supplemental Table S1. Sequence identity of potato specific and general plant apyrases. The protein sequences generated from TC142822 and TC144101 appeared to be partial sequences consisting of 263 and 249 amino acids, respectively.

Gene/TC/ Protein		**Identity of amino acid sequences**									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	
StAPY1	**1**		98.9	98.7	92.6	88.5	49.4	48.8	50.1	50.8	**1**
TC137054	**2**	99.5		99.3	92.6	88.4	48.9	48.8	50.1	50.8	**2**
StAPY3	**3**	99.1	99.6		92.6	88.4	49.4	48.8	50.1	50.8	**3**
StAPY2	**4**	92.0	91.9	91.8		97.9	48.1	51.8	50.4	50.4	**4**
TC134863	**5**	88.4	88.3	91.9	99.0		48.9	46.0	50.3	50.6	**5**
TC142822	**6**	57.9	57.9	61.0	61.7	58.7		59.6	57.1	57.9	**6**
TC144101	**7**	58.6	58.5	59.5	59.8	59.4	77.8		67.9	66.3	**7**
AtAPY1	**8**	53.5	53.5	56.3	57.3	55.0	57.9	61.3		87.3	**8**
AtAPY2	**9**	55.3	55.3	57.8	58.2	55.7	61.5	62.4	80.2		**9**
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	
		Identity of nucleotide sequences									

Supplemental Table S2. Metabolite profile of tubers from the B33-RNAi lines. Results are means ±SE (*n* = 8).

Metabolite	wt	B33:3	B33:10	B33:25
1-Kestose	100 ±7	123 ±16	142 ±14	113 ±13
6-Phosphogluconic acid	100 ±4	133 ±9	108 ±3	121 ±10
Aconitic acid	100 ±3	97 ±4	110 ±6	98 ±5
Alanine	100 ±10	72 ±8	108 ±11	68 ±12
Arginine	100 ±14	75 ±12	75 ±4	92 ±15
Asparagine	100 ±14	141 ±22	116 ±11	119 ±10
Aspartic Acid	100 ±10	146 ±12	112 ±9	111 ±8
Benzoic acid	100 ±6	93 ±6	93 ±6	93 ±8
β-Alanine	100 ±13	76 ±9	98 ±17	88 ±13
Chlorogenic acid	100 ±12	131 ±11	170 ±28	139 ±42
Citric acid	100 ±2	101 ±2	109 ±3	104 ±3
Cysteine	100 ±17	133 ±17	103 ±10	104 ±14
Dehydroascorbic acid	100 ±9	153 ±18	120 ±12	129 ±11
Fructose-6-phosphate	100 ±3	132 ±7	111 ±4	114 ±9
Glucose-6-phosphate	100 ±6	135 ±10	108 ±3	124 ±11
Fructose	100 ±11	103 ±11	90 ±8	135 ±9
Fumaric acid	100 ±8	139 ±25	108 ±11	134 ±15
Galactinol dihydrate	100 ±7	124 ±7	123 ±6	107 ±6
γ-Amino-butyric acid	100 ±12	83 ±5	106 ±13	96 ±9
Gluconic/Galactonic acid	100 ±7	139 ±9	119 ±6	117 ±6
Glucose	100 ±11	141 ±19	115 ±18	92 ±14
Glucose-1-phosphate	100 ±5	93 ±5	110 ±3	98 ±4
Glutamic acid	100 ±16	158 ±19	94 ±8	96 ±6
Glutamine	100 ±7	103 ±9	103 ±7	101 ±3
Glycerate-3-phosphate	100 ±8	156 ±13	99 ±4	131 ±14
Glycerol	100 ±6	90 ±3	102 ±4	87 ±3
Glycerol-3-phosphate	100 ±3	114 ±4	113 ±3	110 ±5
Glycine	100 ±10	77 ±8	109 ±13	93 ±10
Histidine	100 ±18	110 ±10	87 ±10	102 ±13
Homoserine	100 ±5	75 ±4	84 ±8	123 ±12
Myoinositol	100 ±6	88 ±7	89 ±6	93 ±5
Isocitric acid	100 ±7	102 ±9	101 ±8	96 ±4
Isoleucine	100 ±12	86 ±11	105 ±12	79 ±15
Isomaltose	100 ±7	103 ±7	118 ±7	89 ±7
Citrulline	100 ±13	81 ±10	94 ±8	96 ±12
Leucine	100 ±17	74 ±16	92 ±16	60 ±12
Lignoceric acid	100 ±5	109 ±8	125 ±12	102 ±10
Serine	100 ±6	87 ±3	117 ±8	94 ±5
Lysine	100 ±12	71 ±10	90 ±9	88 ±19
Malic acid	100 ±10	160 ±15	121 ±11	142 ±11
Maltose	100 ±6	112 ±9	111 ±7	95 ±4
Methionine	100 ±10	95 ±9	106 ±4	113 ±15
Nicotinic acid	100 ±4	104 ±8	99 ±5	101 ±8
Oxoproline	100 ±12	113 ±13	93 ±11	100 ±5
Phenylalanine	100 ±8	73 ±12	102 ±6	89 ±11
Phosphoric acid	100 ±4	112 ±4	119 ±5	121 ±4
Putrescine	100 ±10	95 ±9	108 ±13	123 ±16
Quinic acid	100 ±8	80 ±9	81 ±4	131 ±10
Raffinose	100 ±5	104 ±14	133 ±11	117 ±15
Ribonic acid	100 ±5	117 ±5	113 ±3	113 ±4
Ribose	100 ±9	123 ±15	103 ±9	85 ±11
Shikimic acid	100 ±8	99 ±9	101 ±4	119 ±10
Spermidine	100 ±10	111 ±11	108 ±9	96 ±7
Succinic acid	100 ±16	66 ±17	106 ±14	46 ±9
Threonic acid	100 ±4	116 ±8	109 ±5	99 ±8
Threonine	100 ±9	92 ±9	110 ±4	100 ±12
Tryptophan	100 ±20	54 ±11	98 ±16	41 ±9
Tyrosine	100 ±17	68 ±13	110 ±16	61 ±14
Uronic acid	100 ±7	95 ±8	92 ±7	111 ±4
Valine	100 ±5	78 ±7	95 ±4	90 ±8

10. Manuscript 1 (MS1)

A cell wall bound adenosine nucleosidase is involved in the salvage of extracellular ATP in *Solanum tuberosum*

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Manuscript submitted to FEBS Letters.

Authors' contributions

The research was conceived and planned by Peter Geigenberger, Alisdair Fernie and David Riewe. The experimental work was done by David Riewe. Lukasz Grosman, Henrik Zauber and Cornelia Wucke assisted as student workers supervised by David Riewe.

10.1 Abstract

Extracellular (e)ATP has recently been implicated in plant development and growth. To investigate the fate of eATP within the apoplast, we used potato-tuber slices as experimental system which enabled direct access to the apoplast. (i) Incubation of tuber slices with eATP resulted in the formation of ADP, AMP, adenosine and adenine. (ii) Analysis of intact tuber slices and fractionated tuber tissue led to the identification of a novel cell wall bound adenosine nucleosidase activity. (iii) Apyrase and adenosine nucleosidase activities were found to be co-regulated in wild-type tubers and in transgenic lines with reduced apoplastic apyrase activity, indicating functional linkage within a shared pathway. (iv) The results provide compelling evidence that an apoplastic ATP-salvage pathway is present in plants.

10.2 Introduction

In plants, growing evidence has emerged that extracellular ATP (eATP) acts as signaling molecule on the surface of plant cells (Day et al., 2000; Jeter at al., 2004; Kim et al., 2006; Wu et al., 2007;.Riewe et al., in press). Recently, the visualization of *in planta* eATP levels via the use of a recombinant cellulose-binding luciferase revealed that it is predominantly present in rapidly expanding tissues (Kim et al., 2006). Consistent with this hypothesis, studies have revealed that *Arabidopsis thaliana* apyrases *AtAPY1* and *AtAPY2* are expressed in such tissues (Wu et al., 2007), whilst localization studies performed in *Medicago truncatula* (Day et al., 2000) and *Solanum tuberosum* (Riewe et al., in press) confirmed the presence of plant apoplastic apyrases. In accordance with the presumed role in the turnover of eATP in plants, both Arabidopsis and potato plants deficient in apyrase expression exhibited reduced growth and altered development (Wu et al., 2007; Riewe et al., in press). Despite these findings there is no clear evidence that directly links the concentration of eATP to the phenotype observed in these transgenic plants. Whilst it is believed that plant eATP triggers a signal transduction event either via an as yet unidentified plasma membrane receptor (Jeter et al., 2004; Roux and Steinebrunner, 2007) or by protein phosphorylation (Chivasa et al., 2005), little evidence has, as yet been provided to substantiate this theory. Moreover, little is known concerning the fate of eAMP, the product of apoplastic apyrase. Whilst it seems likely that AMP, or a breakdown product thereof, is salvaged by re-import into the cell there is currently no direct evidence to support this theory. That said Wormit et al. (2004) have hypothesized that equilibrative nucleoside transporters may be involved in such a salvage pathway and in principle purine permeases (PUP), could also support such a process (Gillissen et al., 2000; Burkle et al., 2003).

To evaluate the fate of apoplastic eATP in the potato, we incubated tuber slices with ATP, ADP, AMP and adenosine and followed the accumulation of reaction products evolved on its consumption. In addition, we determined import rates of radio-labeled adenine in the presence of the other adenylates mentioned above. Utilizing correlation analysis, we found significant evidence that a cell wall-bound adenosine nucleosidase (ANase) activity is tightly co-regulated with apyrase activity in wild-type tubers. On the basis of the apoplastic localization of both ANase and apyrase, the likelihood that the substrate for ANase is a product of apyrase, and the coordinated activity of both enzymes prompts us to suggest that apyrase and ANase are functioning coordinately in the apoplastic adenylate salvage pathway of potato.

10.3 Results

The potato tuber apoplast converts ATP into adenine, ribose and P_i

To obtain insight into the apoplastic metabolism of ATP, we incubated intact tuber slices from eight week old potato plants in buffer containing ATP. Samples were taken over a time course and analyzed by HPLC. The elution chromatogram (Fig. 1A) clearly revealed that ATP was converted into ADP, AMP, adenosine and adenine within the time points chosen for analysis. By incubating the slices with AMP instead of ATP (Fig. 1B), we could demonstrate that both adenosine and adenine were produced from AMP. Incubation with adenosine suggested that adenine is produced from adenosine (Fig. 1C). To confirm the identity of the adenosine and adenine peaks, we spiked some samples with adenosine and adenine (Fig. 1C and 1D). If adenosine was converted into adenine, ribose should be produced in parallel. For this reason, aliquots from tuber slice incubations were also analyzed using GCMS. The only sugar found in considerable amounts in these incubation extracts was ribose (Fig. 1E). Unfortunately, it was technically impossible to quantitatively analyze the amounts of adenosine or adenine using the GCMS protocol adopted for this study. Nevertheless, the combined results of these studies clearly suggest that the potato tuber apoplast possesses the necessary machinery to catabolize the conversion of ATP into adenine, ribose and P_i.

In order to analyze whether ATP can be re-imported as adenine, we studied the inhibition of adenine uptake in the presence of ATP, ADP or AMP. Zeatin was chosen as positive control, since it has been reported that PUP-transporters also transport this adenine derivative (Burkle et al., 2003). As a negative control, sucrose was applied instead of an adenylate/nucleobase. As is clearly visible in Fig. 1F, the presence of ATP, ADP and AMP and zeatin (all 500 µM) clearly led to a marked competitive inhibition of adenine uptake, whereas sucrose did not lead to an inhibition (data not shown). Further experimentation revealed that this transport was likely to be H^+-dependent, since presence of the ionophore CCCP led to a strong inhibition of uptake (data not shown).

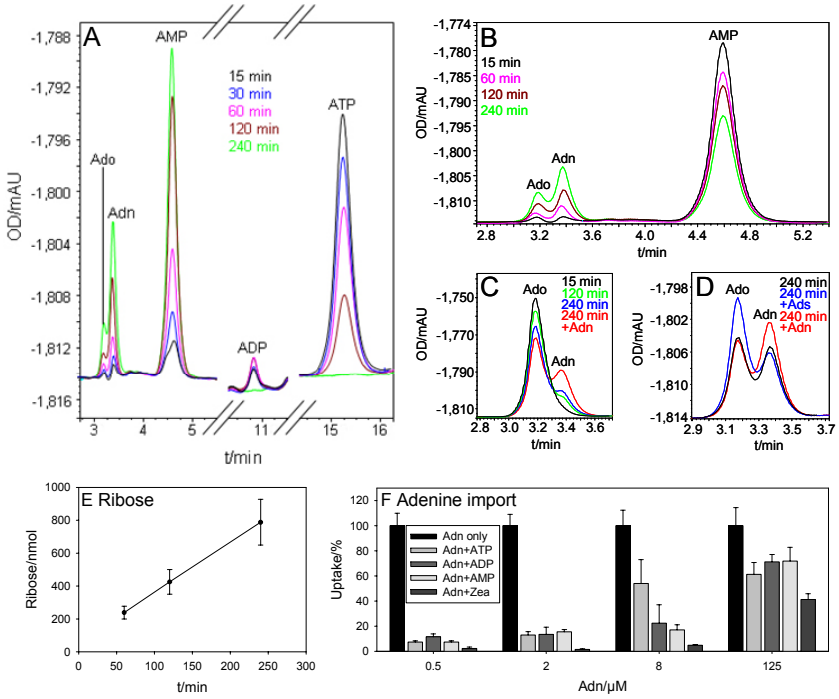

Fig. 1. Fate of ATP when incubated with intact tuber slices. Intact tuber slices in buffer solution were incubated with **(A)** ATP, **(B)** AMP or **(C)** and **(D)** adenosine (Ado) for 15 to 240 minutes and aliquots of the buffer solution were subjected to HPLC analysis. In **(C)** peaks were spiked with adenine (Adn) and in **(D)** with both adenosine and adenine. **(E)** Ribose content as determined with GCMS in aliquots of buffer solution subtracted from tuber slices incubated with Ado (*n* = 6). **(F)** Adenine uptake rates into tuber slices in the presence of adenylates and zeatin (Zea, each 500 µM, *n* = 4). **(A)** to **(D)** are representative results out of six replicates, in the case of **(E)** and **(F)**, data are means ±SE.

In addition to apyrase and phosphatase, the potato tuber apoplast contains a cell wall bound nucleosidase

To confirm the presence of the enzymatic machinery capable of converting ATP to adenine, ribose and P_i within the plant apoplast, we compared the activities in intact tuber slices, enzyme extracts and washed pellets of enzyme extracts containing enzymes associated to insoluble material such as cell wall or starch granules. As all activities were assayed in extracts from identical tuber material, this provides information on the relative contribution of the activities in the apoplast, the cell wall and the soluble fraction to the total cellular activity. In order to determine apoplastic nucleotide hydrolyzing activities, five potato tuber slices were incubated with buffer containing ATP, AMP or the common phosphatase substrate PNP. After several time intervals aliquots were collected and the levels of P_i, nucleotide and nitrophenol were analyzed. To assay adenosine hydrolyzing activity, five slices were incubated with adenosine alone and in the presence of the ionophore CCCP to inhibit substrate/product uptake into the intact slices. Aliquots were collected at intervals during the

incubation and the production of ribose was monitored. For all measurements linearity of product production over time was observed, and for UGPase, apyrase and 5'-nucleotidase additional measurements without substrate were carried out to confirm that product formation was dependent on enzyme activity (Fig. 1E and Supplemental Fig. S1). All activities were also determined both in enzyme extracts and washed insoluble pellets of these enzyme extracts (Table 1). The activity of UGPase in the intact tuber slices was less than 0.3% of the total activity, confirming that these preparations were essentially free of cytosolic activities. In accordance with previous experiments (Riewe et al., in press), apyrase activity detected in tuber slices was ca. 6% of the total activity, with the activity being much higher in the extract than in the pellet fraction.

Table 1. Activities of nucleotide converting enzymes determined in soluble extracts, pellet fractions of soluble extracts and incubated intact tuber slices and adenine uptake rate. Data are means ± SE (n = 6 for activity measurements, n = 4 for uptake measurement) and displayed in mU·gFW^{-1}.

Activity type/Substrate	soluble activity (extract)	insoluble activity (pellet)	total activity	activity in incubated tuber slices	$\frac{tuber\ slice\ activity}{total\ activity}$ (%)
UGPase/PP_i and UDPG	61372 ±4798	n. d.	61372 ±4798	155 ±13	0.3%
Apyrase/ATP	1239 ±363	55 ±12	1294 ±375	73 ±13	5.6%
5'-Nucleotidase/AMP	73 ±6	58 ±7	131 ±13	13 ±0.3	9.7%
Nucleosidase/adenosine	0.9 ±0.2	66 ±12	67 ±12	10 ±1.6	14.9%
Adenine uptake/adenine				0.6 ±0.2	

5'-nucleotidase activity in the intact tuber slices represented 10% of overall activity suggesting that the apoplast contains a significant activity of this type. 5'-nucleotidase in the intact tuber slices did not appear to be specific for nucleoside-monophosphates, since PNP was hydrolyzed with only slightly higher speed (18.6 ±0.5 mU·gFW^{-1}, data not shown). In contrast to the activities found in intact tuber slices, in the pellet fraction hydrolysis of ATP and AMP was equal (Table 1) and PNP-hydrolysis was only slightly higher (83.4 ±8.4 U·gFW^{-1}, data not shown), pointing to a low substrate-specificity of the enzyme. Moreover, the turnover of nucleotides was equivalent most likely due to absence of apyrase and hence exclusive activity of phosphatase in this fraction. From this we conclude that the eAMP was hydrolyzed by a rather unspecific cell wall bound phosphatase than a specific 5'-nucleotidase
As expected from the preceding experiments, a high nucleosidase activity was found in the intact tuber slices. The activity presented in Table 1 (10 ±1.6 mU·gFW^{-1}) was obtained in experiments using 50 µM CCCP to inhibit H^+-dependent ribose uptake, but was only slightly lower without the ionophore (8.9 ±0.4 mU·gFW^{-1}). This activity contributed to 15% of the total

activity. The pellet contained 99% of nucleosidase activity, suggesting that this enzyme is bound to the cell wall, hardly present in the cytosol and not released from the cell wall under the conditions of extraction used here. The low abundance of nucleosidase activity in the soluble fraction of the enzyme extract is in close accordance with recently published work on potato tubers measured via a radiochemical assay (Katahira and Ashihara, 2006). Adenine uptake was determined to be 0.6 mU·gFW^{-1} (one order of magnitude lower than nucleosidase activity). This observation is in keeping with the observed accumulation of adenine if adenosine (or indeed any adenylate) is offered to tuber slices (see Fig. 1A-C). When taken together, these experimental data provide evidence for the existence of a series of three enzymes that are capable to convert ATP to adenine, ribose and P_i. In a similar experiment, Mazelis described enzymic activities converting ATP to adenine in insoluble particles of enzyme extracts prepared from cabbage leafs almost 50 years ago (1959). Mazelis, however, argued for a cytosolic localization of both the insoluble particles and their attached activities.

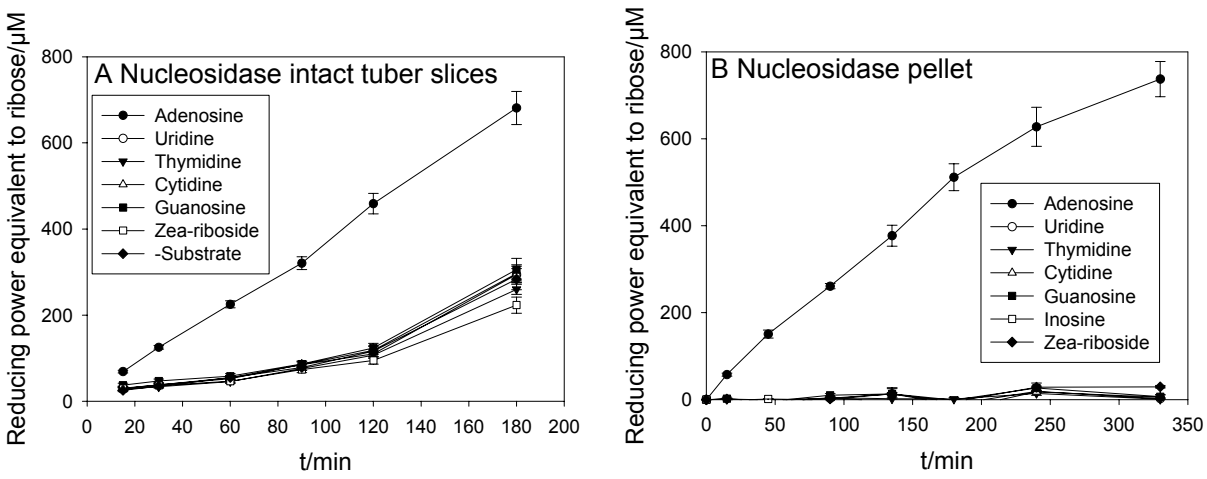

Fig. 2. Substrate specificity of apoplastic nucleosidase. (A) Ribose detected in incubation buffer of intact tuber slices incubated with 1 mM adenosine, uridine, thymidine cytidine, guanosine or zeatin-riboside. **(B)** Ribose detected in buffer incubated with washed pellet fraction and 1 mM adenosine, uridine, thymidine, cytidine, guanosine, inosine or zeatin-riboside. Data are means ±SE (n = 6).

The cell wall bound nucleosidase is highly specific for adenosine

In our previous experiments nucleosidase activity was assayed using adenosine as substrate. To further analyze the biochemical properties of this activity, several putative substrates with structural similarity to adenosine were analyzed. For this purpose we tested the purine nucleosides guanosine and inosine, as well as the pyrimidine nucleosides cytidine, uridine and thymidine and the phytohormone zeatin-riboside. There is considerable indirect

evidence for turnover of cytokinin-ribosides in plants, however, the enzyme(s) responsible have, as yet, not been identified (Auer, 2002). Using the same experimental set-up as defined above, washed intact tuber slices and washed pellets were incubated with different putative substrates, and ribose production was monitored by a Fehling-based assay. In both tuber slices and pellets, ribose was produced following incubation with adenosine but not with uridine, thymidine, cytidine, guanosine, inosine or zeatin-riboside (Fig. 2A and 2B). These results clearly demonstrate that the cell wall bound nucleosidase activity is highly specific for adenosine and can therefore be termed adenosine nucleosidase (ANase).

The cell wall bound adenosine nucleosidase is functionally linked to apyrase

Recently, we have shown that the potato specific apyrase is confined to the apoplast (Riewe et al., in press). Given that we now identified a cell wall bound ANase, it is conceivable that nucleosidase might act together with apyrase and a nonspecific phosphatase in a pathway to convert extracellular ATP into adenine. Given that constituent enzymes of a pathway are often co-regulated at the transcript, protein or activity level, as it has been shown for glycolytic or TCA-cycle enzyme activities (Mitchell-Olds and Pedersen, 1998; Cross et al., 2006), we chose to assess whether this was the case for these enzymes. For this purpose we measured apyrase and ANase in 24 tubers of different developmental stages from individual plants in two independent experiments (n = 18/6). We prepared enzyme extracts and washed pellet fractions from these samples and measured apyrase activity in the soluble extract and ANase activity in the pellet and correlated these activities. As shown in Fig. 3A, the correlation between ANase in the pellet and apyrase in the extract was highly significant ($r = 0.81$, $p = 2 \cdot 10^{-6}$, $n = 24$). However, neither ANase nor apyrase correlated with the cytosolic enzyme UGPase (Fig. 3B, r = 0.06, p = 0.82, n = 18; and data not shown) or the protein content of the enzyme extract (r = 0.11, p = 0.67, n = 18 for ANase vs. protein content; and data not shown). The strong and specific correlation between ANase and apyrase strongly favors the hypothesis that these enzymes are constituents of the same pathway.

To further characterize the co-ordination of these activities, we assayed ANase and apyrase activities in tuber tissue from transgenic potato plants with reduced (B33:25 and 35S:23) or elevated (35S:113) apyrase activity.

As already described previously (Riewe et al., in press), apyrase activity in intact slices was significantly reduced in the RNAi-lines B33:25 and 35S:23 to a level of 43-45% of the wild-type and increased in the overexpressor line 35S:113 to appr. 500% (Fig. 3C). Irrespectively of whether assayed in intact tuber slices or pellets, ANase is significantly reduced to 28-60% of the wild-type activity in both RNAi-lines. This was, however, not the case for the

overexpressor line 35S:113. In this line, ANase was not significantly altered when compared to the wild-type. These data demonstrate that strong reduction of apyrase leads to a reduction of ANase. We can exclude that ANase activity is performed by apyrase, because ANase is not altered in the overexpressing line.

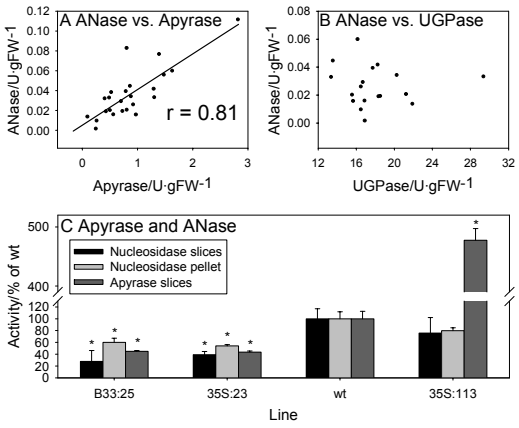

Fig. 3. Coordination of ANase and apyrase activities in potato tubers. Correlation between **(A)** ANase and apyrase (*n* = 24) compared to **(B)** ANase and UGPase (*n* = 18) in wild type tubers. **(C)** ANase activity in slices and pellets and apyrase activity in slices of tubers from wild-type, the apyrase-RNAi lines B33:25 and 35S:23 and the apyrase-overexpressing line 35S:113. For **(C)**, data are means ±SE (*n* =6) and significant differences to the wild type according to the Student's t test are indicated with asterisks ($P < 0.05$).

10.4 Discussion

Our results provide evidence for an apoplastic salvage pathway for extracellular ATP (eATP) in potato tubers. This pathway consists of apyrase, unspecific phosphatase, adenosine nucleosidase (ANase) and an adenine transport system (see Fig. 4). Using intact tuber slices as model system for the potato tuber apoplast, we were able to assay apyrase, phosphatase and an as yet uncharacterized ANase from an identical source in a single experiment. Hence comparison between the absolute activities of these enzymes in the apoplastic space of potato tubers was possible. 5'-nucleotidase and ANase activities were almost identical whilst apyrase activity (when assayed on the basis of the conversion of ATP to AMP instead of P_i-release), was higher but of a similar magnitude (Table 1).

Apyrase appears to be a soluble apoplastic protein enclosed by the cell wall, but we cannot currently exclude the possibility that it is weakly associated to the cell wall or cell membrane (see model, Fig. 4). Free diffusion or related kinetic effects after extraction might explain its relatively high activity in the soluble fraction when compared to the activity determined in intact tuber slices. Unspecific phosphatase and ANase were found to be bound to the cell wall and were not released under native extraction procedure including the nonionic detergent Triton X-100. The cell wall bound phosphatase appeared to have a broad substrate spectrum in accordance with those cell wall phosphatases characterized from other plant species such as mustard (Duff et al., 1991) or white clover (Zhang and McManus, 2000). The nucleosidase identified in the apoplast was highly specific for adenosine, and this implies that

adenosine is converted into adenine *in vivo* and subsequently imported into the cell by an adenine transporter. Our data show that ATP (or ADP and AMP) leads to a competitive inhibition of adenine uptake into potato tuber slices. It is unlikely that this competition is direct, because adenine transport by the Arabidopsis purine permease 1 was not inhibited by adenylates at all and only to a low extent by adenosine (Gillissen et al., 2000). Although speculative, it seems more likely that inhibition was caused by adenine, which was produced by concerted action of apyrase, phosphatase and ANase.

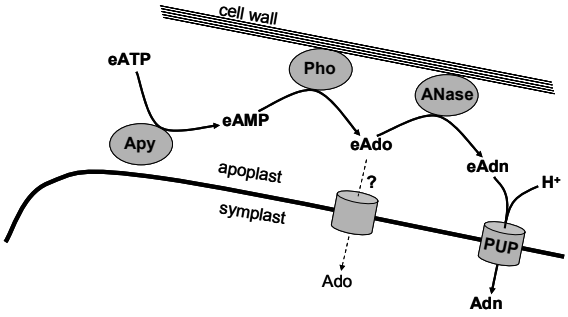

Fig. 4. Model of an apoplastic eATP salvage pathway. ATP (and ADP) is primarily hydrolyzed by apyrase to AMP (and P_i). AMP than is hydrolyzed at the cell wall by an unspecific phosphatase forming adenosine (and P_i). Our data suggest that adenosine is converted into adenine (and ribose) by a highly adenosine-specific cell wall bound nucleosidase and imported into the cell along a proton gradient by a purine permease (PUP). Theoretical import of adenosine by nucleoside transporter is indicated.

Under saturating conditions, AMP or adenosine are converted in significant amounts into adenine by intact tuber slices without relevant diminution of the sum of AMP, adenine and adenosine by uptake processes within 240 minutes (see peak areas in Fig. 1B and 1C). If this applies to the situation *in vivo*, ANase would bypass adenosine uptake and adenine uptake would take place instead (see Fig. 4).

A further indication for an involvement of ANase in eATP salvaging is given by the fact, that apyrase and ANase are coordinately regulated at the activity level. We found a high and significant co-regulation of apyrase and ANase activity in wild-type tubers of different developmental stages. This may be achieved either by shared transcriptional control or by sensing of one of the substrates/products of apyrase, phosphatase or ANase triggering transcriptional or posttranscriptional regulation. The latter scenario may partially explain why reduction of ANase is observed in transgenic lines with very low apyrase activity.

In conclusion, our results show the presence of an adenosine nucleosidase in the apoplast of potato tuber parenchyma cells that is likely to be involved in an eATP salvage pathway.

10.5 Materials and methods

HPLC

A deep frozen aliquot of 80 µl was mixed and thawed with 80 µl 10% (w/v) trichloroacetic acid to stop any enzymatic activity, neutralized with 5 M KOH, and diluted to a final volume of 1600 µl with distilled water. After centrifugation, 20 µl were analyzed as described previously (Geigenberger et al., 1997).

GCMS

A deep-frozen aliquot of 80 µl was lyophylized, resolved in 1600 µl methanol and centrifuged. 100 µl of the supernatant were evaporated, derivatized and analyzed as described by Roessner et al. (2001). The system was calibrated using ribose from 5-5000 pmol per derivatized sample.

Adenine uptake rates

Freshly prepared tuber slices were washed and incubated with 20 mM MES/KOH, pH 6.0. Adenine was applied in concentrations ranging from 0.25 µM to 500 µM and 200 Becquerel [U-^{14}C]adenine (GE Healthcare, Uppsala, Sweden) alone or together with the potential inhibitor in 500 µM concentration in a final volume of 300 µl buffer. After 75 minutes of gentle shaking, the slices were twice shortly washed in buffer. After homogenization, incorporated radioactivity was determined.

Preparation of intact slices, soluble enzyme extracts and pellets for enzyme analysis

Freshly harvested potatoes were sliced using a corkborer (diameter 8 mm) and a sharp knife The slices were either immediately frozen in liquid N_2 or washed 4 times with 20 mM MES/KOH pH 6.0, 1 mM $CaCl_2$. Five slices were transferred to 3 ml of assay buffer for measurement of the enzymatic activity specified below. The N_2-frozen slices were homogenized using a MM301 Retsch mill (Retsch, Haan, Germany). Soluble proteins were extracted as previously described (Riewe et al., 2008) and kept at 4°C prior to assay. The pellets of these extracts were washed one more time with extraction solution and twice with assay buffer. Then the pellet was subsequently suspended in a final volume of 2 ml assay solution.

Enzyme analysis

UGPase: In the soluble extract, UGPase was assayed as described by Zrenner et al. (1993) in a 96-well Anthos ht3 photometer (ASYS Hitech, Eugendorf, Austria) in 200 µl assay mix containing 10 µl of 1:1000 diluted enzyme extract. Intact tuber slices were incubated with assay mixture in a final volume of 3 ml on a shaker. After addition of PP_i to a final concentration of 1 mM, aliquots of 200 µl were subtracted after short time intervals and NADPH-production was determined in a photometer as described for the soluble extracts. Analogous, the pellets were suspended in 2 ml assay mixture and kept on a roller (Karl Hecht KG, Sondheim, Germany). After addition of PP_i in a final concentration of 1 mM, 250 µl aliquots were subtracted in short intervals, shortly centrifuged and immediately analyzed in the photometer as described above.

Apyrase and 5'-nucleotidase: In soluble extracts apyrase/5'-nucleotidase were measured by determination of P_i-liberation as described previously (Riewe et al., in press) using 70 µl 20 mM MES/KOH, pH 6.0, 1 mM $CaCl_2$, 10 µl extract and 2 µl of the substrates ATP or AMP in a final concentration of 1 mM. One Unit was defined as 1 µmol P_i released per minute. Intact tuber slices were incubated with ATP or AMP in 1 mM concentration in a final volume of 3 ml buffer on a shaker. After certain time points, 80 µl of buffer solution was subtracted and tested for both P_i-content or deep frozen and analyzed regarding nucleotide-content using HPLC. The formation of hydrolyzed substrate was converted into P_i-liberation. The pellet fraction was incubated with ATP or AMP in 1 mM concentration in a volume of 2 ml buffer on a roller and P_i-formation was measured in shortly centrifuged 80 µl aliquots collected along a time course.

Unspecific phosphatase: In the soluble extract, unspecific phosphatase was assayed using the discontinuous assay with PNP as substrate (Bergmeyer, 1985). 10 µl enzyme extract was incubated in 200 µl 20 mM MES/KOH pH 6.0, 1 mM $CaCl_2$. The reaction was started by addition of PNP to a final concentration of 1 mM. The reaction was terminated by the addition of 5 µl 3 M NaOH to enable extinction of p-nitrophenol and extinction at 405 nm was immediately determined in the photometer. If a precipitate occurred, the samples were centrifuged for one minute at maximal speed and 100 µl of the supernatant were read in the photometer. Intact tuber slices were incubated in 3 ml of the same buffer on a shaker and the reaction was started with PNP in a final concentration of 1 mM. 200 µl samples were subtracted from the buffer along a time course and analyzed as described for the soluble extracts. The pellets were suspended in the same buffer in a final volume of 2 ml and the samples were kept on a roller. The reaction was started with PNP in a final concentration of 1 mM. 250 µl samples were subtracted along a time course, centrifuged and analyzed as described above.

Nucleosidase: In soluble extracts nucleosidase was assayed using 1900 µl 20 mM MES/KOH, pH 6.0, 1 mM $CaCl_2$, 100 µl soluble extract and started by addition of adenosine in a final concentration of 1 mM. Along a time course, 80 µl aliquots were subtracted and deep frozen and ribose production was analyzed with GCMS. Intact tuber slices were incubated in 3 ml buffer on a shaker and the reaction was started by addition of nucleoside (adenosine, uridine, thymidine, cytidine, guanosine or zeatin-riboside) in a final concentration of 1 mM. Along a time course, either 80 µl aliquots were subtracted, deep frozen and analyzed with GCMS (only adenosine) or 150 µl aliquots were subtracted and analyzed using a Fehling assay described by Parkin (1996). The pellets were suspended in 2 ml assay buffer and incubated with nucleoside (adenosine, uridine, thymidine, cytidine, guanosine, inosine or zeatin-riboside) in a final concentration of 1mM on a roller. Aliquots were subtracted from the reaction mix and either analyzed using GCMS or centrifuged and analyzed using the Fehling assay.

10.6 Acknowledgements

We wish to thank Gareth Catchpole and Aenne Eckard (MPI Molecular Plant Physiology, Potsdam, Germany) for analyzing the GCMS samples. This work was supported by the Deutsche Forschungsgesellschaft.

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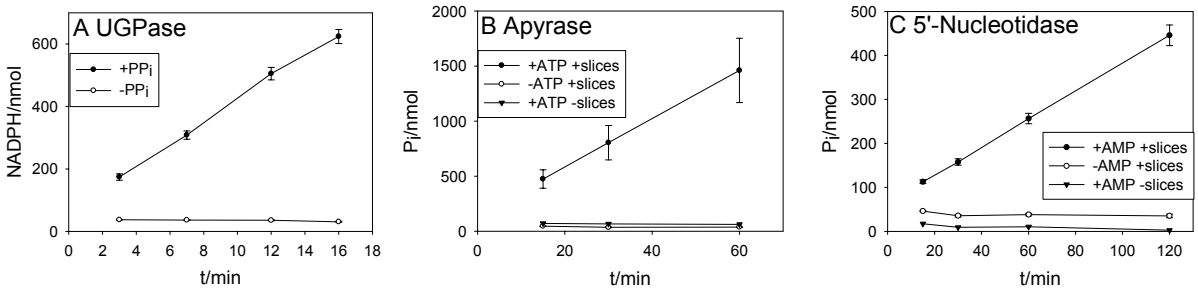
10.8 Supplemental data



Supplemental Fig. S1. Time dependent linear product formation in the incubation buffer of tuber slices incubated with substrates. (A) UGPase was quantified by detection of NADPH in the incubation after the reaction was started with PP_i. **(B)** Apyrase and **(C)** 5'-nucleotidase were measured by detection of P_i in the incubation buffer after addition of the substrates ATP or AMP. Data are means ±SE (*n* = 6).

11. General discussion

The general aim of this work was to investigate the role of adenylates for metabolism and development of heterotrophic potato tubers. The first aim of this work was to identify energy-state regulated sites in central metabolism by temporal or constitutive expression of a heterologous apyrase in the plastid of developing tubers. A second aim was to investigate the localization and functional characterization of enzymes involved in a potential apoplastic ATP-salvage pathway in potato tubers. The third aim was to elucidate the function of one of these enzymes (apoplastic apyrase) via a reversed genetic approach.

Adaptive regulation of tuber metabolism by the energy status

Both inducible and constitutive plastidial expression of an *E. coli* apyrase in potato tubers successfully reduced the energy state in potato tubers. The reduction of the energy state was decoupled from changes in the oxygen availability, hence the experimental system allows studying specifically the adaptive mechanisms to changes in the energy charge without interference of changes in response to oxygen.

When comparing the results of both approaches, it becomes evident that inducible induction led to a reduction in ATP levels and in the ATP/ADP ratio within 24h, while there were only few significant changes in other metabolites of carbon metabolism. Among these, mostly amino acids and intermediates of sucrose to starch conversion, some showed very high and significant positive correlations to ATP. While there was no increase in overall respiration rates, the changes in metabolite levels are indicative of a depression of sucrose metabolism.

In contrast to this, constitutive apyrase expression led to a decrease in the ATP/AMP ratio and an induction of respiration rates that was accompanied by morphological changes such an increase in the surface-to-volume ratio of the tubers. However, the ATP correlated changes in metabolite levels observed for the inducible lines were confirmed. From this we conclude that the inducible apyrase expression allowed the identification of metabolic sites that are more directly affected as a response to short term reductions in ATP, whereas the tubers constitutively expressing apyrase do rather represent a metabolic state after adaptive responses have been taken place. The results also show that a combination of inducible and constitutive expression systems provides an excellent approach to study adaptive responses to metabolic perturbation.

The results presented in this thesis are in confirmation with and extend previous studies on the role of adenylates in potato tubers. There was an increase in starch and amino acid synthesis in potato tubers that were reduced in the activity of the plastidial adenylate kinase (ADK) and having partially elevated levels of ATP and total adenylates (Regierer et al.,

2002). Conversely, reduction of the plastidial ATP/ADP-translocator (AATP) led to a decrease in the levels of starch, amylose (Tjaden et al., 1998) and amino acids (personal communication, Peter Geigenberger, MPI of Molecular Plant Physiology). In tubers with decreased AATP activity there was also an increase in respiration rates and changes in tuber morphology leading to an increased surface/volume ratio (Tjaden et al. 1998; Geigenberger et al. 2001).

Enzyme activities involved in starch synthesis were found to be altered in a way that is consistent with the changes in starch metabolism. There was a decrease in the activity of AGPase, the key enzyme of starch synthesis, via post-translational redox-regulation. This indicates a possible link between energy state and redox-regulation of AGPase. In accordance with this, there was an increased redox-activation of AGPase in the ADK-antisense lines (Regierer et al., 2002) and a reduced redox activation of AGPase in the AATP-antisense lines (Tjaden et al., 1998). Furthermore, low-oxygen treatment, which is inevitably accompanied by a decrease of the energy charge, also led to a decrease in the redox-activation of AGPase (Anke Langer, MPI of Molecular Plant Physiology, manuscript in preparation). Overall, these results indicate that AGPase is redox-regulated in response to changes in the energy state of potato tubers.

We found a clear switch from granule-bound starch synthase (GBSS) to soluble starch synthase (SSS) activity in the tubers with a long-term decrease in the energy charge. This switch was highly correlated to a shift from amylose to amylopectin in these tubers. A similar decrease in the amylose/amylopectin ratio has also been observed in the AATP-antisense tubers (Tjaden et al. 1998). It is known that flux through these enzymes is regulated by the different affinities of SSS and GBSS towards ADP-glucose (Lloyd et al., 1999), but not much is known about translational or posttranslational regulation of these enzymes. Lloyd et al. (1999) showed that GBSS accumulates on starch particles when ADP-glucose is low and postulated that this is a countermeasure to increase amylose production. Our data rather suggests an adjustment of the different activities to a lower ADP-glucose concentration or lower ATP-availability. Although the regulatory mechanisms leading to this switch in activites could not be elucidated from our data, we found evidence that the amylose/amylopectin production is not solely dependent on substrate supply, but also on the regulation of the maximal activities of GBSS and SSS. It has been shown that starch synthases are regulated posttranslationally by phosphorylation and oligomerization. Possibly, this regulation is dependent on the energy status.

The activities of glycolytic enzymes were hardly altered, which led to the speculation that they are rather regulated on the posttranscriptional level. Although the maximal activity of pyruvate kinase was slightly reduced, the increase in respiration and the decrease in PEP-pyruvate ratio clearly indicate that PEP to pyruvate conversion must have been activated *in*

vivo. By correlation analysis we found that the ATP/AMP ratio could control the turnover of PEP to pyruvate. Up-regulation of PEPase appears to be paradox, since conversion of PEP to pyruvate by this enzyme would waste ATP. It has been suggested that PEPase could bypass the ADP-limited pyruvate kinase (Plaxton, 1996). Since ADP was reduced in all transgenic lines, elevated activity of PEPase *in vivo* could make pay off if fast flux through glycolysis is required to produce ATP by oxidative phosphorylation. However, since PEPase is probably located in the vacuole, the role of this enzyme in plants is not clear.

Some of the responses to the decreased energy state were also observed to occur under low oxygen. Short term reduction of oxygen led to a reduction in the synthesis of starch, protein and amino acids in potato tubers (Geigenberger et al., 2000; Geigenberger, 2003). In combination with our data we can now say that at least the reductions in starch and amino acid synthesis could have also been caused by concomitant reductions in the energy charge in this previous short term experiment. However other responses were not observed under low oxygen: Carbon fluxes through glycolysis, TCA-cycle and respiratory chain were up-regulated in the tubers with decreased energy status in the long-term. This contrasts with hypoxic tubers, where respiration and glycolysis were down-regulated in response to low oxygen (Geigenberger 2003). These findings suggest that energy metabolism can be activated by changes in the energy status, independent of changes in oxygen availability, and that the inhibition of respiration by oxygen overrides energy effects. Furthermore, incubation of potato tubers with reduced energy status with superambient oxygen concentrations (40%) leads to an even higher activation of energy metabolism and a further increase in respiration, while this was not the case for wild type tubers (data not shown). If oxygen is limiting, however, the flux through these pathways is reduced although the energy status is low, and this also reflected in an increased PEP/pyruvate ratio then (Anke Langer, MPI of Molecular Plant Physiology, manuscript in preparation). More studies are needed to investigate the mechanisms leading to an inhibition of respiration by low oxygen.

Fermentative enzymes (ADH and LDH) were not activated in the tubers expressing apyrase constitutively, with the exception of pyruvate decarboxylase (PDC). This is different to the situation under low oxygen, which leads to an activation of the enzyme alcohol dehydrogenase in most plant species and lactate dehydrogenase in some (Drew, 1997). This suggests that the expression of ADH and LDH is not controlled by the energy state in a direct way. We assume that the induction of PDC could be a preceding adaptation to a situation when fermentation is likely to be rapidly required.

The tubers expressing apyrase constitutively developed a strong phenotype, having a higher surface-to-volume ratio and producing a massive amount of side-tubers in the very strong lines. Similar morphological changes were also observed and documented for tubers long-term incubated with low oxygen in two independent experiments (Anke Langer, MPI of

Molecular Plant Physiology, manuscript in preparation, although this feature was conditional and was not observed in the experiment described in P1). Tubers incubated under low oxygen also developed enlarged lenticells, which was not the case for the tubers with low energy due to long-term apyrase expression. This suggests that lenticell formation is dependent on oxygen-dependent mechanisms whereas the changes in tuber morphology are under control of the energy charge. Even aeration with 40% oxygen did not reverse the tuber-morphology-phenotype in the strongest line expressing apyrase constitutively, again showing that oxygen has not a direct influence on tuber shape. A similar tuber phenotype was also found for the tubers with reduced AATP activity (Tjaden et al., 1998), which supports the notion that the morphological phenotype is energy-regulated.

Evidence for a novel apoplastic ATP-salvage pathway

We characterized a complete ATP-salvage pathway in the apoplast of potato tubers. This pathway comprised apyrase, unspecific phosphatase, adenosine nucleosidase (ANase) and an adenosine transport activity.

Previous reports showed that also the apoplast contains significant amounts of ATP (eATP) (Kim et al., 2006; Song et al., 2006), which is supposed to be catabolized by apoplastic apyrases to ADP and AMP (Steinebrunner et al., 2000; Song et al., 2006). It has been reported that potato contains specific apyrases, potentially in addition to apyrases that more closely resemble those characterized from other plant species like Arabidopsis or *Oriza sativa* (Roberts et al., 1999) and EST-analysis confirmed this assumption (see P2). According to our results, also the potato specific apyrases reside in the apoplast and account for a large part of total ATP-phosphatase activity, when compared to other plant apyrases. Our data suggest that in contrast to all previously characterized plant apyrases, the potato specific apyrases do not appear to be attached to the cell membrane, but we cannot fully exclude a weak interaction with any other macromolecular structure. It is surprising that only little information is available documenting the fate or salvaging route of eAMP. Wormit et al. (2004) discussed that eAMP formed by apyrase could be reimported in the form of adenosine after dephosphorylation by already characterized unspecific cell wall bound phosphatases (Stephens and Wood, 1974; Tu et al., 1988). In fact we measured significant apoplastic cell wall localized phosphatase activity in the apoplast, which is in accordance to the previous reports. Unexpectedly, we found evidence that in the potato tuber apoplast, adenosine is actually further hydrolyzed to adenine and ribose by a cell wall bound adenosine specific nucleosidase (ANase), prior import in the form of adenine. This was surprising since it disagrees with the import route found in mammal systems, which is considered to be adenosine (Che et al., 1992; Ohkubo et al., 2007). Our data also show that apyrase and

ANase are coordinately regulated on the activity level. This provides evidence for a functional linkage, supporting a concerted action of both enzymes in the ATP salvage pathway. Unfortunately, no plant ANase has been cloned to date, making an identification of this enzyme at the genetic level relatively difficult. Cloning of a *Coffea arabica* ANase is probably underway, because it has been purified and characterized some time ago (Campos et al., 2005). Interestingly, like apyrase, this ANase also showed its maximum activity at pH 6, supporting that it could be localized in the more acidic apoplast. The salvage way is completed by a H^+-dependent adenosine-transporter, which could belong to the class of purine permeases due to its characteristics (see MS1).

It has been reported that ATP could enter the apoplast by secretion, wounding, transport, touch or osmotic stress (for review, see Roux and Steinebrunner, 2007), and the existence of this salvage pathway provides indirect evidence for the occurrence of significant amounts of eATP in the apoplast. Emerging evidence suggest that eATP is activating a signal cascade through binding to an as yet uncharacterized purinergic receptor at the cell membrane and is deactivated by the hydrolysis through apoplastic apyrases (see P2) Consequently, this leads to the question, whether eATP also has a signaling function in the potato tuber apoplast or may just occur outside the cell as a consequence of leakage without any signaling function.

The role of apoplastic apyrase in potato tubers

Transgenic alteration of the potato specific apyrase was used to obtain information whether this enzyme might have a function restricted only to the conversion of ATP to ADP and AMP with some metabolic importance, or whether this conversion could also be linked to purinergic signalling. Due to its apoplastic localization we expected that alterations in the level of this enzyme would also affect the concentrations of the adenylates in this compartment. Reduction of apyrase using the ubiquitous 35-promotor led to a reduction in whole plant growth and changes in tuber and flower development (see P2). Similar observations regarding plant growth were reported for a conditional Arabidopsis *AtAPY1* and *AtAPY2* double knock-out, which is not vital at a later stage of development (Wolf et al., 2007; Wu et al., 2007). The 35S-overexpressors had the opposite flower morphology and an earlier onset of flowering, suggesting that apyrase may have a more direct effect on flower development, an assumption supported by its high degree of expression in this tissue. Previous studies in Arabidopsis also suggested that apyrases could play a role in sexual reproduction because inhibition of *AtAPY1* and *AtAPY2* inhibited pollen germination (Steinebrunner et al., 2003). However, such subtle phenotypes are difficult to interpret due to a magnitude of pleiotropic effects caused by the ubiquitous reduction. We therefore focused on plants with reduced apyrase in the tubers only. These lines looked indifferently to the wild

type with exception of the tubers, which exhibited the same increased longitudinal growth like tubers from the 35S-RNAi-lines. Hence we assume that the tuber phenotype is a direct consequence of apyrase reduction in the tubers and not caused by apyrase reduction elsewhere in the plant. Metabolite profiling revealed that reduction of apyrase in the tubers had no significant effect on primary and in particular adenylate metabolite levels, suggesting that its ATP-hydrolyzing activity is not directly linked to the major adenylate pools within the cell. The fact that substrates (ATP/ADP) or product (AMP) of apyrase did not accumulate/decrease in the overall levels indicates that flux through this enzyme is – in contrast to its maximal activity – probably not very high *in vivo*. From this point of view it appears unlikely that the influence of apyrase on plant development is caused by limitation of apoplastic adenylate salvage activities alone, if at all.

It was found that tubers from these plants produced more starch per plant due to a higher starch concentration and a higher tubermass per plant. Transcript profiling revealed that in fact many genes involved in starch production, such as sucrose synthase, the plastidial glucose-6-phosphate translocator, ATP/ADP-translocators, ADP-glucose pyrophosphorylase and adenylate kinase were regulated in a way that would promote a higher starch production (see above). This, in analogy to the changes found regarding the flowers, can also be interpreted as an influence on the reproduction strategy (see also P2). However, the largest proportion of differentially expressed genes belonged to the class of extensins and related proteins. These findings indicate that apyrase affects plant development by alterations in nuclear gene expression. Extensins are regulators of polar growth and it has been shown that also eATP has an influence on growth activities (see P2). An influence of apyrase on polar growth would explain the relatively strong changes in tuber morphology, which had no metabolic trigger according to our analysis. Although it was technically not feasible to confirm an influence of apoplastic apyrase on eATP in tubers (or leafs), it is tempting to speculate that eATP is accumulating in the potato tuber apoplast of the apyrase-reduced lines. This could lead to a higher activation of purinergic receptors or so far unknown signaling components leading to alterations in nuclear gene expression like an increase in the expression of extensin genes. It was shown that plant cells specifically respond to eATP or non-hydrolyzable ATP-analoga (Jeter et al., 2004; Song et al., 2006). This response is characterized so far by an increase in the level of cellular Ca^{2+}, the production of reactive oxygen species and an increase in the transcript levels of genes associated with wounding in Arabidopsis. Although such a purinergic receptor has not been identified yet, the responses to eATP were prevented by inhibitors of mammalian purinergic receptors (Song et al., 2006; Wu et al., 2008), favoring the existence of a receptor with certain structural similarity to the mammal receptor.

Although more evidence, in particular the cloning of a purinergic receptor, will be needed to establish the concept of purinergic signaling in plants, our results are supportive to a role of potato specific apyrase in the termination of ATP-signaling in the apoplast, linking this signaling to differential expression of genes involved in polar growth.

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13. Acknowledgments

First, I would like to thank Dr. Peter Geigenberger for the excellent supervision of my work. His advice, his relaxed attitude and the generous support made science often being a pleasure than hard work only. I would also like to thank Dr. Alisdair Fernie, who conceived this work together with Peter and me and advised me throughout my PhD, not to mention the benefit regarding the English. I would like to acknowledge Prof. Lothar Willmitzer and Prof. Bernd Müller-Röber for the critical evaluation of my work during my PhD. I should have better heard on them in some respect. Many experiments were simply not realisable without the help and the autonomous work of Lukasz Grosman, Cornelia Wucke, Henrik Zauber and Chutima Ploychankul, who assisted in many experiments as student workers or practical students. I would also like to thank Dr. Eva Urbanczyk-Wochniak and Dr. Matthew Hannah for their support on the Microarray analysis, Dr. Ulrike Haensel and Dr. Peter Immerzeel for their help with the cell wall analysis and Dr. Gareth Catchpole and Ilse Balbo for help with the GC-MS analysis. I am very thankful to the people from the infrastructure, in particular I would like to mention here: Dr. Karin Koehl, Helga Kulka and Dirk Zerning (Greenteam), Romy Baran and Brigitte Buchwald (Tissue Culture), Sven Bornegräber (IT), Josef Bergstein (Photodocumentation) and Renate Bathe (Media Kitchen).

14. *Curriculum Vitae*

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Name: David Riewe
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Education

2003-present: PhD in Biochemistry at the Max-Planck-Institute of Molecular Plant Physiology in Potsdam, Department of Prof. Mark Stitt, Group of Dr. Peter Geigenberger on "Molecular Physiology, Energetic and Regulation of Plant Primary Metabolism", funded by the German Science Foundation. Working title: "*The relevance of adenylates and adenylate converting enzymes on metabolism and development of potato (Solanum tuberosum L.) tubers*".

1997-2003: Study of Biochemistry at the University of Potsdam, Diploma at the Max-Planck-Institute of Molecular Plant Physiology in Potsdam. Title: „*Herstellung und Charakterisierung von Pflanzen mit veränderter Apyrase Aktivität*". Grade Excellent (1,0 on a Scale of 1-4).

1996: Graduated at the Andreas-Vesalius-Gymnasium in Wesel, A Level Biology and English, Grade 1,9 (Scale 1-6).

Conferences

2006: 8th International Congress of Plant Molecular Biology, Sydney, Australia. Poster: "Potato apyrase regulates plant growth and development by modulating purinergic signals within the apoplast".

2006: 3rd International Symposium on Signals, Sensing and Plant Primary Metabolism, Potsdam, Germany. Poster: "Heterologous overexpression of apyrase leads to a decrease in cellular energy charge without changing the internal oxygen status of growing potato tubers resulting in various metabolic and morphological adaptations".

2004: 15th Havel-Spree-Kolloquium, Berlin, Germany. Presentation: “Importance of Adenylates in different subcellular Compartments”.

2004: 15th International Conference on Arabidopsis Research, Berlin, Germany. Poster: “Altered Apyrase activity influences potato (*Solanum tuberosum*) plant development and tuber yield”.

15. Publication list

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