Metabolic and Developmental Adaptations of Growing Potato Tubers in Response to Specific Manipulations of the Adenylate Energy Status

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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 (Solanum tuberosum) plants under the control of either inducible or constitutive promoters. Inducible apyrase expression in potato tubers, for a period of 24 h, 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 of amylose in these tubers. Furthermore, the levels of almost all amino acids were decreased, although soluble sugars and hexose-Ps 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 posttranslational redox activation of ADP-glucose pyrophosphorylase and a shift in the ratio of soluble 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.

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 (Solanum tuberosum) 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 Suc and its subsequent metabolism within starch biosynthetic (Viola et al., 2001; Geigenberger, 2003a; 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, whereas 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 that lead to translational or posttranslational regulation (Berg et al., 2002). Uridine nucleotides are involved as substrates in the pathway of Suc degradation via Suc synthase and UDP-Glc pyrophosphorylase (Geigenberger and Stitt, 1993), whereas adenine nucleotides are involved in the conversion of hexoses to hexose-Ps by hexokinase and their subsequent use, by ADP-Glc pyrophosphorylase

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Escherichia coli expressing an ADP, and AMP in growing potato tubers. For this specifically alter the ratios between the pools of ATP, and uridine nucleotides (Loef et al., 1999; Geigenberger et al., 2005) are colimiting for respiration and starch synthesis in growing potato tubers.

Although 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, 2003b). 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). Although 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, whereas it remains unclear whether these are due to direct oxygen signaling or to indirect signaling effects via changes in the adenylate energy status (Geigenberger, 2003b; 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 over-expressing an *Escherichia 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, whereas 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 h led to decreased ATP contents and specific alteration in metabolite profiles of growing tubers, indicating a general depression of Suc 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 that 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.

**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 no. AJ315184; Santapaola et al., 2002) omitting the first 70 nt that encode the endogenous N-terminal periplasma targeting sequence. This fragment was fused in-frame N terminally with the transit peptide of the spinach (*Spinacia oleracea*) ferredoxin-NADP⁺-reductase (FNR; Jansen et al., 1988) by cloning it into pART33-FNR, which possesses the tuber-specific B33-promotor (Rocha-Sosa et al., 1989; 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 promoter. The region from the alc promoter 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 Supplemental 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 because 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, 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 borehole (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 24 h, this procedure led to reproducible induction of transgene expression in a concentric region of 2 to 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.

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 24 h after treatment with acetaldehyde, whereas 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 because no leaky expression took place in tubers that were not treated.

The FNR transit peptide, which was fused to the apyrase, has been successfully used to direct enzymes into the plastid in previous studies (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 transit peptide, the size of the processed protein was determined using PAGE. The predicted size of the plastidial *E. coli* apyrase is 281 amino acids/31 kD for the unprocessed protein and 223 amino acids/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-kD protein (Fig. 1B). Furthermore, C-terminal His-tagged *E. coli* apyrase with a molecular mass of 233 amino acids/26 kD after processing in the periplasma was expressed in *E. coli*. This recombinant protein migrated approximately 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.

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 nontreated 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, whereas the adenylate energy charge and the ATP-AMP ratio were not altered (Fig. 2, E and G).

**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 24 h, 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 gas chromatography-mass spectrometry.

**Figure 2.** Adenylate levels in potato tuber tissue after apyrase induction for 24 h. A to C, ATP (A), ADP (B), and AMP (C). D, Total adenylates and corresponding adenylate energy-dependent parameter. E to G, Adenylate energy charge (E), ATP-ADP ratio (F), and ATP-AMP ratio ($n = 6–8$; G). Significant differences to the wild type according to the Student’s $t$ test are indicated with asterisks ($P < 0.05$).
(GC-MS) and enzymatic assays. The data are summarized in Figure 3, showing the changes in metabolite levels in 24-h-induced tubers of transgenic lines compared to wild-type tubers identically treated as control. In Table I, individual metabolites were correlated to ATP using the Spearman algorithm. Results of this correlation analysis are also indicated in the color code of Figure 3.

Induction of apyrase led to a consistent but nonsignificant decrease of Suc, hexoses, and UDP-Glc and a significant decrease of G1c-1-P in all transgenic lines, indicating inhibition of Suc transport or faster metabolism. Labeling studies using [14C]Suc, which was injected into the fine borehole of the tubers 24 h after induction showed that Suc metabolism was minorly, albeit, insignificantly inhibited to rates between 77% and 97% of the wild-type level in the transgenic lines (data not shown).

Although the levels of Glc and Fru did not correlate with ATP using the Spearman test (see Table I), the strong positive correlation between UDP-Glc and ATP indicates that Suc degradation via Suc synthase rather than invertase has been inhibited, which is in agreement with a more profound role of Suc synthase compared to invertase in growing tubers (Zrenner et al., 1995). These findings indicate that the availability of ATP regulates Suc degradation processes in growing tubers. Apyrase expression also led to a consistent but nonsignificant decrease in the level of ADP-Glc, 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-Glc and ATP shown in Table I.

Induction of apyrase also led to changes in metabolite intermediates involved in glycolysis and tricarboxylic acid (TCA) cycle. There was an increase in the levels of 3-phosphoglyceraldehyde (PGA), significantly in one out of three lines, which may be due to a shift in the glycolytic reaction of glyceraldehyde 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), whereas the levels of metabolites further downstream of the TCA cycle such as succinate, fumarate, and malate if anything decreased, marginally (although nonsignificantly). 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 I. The reaction product of succinate oxidation, fumarate, showed a high negative correlation to ATP (Table I). 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 Asp, pyruvate, or Ser family (Fig. 3). There was a relatively strong correlation between ATP and the levels of many amino acids (Table I). These correlations were highest for amino acids that are mainly synthesized in the plastid rather than the cytosol, such as Trp, Tyr, Phe, Met, Lys, Thr, and Ile. Only very minor influence of ATP was found on the levels of Asp, Asn, Glu, Gln, Gly, Val, Arg, and Pro. Given that Asp, Asn, Glu, and Gln are imported from the shoot rather than produced de novo in tubers (Karley et al., 2002; Koch et al., 2003), only the production of Arg, His, and Pro demands further ATP consumption for their synthesis starting from intermediates of glycolysis and the TCA-cycle. In contrast to this, eight out of the 12 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 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 His, Ile, Leu, Met Phe, Lys, Trp, and Tyr. 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 this 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 periplasmic targeting sequence.

The plasmid B33-apy was produced by cloning the region from the B33 promoter to the terminator from
Figure 3. (Legend appears on following page.)
the plasmid pART33-FNR-apy (see above) into pART27 (Gleave, 1992; for vector map, see Supplemental 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 (number 1) and 1330 (number 8) than in lines 1330 (number 45) and 1330 (number 32; Fig. 4A). Surprisingly, there was no change in the ATP content of tubers from the transgenic plants with the exception of the strongest line 1330 (number 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 three out of four of the transgenic lines, and the ATP-ADP ratio was significantly increased in all lines (Fig. 4G).

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 Figure 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 four 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 ($r = -0.85, P < 0.000001, n = 39$).

Potato starch is composed of two different macromolecules: amyllose, which is an α-1,4-linked linear Glc chain, and amylopectin, which also consists of α-1,6-

Table 1. Metabolites with significant Spearman correlation to ATP after apyrase induction for 24 h

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$r$</th>
<th>$P$</th>
<th>FDR $P$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-Glc</td>
<td>0.850</td>
<td>$&lt;0.000001$</td>
<td>$&lt;0.00005$</td>
<td>27</td>
</tr>
<tr>
<td>ADP-Glc</td>
<td>0.816</td>
<td>$&lt;0.000001$</td>
<td>$&lt;0.00005$</td>
<td>27</td>
</tr>
<tr>
<td>GTP</td>
<td>0.737</td>
<td>0.000008</td>
<td>0.0015</td>
<td>28</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.734</td>
<td>0.000013</td>
<td>0.0018</td>
<td>27</td>
</tr>
<tr>
<td>Ribonic acid</td>
<td>0.687</td>
<td>0.000054</td>
<td>0.0060</td>
<td>28</td>
</tr>
<tr>
<td>Suc</td>
<td>0.690</td>
<td>0.000069</td>
<td>0.0061</td>
<td>27</td>
</tr>
<tr>
<td>UTP</td>
<td>0.687</td>
<td>0.000076</td>
<td>0.0061</td>
<td>27</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.670</td>
<td>0.000179</td>
<td>0.00125</td>
<td>26</td>
</tr>
<tr>
<td>Fumarate</td>
<td>-0.622</td>
<td>0.000406</td>
<td>0.00253</td>
<td>28</td>
</tr>
<tr>
<td>Met</td>
<td>0.606</td>
<td>0.000803</td>
<td>0.00450</td>
<td>27</td>
</tr>
<tr>
<td>Threonate</td>
<td>0.599</td>
<td>0.000965</td>
<td>0.00491</td>
<td>27</td>
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<tr>
<td>Phenyl-Ala</td>
<td>0.595</td>
<td>0.001056</td>
<td>0.00493</td>
<td>27</td>
</tr>
<tr>
<td>Glycerol-1-P</td>
<td>0.572</td>
<td>0.001473</td>
<td>0.00635</td>
<td>28</td>
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<tr>
<td>Lys</td>
<td>0.580</td>
<td>0.001890</td>
<td>0.00756</td>
<td>26</td>
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<td>Leu</td>
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<td>0.002666</td>
<td>0.00994</td>
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<tr>
<td>Trp</td>
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<td>0.002841</td>
<td>0.00994</td>
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<td>Thr</td>
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<td>0.003856</td>
<td>0.01270</td>
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<td>Glycerol</td>
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<td>0.008909</td>
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<td>Iso-Leu</td>
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<td>0.014191</td>
<td>0.03973</td>
<td>27</td>
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<td>0.018000</td>
<td>0.04800</td>
<td>27</td>
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<tr>
<td>Glc-6-P</td>
<td>0.440</td>
<td>0.019273</td>
<td>0.04906</td>
<td>28</td>
</tr>
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</table>
branched glucan residues. The amylose-amylopectin ratio of potato tuber starch has been shown to be approximately 20% under normal conditions (Lloyd et al., 1999b). To investigate whether apyrase expression affects the amylose-amylopectin ratio, tuber starch was analyzed 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., 1999a).

Constitutive Expression of E. coli Apyrase Leads to 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.

**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...
apyrases, indicating that apyrases 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 Figure 8, showing the changes in metabolite levels or ratios in the transgenic lines compared to wild type.Suc was only slightly, but significantly increased in three out of four lines, whereas the levels of its degradation products such as Glc, Fru, and hexose-Ps increased significantly in at least three of the four lines with increasing apyrase activity (Fig. 8). In contrast, the level of UDP-Glc was not significantly changed.

Compared to wild type, metabolite levels at the end of glycolysis such as 3-PGA and phosphoenolpyruvate (PEP) were significantly decreased in all transgenic lines, with the exception of the strongest line 1330 (number 8), which had concentrations of these metabolites significantly above the wild-type level (Fig. 8). The decrease in PEP in lines 1330 (number 45), 1330 (number 32), and 1330 (number 1) was accompanied by a minor yet nonsignificant increase in pyruvate levels and a consequent significant decrease in the deduced PEP-pyruvate ratio in three out of 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 (number 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 because malate and Asp showed an almost identical pattern of change as PEP.

Constitutive apyrases 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 the 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 apyrases led to a strong decrease in the overall cellular redox state (Fig. 8). Fermentation products such as lactate, Ala, or glyceral-1-P displayed few changes that were conserved across the transgenic lines, indicating that fermentative metabolism was not induced in the transgenic tubers.

Expression of apyrases led to a marked decrease in the amino acid content of the tubers (Fig. 8). There was a strong decrease in Asn and Gln, significant in three and two lines, respectively. These are the predominant amino acids in potato tubers that account for approximately 40% of total tuber amino acid content (Koch et al., 2003; Stiller et al., 2007). The contents of most amino acids deriving from glycolytic intermediates were decreased (although the statistical significance of these decreases varies from metabolite to metabolite), with the exception of Ala and Ser, which were significantly increased (in at least two of the transgenic lines). In addition, two of the aromatic amino acids, Trp and Tyr, 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, Phe remained unchanged and there was a significant increase in the levels of phenylpropanoids such as caffeic acid (in two lines) and

Figure 7. Respiration rates and oxygen tensions in tubers constitutively expressing plastidial apyrases. 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 \( <30 \mu m \) \( (n = 15–30) \). Significant differences to the wild type according to the Student’s \( t \) test are indicated with asterisks \( (P < 0.05) \).
Figure 8. (Legend appears on following page.)
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), whereas PEP phosphatase activity significantly increased in two lines (Fig. 9B). No significant changes were observed in the activities of UDP-Glc pyrophosphorylase and hexokinase (data not shown). This pinpoints the importance of posttranslational 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 analyzed 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).

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 posttranslational redox activation of 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 dithiothreitol [DTT]; Fig. 9F) and optimal assay conditions (including 5 mM DTT; Fig. 9G), and is shown in Figure 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, 2003; Hendriks et al., 2003). The strong positive correlation between AGPase redox-activation state and starch content \( (r = 0.66, P = 0.0001, n = 37) \) and the high negative correlation between AGPase redox state and Glc-1-P \( (r = -0.61, P = 0.0001, n = 34) \) is in support of an important role of this redox-based posttranslational 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, whereas soluble starch synthase (SSS) activity progressively increased significantly in all lines (Fig. 9, I and J). This provides a possible explanation for the decrease in starch amyllose content in response to apyrase expression (see Fig. 6F). Previous studies with transgenic tubers showed that GBSS is crucial for the synthesis of amyllose, whereas SSS is mainly involved in amylepectin synthesis (Visser et al., 1991; Lloyd et al., 1999a).

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 con-

Figure 9. Enzyme activities associated with glycolysis, fermentation and starch synthesis in tubers constitutively expressing apyrase. A to J, Pyruvate kinase (A), PEP phosphatase (B), LDH (C), PDC (D), ADH (E), AGPase nonreductive assay (Vs; F), AGPase reductive assay (Vmax; G), AGPase redox-activation state (Vx/Vmax; H), GBSS (I), and SSS (J; n = 7–8). Significant differences to the wild type according to the Student’s t test are indicated with asterisks (P < 0.05).
cerning the direct consequences of a specific change in energy parameters in plants. In this article 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 transit peptide was cleaved off from the apyrase (Fig. 1). This can only happen within the plastid and argues that apyrase was properly targeted into this organelle. Because 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., 2002) and inconsistent with patterns obtained when the expression of apyrase is altered in nonplastidial compartments (D. Riewe, L. Grosman, A.R. Fernie, C. Wucke, and P. 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 24 h (Fig. 2, A 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 that are subsequently transformed to the cytosol and the mitochondrial 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 *Arabidopsis thaliana* 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 Suc degradation and metabolism, indicated by a decrease in the levels of Suc, Suc degradation products, organic acids involved in the TCA cycle, and most amino acids deriving from these pathways (Fig. 3). As the reduction in Suc was apparently not caused by an increased metabolism, it is highly likely that Suc 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 I), because most of the metabolites that were found to be significantly correlated to ATP showed positive correlations, whereas only two out of 22 showed negative correlation coefficients (fumarate and dehydroascorbate). Positive correlations to ATP were found for (1) UTP and GTP, indicating that adenine, uridine, and guanine nucleotide pools are interlinked via nucleoside-diphosphokinase, (2) Suc, UDP-Glc, and Glc-6-P, indicating that Suc degradation is linked to ATP, (3) ADP-Glc, indicating a link between ATP and AGPase in the plastid, (4) organic acids such as succinate and fumarate, indicating a link between ATP and succinate dehydrogenase in the TCA cycle, and (5) aromatic amino acids (Tyr, Trp, and Phe), amino acids of the Asp family (Met, Lys, Leu, Thr, and Ile) as well as His, 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-Glc 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, 2003b), wheat (*Triticum aestivum*) and rape seeds (*Brassica napus*; Vigeolas et al., 2003; van Dongen et al., 2004), and rice (*Oryza sativa*) 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 signaling. In this context, it remains to be determined whether ATP is acting as substrate, allosteric effector, or a signal in its own right.

Although a general inhibition of ATP-consuming processes such as Suc 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 cofor-mycin (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 (number 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 subcellular 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 Asp family, were manifested under long-term expression of ATP. Specifically the stimulation of respiration led 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 (number 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 subcellular compartments.

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-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 8 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 Supplementary 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 posttranslational 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., 2008). 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, whereas 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-Glc concentrations in the plastid, which will inhibit GBSS much stronger than SSS due to the differences in their $K_v$ values for ADP-Glc (Baba et al., 1990; Lloyd et al., 1999b). 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 pro-
tein phosphorylation and protein complex formation (Tetlow et al., 2004, 2008). 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, Ala, or glyceral-1-P in response to apyrase expression, indicating that fermentation is rather a feature of hypoxia, but is not activated in a low energy state. In addition, 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 (Nicotiana tabacum) leaves does not alter production of acetaldehyde or ethanol under normoxic conditions, but under low oxygen the production of both metabolites accumulated 8- to 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 Suc metabolism to avoid ATP-consuming processes, and maintenance of the energy charge, long-term responses also involve changes in carbon partitioning in favor of respiration, large changes in tuber morphology, and growth resulting in an increased surface-volume ratio.

MATERIALS AND METHODS

DNA Manipulation

Constitutive Tuber-Specific Plastidial Apyrase (B33-apy)

The DNA sequence corresponding to the mature protein sequence of the Escherichia coli apyrase gene (GenBank accession no. AJ35188) ranging from 70 to 741 nt (without periplasma targeting sequence) was amplified from the virulence plasmid pINV (kindly provided by Mauro Nicoletti) isolated from E. coli strain HN280 (Santapaula et al., 2002) using pfu polymerase and the primers eapyHolof (5'-CTG AAG GCA GAA GGT TCT CTC AC-3') and ecoliapyr (5'-CTG TGC ATC TGT GGC TCA GGG TAG GAT G-3'), the latter being flanked by a BamHI restriction site. The pART derivative pART3-FNR-PHAG (kindly provided by Dr. Volker Mittendorf), was digested with Ncol and blunt-ended by incubation with Klenow enzyme and dNTPs. After digestion of both the PCR product and the linearized plasmid with BamHI, the PCR product was ligated in frame to form a chimeric open reading frame (ORF) consisting of 165 nt coding for the spinach (Spinacia oleracea) FNR transit peptide (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 Ncol restriction sites.

Ethanol-Inducible Tuber-Specific Plastidial Apyrase (B33-alc-apy)

The sequence of the E. coli apyrase fused to the transit peptide of the spinach FNR 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 BamHI. The PCR product was digested with BamHI and ligated into the BamHI digested plasmid pUC19-alcA (obtained by Dr. Bjorn Jerskey). Accuracy of the coding sequence was confirmed by sequencing. The obtained plasmid was digested with HindIII to obtain the cassette containing the alc promoter, the chimeric ORF, and the 35S-terminator. This cassette was cloned into B33-alc-GUS, which was digested with HindIII to excise the region coding for the alc promoter, GUS, and nopaline synthase terminator.

Lactose/Isopropylthio-β-Galactoside-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<sup>+</sup>-3'f (5'-AAA ACC AAA AAC TTT CTT CTT TTT TG-3') and eapy s<sup>+</sup>-3'r (5'-TCC ACG ATC TCT GCG TCA GTT CAT TGC TAG GAC TA-3'), the latter being flanked by a BamHI restriction site. The plasmid pQE60 (QUIAGEN) was digested with Ncol and blunted with Klenow enzyme. After digestion of the PCR product and linearized plasmid with BamHI, 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 (Bio-Rad) as described by Mattanovich et al. (1989). The recombinant Agrobacteria were used to transform sterile, freshly injured leaves of potato (Solanum tuberosum) 'Desiree' by the method established by Rocha-Sosa et al. (1989).

Plant Growth Conditions

Potato plants 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 day/night [d/n], 22°C/16°C d/n, 150 μmol s<sup>-1</sup> m<sup>2</sup> light intensity, 16/18 h 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°C/16°C d/n, 350 μmol s<sup>-1</sup> m<sup>2</sup> light intensity, 16/18 h d/n) for eight or 10 weeks for the biochemical analysis, or for 13 weeks in an uncontrolled greenhouse for phenotypical characterization.

Induction of Apyrase in Tubers in Planta

Tubers attached to plants grown for 8 weeks were pierced with a coaxial needle (diameter, 0.9 mm; HS Hospital Service, Apriila, Latina, Italy). The duct was filled with 50 μL of 0.2% acetaldehyde solution and the pipette tip remained sticking in the duct filled with 150 μL of acetaldehyde solution. This allowed further supply with acetaldehyde and identification of the duct after the tuber was covered again with soil. After 24 h, only the part surrounding the duct was sampled with a cork borer (6-mm diameter) and immediately frozen in liquid nitrogen.

Incubation of Tubers with Different Oxygen Concentration in Planta

After 2 weeks of growth in a phytotron and 1 week of growth in the conditioned greenhouse, the pots were transferred into boxes (Allibert-Buckhorn; five plants per box) with water supply and grown for 2 weeks to allow the shoot to grow through a 0.5 cm high duct consisting of a round tissue culture vessel (6.5-cm diameter, Greiner Bio-One) 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 2 weeks the boxes were aerated constantly for another 5 weeks with gas with different oxygen concentration and (4%, 21%, and 40% oxygen, 0.005% carbon dioxide, rest nitrogen) mixed by a flow...
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) following the manufacturer’s instructions.

Real-Time PCR

RNA was extracted from 20 mg fresh weight using the RNeasy Plant Mini Kit (QUIAGEN) and DNA was digested on column as suggested by the supplier. RNA (200 ng) was used to produce complementary DNA (cDNA) in a final volume of 130 μL. For cDNA quantification, 2 μL were used as a template in a real-time PCR reaction mixed with 10 μL of Power SYBR-Green (Applied Bioscience) and 10 μL of primer mix (forward and reverse primer, each 0.5 μM). The following primer mixes were used for quantification: eapy (forward, 5'-GAG GCT GGC CCT TTA ATG GGA G-3'; reverse, 5'-GGG GTG AGT TCA TTG GTA GGA GT-3'), SIGAPDHS (forward, 5'-AAG GAC AAG GCT GCT GCT CAC-3'; reverse, 5'-AAC TCT GCC TGT TAT CTA TTC TCG-3'), and SIGAPDHE (forward, 5'-TTC AAC ATC ATC CCT GAG ACT 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 cycle values (±1) for the GAPDH cDNA 5’ and 3’ ends were considered for analysis.

PAGE and Western Blotting

Nonreducing 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 polyvinylidene difluoride membrane (Roche), the membrane was probed with a serum directed against E. coli apyrase (kindly provided by Francesca Berlutt). Binding was detected enzymatically using a secondary antibody conjugated to horseradish peroxidase (Bio-Rad).

Metabolite Analysis

GlC, FrU, Suc, hexose-Ps, UDP-Glc, 3-PGA, PEP, pyruvate, and starch were extracted or precipitated with 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 extracted or precipitated with TCA extracts as described by Jelitto et al. (1992) and preceding extraction and derivatization was performed as described by Roessner et al. (2001) using either a GCMS system consisting of a GC 8000 gas chromatograph and a Voyager quadrupole mass spectrometer (ThermoQuest) for the analysis of the plants expressing plastidial apyrase, or a GC 6890 gas chromatograph and a Pegasus II time-of-flight mass spectrometer (Leco) for the analysis of the plants expressing inducible apyrase.

Enzyme Analysis

Enzymes were extracted as described previously by Geigenberger and Stitt (1993) with Pefabloc and polyvinylpyrrolidone instead of phenylmethylsulfonyl fluoride in the extraction buffer and omitting bovine serum albumin. Enzymes were extracted from 30 to 100 mg of frozen homogenized tissue using 1,500 μL of 50 mM HEPES/KOH, pH 7.4, containing 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 10% glycerol, 2 mM benzamidine, 2 mM γ-aminocaproic acid, 0.5 mM Pefabloc, 5 mM DTT, and 0.1% polyvinylpyrrolidone. 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 X-100 was omitted.

Apyrase

Apyrase was measured via Pi release from ATP as described by Ames (1966) using a buffer optimized for the E. coli apyrase (50 mM HEPES/KOH, pH 7.4, 1 mM EDTA, 1 mM EGTA). One unit is defined as liberation of 1 μmol Pi per minute.

AGPase

AGPase was assayed in the absence or presence of DTT as described by Tiessen et al. (2002). Oxygen in extraction and assay buffer was displaced by N₂ before use. Extraction and subsequent measurement did not exceed 30 min.

UDP-Glc Pyrophosphorylase

UDP-Glc pyrophosphorylase 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.

LDH

LDH was quantified as previously described by Bergmeyer (1985).

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.

ADH

ADH activity was determined as described by Bergmeyer (1985).

SSS

SSS was extracted from 30 mg fresh weight without Triton X-100 and was assayed by determination of incorporation of [U-14C]ADP-Glc into amylopectin as described by Abel et al. (1996).

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, and 2 mM DTT. The pellet was then suspended in 500 μL of buffer and the reaction was started by the addition of [U-14C]ADP-Glc in a final concentration of 1 μM and a specific activity of 3.5 kBq/μmol. After several time points, aliquots of 100 μL were subtracted and the incorporation of [U-14C]ADP-Glc into amylose was determined as described for SSS.

Analysis of Respiration Rates

Respiration rates of two freshly prepared tuber discs (diameter, 8 mm; thickness, 2 mm) were analyzed in an oxygen electrode (Hansatech) filled
with 1 mL of 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) without previous washing with water. Without delay a MikroTX2 oxygen microsensor with a tip diameter of 30 μm (Presens; van Dongen et al., 2003) was phase-in, to a depth of 4 mm under the tuber skin, and the oxygen concentration was recorded until it was stable.

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

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Plasmids used for expression of apyrase in S. tuberosum and E. coli.

Supplemental Figure S2. Growth performance of tubers exposed to different oxygen concentrations.

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LITERATURE CITED


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Loef I, Stitt M, Geigenberger P (1999) Orotate leads to a specific increase in uridine nucleotide levels and a stimulation of sucrose degradation and starch synthesis in discs from growing potato tubers. Planta 209: 314–323

Loef I, Stitt M, Geigenberger P (2001) Increased levels of adenine nucleotides modify the interaction between starch synthesis and respiration when adenine is supplied to discs from growing potato tubers. Planta 212: 782–791


concentrations within developing seeds of oilseed rape. Plant Physiol 133: 2048–2060


