Interaction between phosphate starvation signalling and hexokinase-independent sugar sensing in *Arabidopsis* leaves

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**SEARCH**

Search Scope
Expression of selected genes in relation to phosphate (P$_i$) starvation and sugar sensing was studied in leaves of *Arabidopsis*. Excised leaf segments with different P status were supplied with combinations of P$_i$ and sugars. Sugar-inducible genes, encoding β-amylase (β-AMY) and chalcone synthase (CHS), were also induced by P deficiency, and were more strongly regulated by sugars when leaf segments originated from P-starved plants. Furthermore, transcript levels of the P-starvation-inducible genes ACP5 (encoding an acid phosphatase), RNS1 (encoding a ribonuclease), and IPS1 (unknown function) increased in response to exogenously applied sugars. Supply of P$_i$ to the leaf segments reversed both P-starvation-induced and sugar-induced gene expression. These interactions reveal a close relationship between P and sugar sensing. To differentiate between hexokinase-dependent and hexokinase-independent sugar sensing the effect of the glucose analogue 2-deoxyglucose and gene expression in the hexokinase-1 deficient mutant, gin2-1, were studied. Both β-AMY and CHS were induced by supplying sucrose to excised leaves but not by 2-deoxyglucose, confirming that these genes are regulated by hexokinase-independent sugar sensing. In the gin2-1 mutant both β-AMY and CHS responded clearly to P starvation excluding that hexokinase-1 mediates the response to P. Similarly, the P-responding genes, IPS1 and RNS1 were repressed by addition of P$_i$ also in the gin2-1 mutant. In
conclusion, several phosphate starvation-induced genes are also sugar-induced and hexokinase-independent sugar sensing in Arabidopsis is strongly intensified by phosphate starvation.

- Abbreviations

MYB

Myeloblastosis

PAR

photosynthetic active radiation

RT-PCR

reverse transcriptase-polymerase chain reaction

2-DOG

2-deoxyglucose.

Introduction

Phosphorus (P) plays a key role in plant growth and metabolism. P is taken up and assimilated as inorganic phosphate (P\textsubscript{i}). In the lithosphere, a considerable amount of P can be bound either to soil constituents or in organic matter. Therefore, in many soils P\textsubscript{i} is not readily accessible to plants and becomes limiting to growth (Holford 1997). As a consequence, plants have evolved strategies to obtain adequate P\textsubscript{i} and to use it more efficiently during P limitation. These include changes in root to shoot ratio, root morphology, uptake capacity, modification of carbohydrate metabolism, and secretion of phosphatases, protons and organic acids (Abel et al. 2002, Hammond et al. 2004).

These adaptations rely on regulation of gene expression in response to P starvation, and microarray analysis demonstrated extensive changes in expression pattern after withdrawing P (Hammond et al. 2003, Wu et al. 2003). Several genes induced by P starvation are directly involved in mechanisms to increase P uptake and remobilization. These are exemplified by genes coding for high- and low-affinity P\textsubscript{i} transporters (Raghothama 2000) and by acid phosphatases and ribonucleases (Bariola et al. 1994, del Pozo et al. 1999), which assist in releasing P\textsubscript{i} from both cellular and extra-cellular organic compounds. Other strongly induced genes, such as IPS1 (Martin et al. 2000) and At4 (Burleigh and Harrison 1999) have unknown function, but can be used as markers for P deficiency.

Although transcriptional regulation in response to P starvation is widely documented, there is limited information on the molecular mechanisms that sense and signal P\textsubscript{i} status. The discovery of a MYB-related transcription factor, PSR1, involved in P signalling in the algae Chlamydomonas reinhardtii (Wykoff et al. 1999) was an important step forward. In Arabidopsis,
a family of putative transcription factors has homology to CrPSR1. One member, PHR1, has been shown to mediate P-dependent gene regulation (Rubio et al. 2001), and this is the first P-starvation-associated transcription factor identified in higher plants. PHR1 appears to mediate a subset of P starvation responses (Rubio et al. 2001, Franco-Zorrilla et al. 2004). In soybean, binding of homeodomain leucine zipper proteins to a motif in a phosphate response domain suggests their involvement in P-modulated gene expression (Tang et al. 2001). Recently, the phenotype of an Arabidopsis mutant, pdr2, implied that the mutated gene is involved in monitoring environmental P status (Ticconi et al. 2004), but the PDR2 gene remains to be isolated. Simultaneous responses to P starvation might suggest co-ordinated expression of genes by a common regulatory system (Vance et al. 2003). However, the identification of sets of genes with maximal repression or activation at different time points (Hammond et al. 2003, Müller et al. 2004), and the observation of different response patterns in roots and shoots (Müller et al. 2004) indicate more than one signalling system involved in P sensing. Discovering the components of these signalling systems is an important challenge.

P₃ assimilation is closely integrated with carbohydrate metabolism. Most of the intermediates in carbohydrate metabolism are phosphorylated and P₃ is an allosteric regulator of key enzymes in primary metabolism. Gene regulation in response to sugar accumulation or depletion, sugar sensing, is essential to plant growth and development (Rook and Bevan 2003). Key components of a glucose signalling pathway have been identified (Smeekens 2000), and there is now evidence that hexokinase-1 (HXK1) is directly involved in sugar sensing in plants (Jang et al. 1997, Moore et al. 2003). Experiments with sugar analogues, transgenic plants and mutants selected for altered sugar sensing have revealed at least three distinct sugar signalling pathways in plants (1) the HXK1-dependent mechanism, in which gene expression is mediated by a HXK1 signalling function (2) a glycolysis-dependent mechanism influenced by the catalytic activity of HXK1 and (3) a HXK1-independent mechanism (Smeekens 2000, Xiao et al. 2000, Moore et al. 2003).

Sugar sensing interacts with phytohormones (León and Sheen 2003), with environmental response pathways, such as light response (Brocard-Gifford et al. 2003), and with responses to essential nutrients, such as nitrogen (Coruzzi and Bush 2001). It is reasonable also to expect an interaction between sugar sensing and regulation of gene expression by P. However, only few examples of cross talk between sugar- and P-response pathways are documented. In soybean, sugar-induced expression of VspB, a gene encoding a vacuolar acid phosphatase, was inhibited by P₃. The regulation of VspB by sucrose and P₃ suggests a common mechanism of transcriptional control, and promoter analysis identified a domain that mediated both sugar induction and P₃ repression. Several other sugar-inducible genes were repressed by P₃ in the absence of exogenous sucrose (Sadka et al. 1994). In tobacco seedlings, P₃ starvation promoted the expression of the gene encoding AGPase small subunit (AGPS2) and this effect was more prominent when sucrose was added. Feeding P₃ to excised leaves also decreased AGPS2 transcript level by antagonizing the induction by sucrose (Nielsen et al. 1998). In Arabidopsis, UDP-glucose pyrophosphorylase and the small subunit of ADP-glucose pyrophosphorylase (AGPase) were induced during P starvation (Ciereszko et al. 2001a, b). Finally, sugar induction of a P₃ transporter was demonstrated in Arabidopsis roots (Lejay et al. 2003). These studies call for a closer examination of the interaction between sugar- and P-dependent gene regulation.
A previous study of *Arabidopsis* (Müller et al. 2004) demonstrated recovery from P starvation as a valuable system to study P-dependent gene regulation. Plants were grown on limiting Pi concentrations and then re-supplied with Pi over short time periods. This provided plant material of similar development but with different Pi content. It was further demonstrated that isolated leaves responded to Pi supplied during short-term incubations, and similar experiments can be used for testing the interaction with exogenously supplied sugars.

The present study exploits this experimental approach to test the effect of sugars and Pi in combination. Our results reveal a close interaction between Pi- and sugar-dependent gene regulation. We demonstrate that genes known to respond to sugar accumulation are strongly induced during P deficiency, whereas feeding Pi to the leaf segments reverses sugar-induced expression. Furthermore, several P-starvation-inducible genes are induced by sugars. To discriminate between different sugar sensing mechanisms, the gin2-1 mutant (Moore et al. 2003) and incubation with the glucose analogue 2-deoxyglucose (2-DOG) were included, and the data reveal a close interaction between phosphate starvation signalling and sugar sensing.

**Materials and methods**

**Plant material and cultivation conditions**

Seeds of *Arabidopsis* ecotypes Columbia and Landsberg erecta (wild-type and gin2-1 mutants) were germinated on soil, first in the dark for 2 days and then transferred to a growth chamber [20°C, 70% relative humidity, and 120 µmol m⁻² s⁻¹ photosynthetic active radiation (PAR), over an 8-h photoperiod]. After 2–3 weeks the plants were transferred to 40 ml rockwool cubes (Rockwool, Hedehusene, Denmark). The plants were supplied with nutrient solution with limiting Pi concentration (0.05 mM) as described by Müller et al. (2004). Three weeks after transferring the plants to rockwool, one part of the plants was supplied with 4 mM for one week (high Pi), while the other plants remained on 0.05 mM Pi (low Pi). For the experiment with intact gin2-1 plants, control plants were grown at 1 mM Pi.

**Incubation of leaf segments**

The tip and the base of each leaf were excised and the remaining middle sections were immediately transferred to water. After collecting the leaf segments, they were floated for 16 h at 20°C under dim fluorescent light (50 µmol m⁻² s⁻¹) on solutions with different combinations of sucrose (100 mM), Pi (5 mM), 2-DOG (0.9 mM) and glucose (50 mM) or on just water.

**Experimental design**

All presented experiments were repeated twice, using plant material from independent biological experiments. Each sample represented plant material from 5 to 10 plants. Pi, P and sugar values reported are means of three (n) independent extractions.

**Extraction and determination of Pi, total P, anthocyanins, sugars and starch**
P, total P, anthocyanins, were extracted and quantified as described by Müller et al. (2004). Soluble sugars and starch were extracted as described by Nielsen et al. (1991), and sugars were quantified by enzyme-linked reduction of NAD\(^+\) as described by Beutler (1984) and Kunst et al. (1984). Assays were performed in microplates, and the absorbance was followed at 340 nm.

RNA extraction and reverse transcriptase-polymerase chain reaction

RNA was isolated and DNAase treated according to Müller et al. (2004). Synthesis of cDNA was conducted using Biorad reverse transcriptase-polymerase chain reaction (RT-PCR) kit according to manufacturer's specification using oligo (dT)\(_{15}\) as primer. PCR was performed as described by Müller et al. (2004) and the optimal number of cycles during PCR was determined as listed in Table 1. PCR-products were cloned and sequenced as described by Müller et al. (2004) and sequence analysis verified that products amplified by RT-PCR were as indicated in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene ID</th>
<th>Forward-primer 5′ → 3′</th>
<th>Reverse-primer 5′ → 3′</th>
<th>Product size</th>
<th>Cycles Nr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACP</td>
<td>At3g177</td>
<td>CTTTAGTATCTCCATGATGTCGTTGATGCAGAGTTGTAAGGAG</td>
<td>236</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>90</td>
<td>CC</td>
<td>TAAACCGGAAAAACAGTAAACACG</td>
<td>462</td>
<td>22</td>
</tr>
<tr>
<td>At4</td>
<td>At5g035</td>
<td>GAAAACCCCTAAAATGTCGCTAAAGAAGAATCGGACACG</td>
<td>439</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>IPS1</td>
<td>At3g099</td>
<td>GGGGATGGCCCTAATACAAATGAAGC</td>
<td>430</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>RNS1</td>
<td>At2g029</td>
<td>TGGGCTA ACT AAAAAAAGATGAAACGTGAAAATAG</td>
<td>820</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>β-AMY</td>
<td>At4g152</td>
<td>GCGGACCCAGAAAAAGTGAACACCATCaAATCCACAGAAACAG</td>
<td>441</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>CHS</td>
<td>At5g139</td>
<td>TTGGC ACT GCTAACCCTGAGGACC</td>
<td>501</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>CAB</td>
<td>At3g276</td>
<td>ATGGCCACTTCAGCAATCCAAACATTGACACGTGACCCAT</td>
<td>405</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>AGP</td>
<td>At5g483</td>
<td>CGAAGACCGCTTCTCCGATGAAACACTGACC</td>
<td>448</td>
<td>26</td>
<td></td>
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<tr>
<td>SPS</td>
<td>At5g202</td>
<td>GGGTGGATAGAGGGTGAGGTTGAGGTAGGTTG</td>
<td>435</td>
<td>24, 26</td>
<td></td>
</tr>
<tr>
<td>ACTN</td>
<td>At2g376</td>
<td>TGGGAACCTGGAATGTTAGAAGT</td>
<td>435</td>
<td>24, 26</td>
<td></td>
</tr>
</tbody>
</table>

Moore et al. (2003).
Results

Expression studies of genes involved in sugar metabolism

Plants with similar size but different phosphate status were produced by growing plants on limiting P$_i$ (0.05 mM), or by re-supply of starved plants with high P$_i$ (4 mM) for 7 days. Analysis of transcript levels by RT-PCR showed that the expression of β-AMY, and of the P-starvation markers IPS1 and RNS1 was higher in shoots of P-starved plants compared to plants supplied with 4 mM P$_i$ (Fig. 1). The gene encoding β-amylase (β-AMY) is also known as a sugar-regulated gene. To further investigate the effect of sugars and phosphate in combination, excised leaf segments derived from P-starved and P-supplied plants were incubated in solutions of sucrose, 2-DOG and P$_i$. Additional experiments included the hexokinase-deficient mutant, gin2-1.

Figure 1. Transcript levels of selected P-starvation-inducible or sugar responsive genes in starved or P-re-supplied Arabidopsis plants. Plants were grown for 4 weeks in inert media (rockwool) and supplied with a full nutrient solution containing a limiting level of P$_i$ (0.05 mM). Half of the plants were supplied with 4 mM P$_i$ for the last 7 days whereas the other half remained at limiting P$_i$. Levels of mRNA were determined by RT-PCR using primers specific for the genes IPS, RNS1, β-AMY, ACTIN as listed in Table 1.

Sugar and P$_i$ incubations of wild-type leaves

Initially, it was verified that leaf segments took up P$_i$ (Fig. 2A) and sugars (Fig. 2B) during the incubations. Leaf segments floated on 100 mM sucrose contained up to 30-fold more soluble sugars than leaf segments floated on water. A large fraction of the accumulated sugars was recovered as glucose and fructose. The experimental system provided three levels of P in the leaf segments. Leaves of plants grown on high P$_i$ for 1 week also had the highest P$_i$ content (12.4 ± 4.0 µmol g$^{-1}$ FW), whereas leaf segments of plants, which were P-starved during growth had a low P$_i$ content (0.15 ± 0.06 µmol g$^{-1}$ FW). Incubation of P-starved leaf segments in a solution with 5 mM P$_i$ resulted in an intermediary P$_i$ content (2.5 ± 0.8 µmol g$^{-1}$ FW).

Figure 2. Levels of P$_i$, soluble sugars, and mRNA in isolated leaf segments of P-starved Arabidopsis plants or P-re-supplied plants. Plants were grown as described in Fig. 1. At the end of the growth period, leaf segments were excised and incubated for 16 h in solutions containing different combinations of no sucrose (–Suc), 100 mM sucrose (+Suc), no P$_i$ (–P) or 5 mM P$_i$ (+P). (A), inorganic phosphate (black, P$_i$) and total phosphate (grey, P total). (B), Glc (black), Fru (light grey), and Suc (dark grey). Error bars indicate standard deviation (n = 3). (C), Levels of mRNA were determined by RT-PCR using gene specific primers (Table 1).

As expected, the P-starvation-induced genes ACP5, RNS1, At4 and IPS1 were all highly expressed in P-starved leaves in comparison with leaves from P-supplied plants. These genes
were also repressed by addition of \( P_i \) during incubation of the leaf segments (Fig. 2C). The data verify that the experimental procedure reveals starvation-dependent responses.

Sucrose incubation increased the transcript level of all four genes, \( ACP5 \), \( RNS1 \), \( At4 \), and \( IPS1 \). For \( ACP5 \), \( IPS1 \) and \( At4 \), this was most obvious at the intermediate \( P \) level (starved leaves incubated in presence of \( P \)). For \( RNS1 \) a clear induction was observed in leaf segments with low \( P \) levels.

The gene \( \beta\text{-AMY} \) was clearly induced in response to sucrose in leaf segments of plants grown on limiting \( P \). In plants re-supplied with high \( P \) during growth, \( \beta\text{-AMY} \) was also sugar-induced, but level of induced expression was much lower than in \( P \)-starved leaf segments (Fig. 2C). The genes \( AGPS \) and \( SPS \), coding for enzymes regulating starch and sucrose synthesis, were induced in response to sucrose incubation, independently of the \( P \) level of the tissue, and in contrast to the other genes, \( AGPS \) and \( SPS \) did not respond strongly to \( P \) starvation.

\( P_i \) provided either during the growth period or during the incubation reversed \( P \)-starvation response, and also prevented the sugar-induced expression of both \( \beta\text{-AMY} \) and the \( P \)-starvation-induced genes.

### Gene expression in response to 2-DOG

To investigate whether hexose metabolism or hexokinase-mediated signalling is required for the observed sugar-induced gene expression, \( P \)-starved leaf segments were incubated in presence of the sugar analogue 2-DOG, which is phosphorylated by hexokinase but not further metabolized (Jang and Sheen 1997, Klein and Stitt 1998).

The marker genes \( IPS1 \), and \( RNS1 \) and to some extent also \( ACP5 \) showed a clear repression in response to addition of \( P_i \), and both \( IPS \) and \( RNS1 \) were slightly induced (about 30% increase according to quantification of band intensities) by addition of sucrose to \( P \)-starved leaf segments (Fig. 3). The sugar responsive genes \( \beta\text{-AMY} \) and chalcone synthase, \( CHS \), were both induced in response to \( P \) starvation. In the absence of \( P_i \) in the incubation solution, addition of 2-DOG to the incubations did neither induce expression of the \( P \)-starvation-induced genes (\( ACP5 \), \( IPS1 \), and \( RNS1 \)) nor of the sugar responsive genes \( \beta\text{-AMY} \) and \( CHS \). On the contrary, addition of 2-DOG repressed the expression of these genes (Fig. 3). For \( \beta\text{-AMY} \) and \( CHS \) at low \( P \) content, transcript level in response to 2-DOG was even lower compared to both sucrose- and water-incubated leaves. In the presence of \( P \) in the incubation solution, \( \beta\text{-AMY} \) and \( CHS \) were clearly induced in response to sucrose, but not to the same extent by 2-DOG. Thus, neither on low or high \( P \) level, \( \beta\text{-AMY} \) and \( CHS \) were induced by 2-DOG.
In principle, sucrose and 2-DOG could influence P content of the leaf segments. At low P\textsubscript{i}, incubation in sucrose solution resulted in a considerable decrease of P\textsubscript{i} level in the leaf segments (Table 2). In opposition to this, 2-DOG rather resulted in an increase in P\textsubscript{i} in the leaf segments. At high P\textsubscript{i} the relative differences in measured P levels were small for both sucrose and 2-DOG.

Table 2. P content (P\textsubscript{i} and P total) of Arabidopsis leaf segments. Plants were grown and incubated in solutions of sucrose and 2-DOG as described in Fig. 3.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>P\textsubscript{i} (µmol g\textsuperscript{-1} FW)</th>
<th>P total (µmol g\textsuperscript{-1} FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM P\textsubscript{i}</td>
<td>water</td>
<td>0.17 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>100 mM sucrose</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>0.9 mM 2-DOG</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>5 mM P\textsubscript{i}</td>
<td>water</td>
<td>3.24 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>100 mM sucrose</td>
<td>2.55 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>0.9 mM 2-DOG</td>
<td>2.56 ± 0.23</td>
</tr>
</tbody>
</table>

As a control to verify that 2-DOG had triggered sugar signalling, the expression of the gene coding for chlorophyll-a binding protein (CAB) was analysed. In the presence of P, the transcriptional changes of CAB in response to sucrose and 2-DOG confirmed both sucrose- and 2-DOG-dependent repression. In the leaf segments incubated without P\textsubscript{i}, CAB was only slightly repressed in response to sucrose, but clearly repressed in response to 2-DOG.

The genes encoding enzymes regulating sucrose and starch biosynthesis, SPS and AGPS, were very slightly induced in response to sucrose. In contrast to β-AMY and CHS, the P-level had only little effect on the degree of sucrose induction.

**Characterization of gin2-1 mutants – intact plants**

Wild-type and gin2-1 were grown on limiting P\textsubscript{i} (0.05 mM), or sufficient P\textsubscript{i} (1 mM), and half of the P-starved plants were supplied with high P\textsubscript{i} (4 mM) for 1 week. Both the wild-type and the gin2-1 mutant showed a 76% reduction in shoot fresh weight in response to low P\textsubscript{i} (0.05 mM) in comparison with high P\textsubscript{i} (1 mM). Both wild-type and the gin2-1 mutant contained less P\textsubscript{i} and a higher level of anthocyanin, more sugars and starch in response to P starvation. When plants were grown at low P\textsubscript{i}, the gin2-1 mutant contained slightly more anthocyanin, sugars and starch and a higher level of P\textsubscript{i} in comparison with wild type. However, these differences were minor, and mutant and wild-type plants had quite similar phenotypes and fresh weights (data not shown).

Transcript levels of RNS1 and IPS1 were high in P-starved gin2-1 mutants as well as wild-type plants. RNS1 and IPS1 transcripts were not detectable either in plants re-supplied with P\textsubscript{i} for 1 week or in plants grown on 1 mM P during the whole cultivation period (Fig. 4). The genes β-AMY and CHS were highly expressed in the P-starved plants, and for β-AMY the extent of up-regulation was even higher in gin2-1 mutants than in wild-type plants (Fig. 4). β-AMY and CHS transcript levels were moderate in plants re-supplied with high P. No β-AMY transcript was
detectable in plants grown on 1 mM P, and CHS transcript was only weakly expressed in these plants. In wild-type plants, the expression of CAB decreased in response to P starvation, whereas in the mutant gin2-1 this gene was constitutively expressed (Fig. 4).

Figure 4. Transcript levels of selected genes in Arabidopsis wild-type plants and gin2-1 mutants. Plants were grown for 4 weeks in inert media (rockwool) and supplied with a full nutrient solution containing a limiting P_i (0.05 mM). Half of the plants were supplied with 4 mM P for the last 7 days of the cultivation period. Control plants were supplied with 1 mM P_i during the whole growth period. Level of mRNA was determined by RT-PCR using gene-specific primers (Table 1).

Glucose incubation of gin2-1 mutants – leaf segments

To further investigate P starvation and sugar-induced gene expression in gin2-1 mutants and wild-type plants, we analysed transcript levels in response to glucose and P_i added during incubation of leaf segments. Analysis of P, sugars and starch verified that the leaf segments efficiently took up P_i and glucose, and that a large fraction of the glucose was metabolized into sucrose, fructose and starch in both gin2-1 mutants and wild-type plants. Leaf segments harvested from plants re-supplied with high P during growth had clearly lower content of soluble sugars and starch than those from P-starved plants (data not shown).

The P-responsive genes IPS1 and RNS1 were clearly induced during P starvation of mutants and wild-type plants, both with and without glucose incubation (Fig. 5A). The genes coding for β-AMY and CHS were clearly sugar-induced in both mutants and wild-type plants grown on 0.05 mM P and the expression levels were increased in response to P starvation in both gin2-1 mutants and wild-type plants (Fig. 5A). Expression level of CAB was higher in leaves originating from P-starved than from P-supplied plants, both for wild-type and mutants. Anyway, exogenous glucose repressed the gene coding for CAB in wild-type plants, while glucose incubation did not alter transcript level in gin2-1 mutants neither at high nor at low P_i level (Fig. 5B).

Figure 5. Transcript level of selected genes in leaf segments of Arabidopsis wildtype plants and gin2-1 mutants. Plants were grown at limiting P_i (0.05 mM) for 4 weeks. Half of the plants were supplied with 4 mM P for the last 7 days of the cultivation period. Excised leaf segments were incubated for 16 hours in water (–Glc) or 50 mM glucose (+Glc). The level of mRNA was determined by RT-PCR using gene specific primers (Table 1). Panels A and B represent independent RNA isolations from the same plant material.

Discussion

This study reveals a close interaction between P_i and sugar sensing in Arabidopsis. We observed that both sugar-inducible genes respond to P_i and that P-starvation-inducible genes respond to
sugars. High P<sub>i</sub> concentration in the tissue reverses not only P-starvation responses but also sugar-induced expression of specific genes. P<sub>i</sub> and carbon assimilation are closely integrated, not the least in photosynthetic tissues. All the metabolic intermediates in carbohydrate metabolism are phosphorylated, and free P<sub>i</sub> needs to be constantly provided for ATP production. During P starvation the need for efficient recycling of the limited P pools will be imperative, and although P<sub>i</sub> is mobilized from the vacuolar pool to keep homeostasis in other cellular compartments (Foyer and Spencer 1986, Mimura 1999), P starvation has a profound effect on photosynthesis, growth and on carbon accumulation. Even in well-supplied plants P<sub>i</sub> can become limiting to photosynthesis (Sivak and Walker 1986). This imposes a strict demand for metabolic coordination, and P<sub>i</sub> itself is an allosteric regulator of key enzymes in the carbon flow for sucrose or starch biosynthesis. Regulation of gene expression represents another level of metabolic adaptation, and sugar-regulated expression is known to influence photosynthetic carbon metabolism (Paul and Pellny 2003). Recently, the mutant pho3 was shown to be defective in the AtSUC2 transporter (Lloyd and Zakhleniuk 2004). The mutant was originally identified on basis of its altered activity of acid phosphatase and an apparent P-deficient phenotype (Zakhleniuk et al. 2001). The observation that this phenotype relies on a deficient sugar transport verifies a direct relationship between P and sugar metabolism. Therefore, it is reasonable to expect coordination between P- and sugar-dependent gene regulation. Our hypothesis was that a considerable cross talk between P and sugar sensing exists, and that P<sub>i</sub>-status of leaf cells will be important to, and potentially directly involved in sugar sensing.

Therefore, we included selected genes known to be regulated by sugar sensing in our studies of P-starvation responses. One of these genes, encoding β-amylase, was strongly expressed during P deficiency and repressed by feeding P<sub>i</sub> to intact starved plants (Fig. 1). Arabidopsis has nine β-amylase or putative β-amylase genes. The gene included in this study (At4g15210) is known to be induced in response to sugar accumulation (Mita et al. 1995). These observations prompted us to further investigate the relationship between sugar- and P<sub>i</sub>-sensing, using a more controlled approach with detached leaf segments.

**Effect of sucrose and P<sub>i</sub> in combinations**

Quantification of P in the incubated leaf segments confirmed low and high levels of P<sub>i</sub> in starved and re-supplied plants, respectively. During incubation a considerable amount of P was taken up by the leaf segments, resulting in intermediate levels of P<sub>i</sub> (Fig. 2A). Quantification of soluble sugars (Fig. 2B) verified efficient uptake and metabolism of sucrose supplied during incubation. In addition, content of soluble sugars was increased two-fold in P-starved leaves compared with plants re-supplied with high P during the growing period. The selected genes ACP5, At4, IPS1 and RNS1 were all highly expressed in P-starved plants, and repressed by feeding P<sub>i</sub> during incubation or during the previous growth period (Fig. 2), which is in accordance with previous findings of other groups that these genes are P-starvation inducible (Bariola et al. 1994, Burleigh and Harrison 1999, del Pozo et al. 1999, Martin et al. 2000). All together, these data show that the experimental system is suitable for manipulating P<sub>i</sub> and sugar content in the leaf segments and for observing changes in gene expression in response to altered P and sugar content.

Interestingly, the P-responsive genes were also induced by sucrose incubation (Fig. 2C). For RNS1 this was most prominent in the leaf segments with low P content, and for ACP5, At4 and
IPS1 this was particularly clear at the intermediate levels of P in P-incubated leaves. This result was verified by several independent experiments (e.g. Figs 3 and 5A) and might be explained by the fact that leaf segments experience a decreased level of cellular P due to metabolic activity in response to sugar accumulation. For soybean, Sadka et al. (1994) also suggested that sugar-inducible gene expression in plants might be caused by a reduction in the cellular level of free P. However, in the present experiment this argument is weakened by the observation of sugar-induced expression on intermediate P level resulting from P incubation. In these leaf segments, the content of P was not reduced in sugar-incubated leaves compared with water control (Fig. 2A). This suggests that the observed sugar induction of P-responsive genes is a direct response to sucrose or another molecule that signals altered metabolism, rather than a response to a reduction of cellular level of free P.

The gene β-AMY was clearly induced in response to sugar incubation (Fig. 2C), and this response was strongly amplified in P-starved leaves. This result was verified by several independent experiments (e.g. Figs 3 and 5A) and a very similar response was observed for CHS, which is another gene supposed to be regulated by hexokinase-independent sugar sensing.

This suggests that either P or assimilation influences sugar signalling directly, or that P starvation is conditioning the tissue for a stronger signalling. Interestingly, the sugar-dependent induction of both the P-starvation-responsive genes and the sugar-responsive gene β-AMY was much less pronounced at the highest levels of P. This effect was most prominent for RNS1, IPSI and β-AMY, and clearly documents a close interaction between P and sugar sensing.

**Gene expression in response to 2-DOG**

Addition of glucose or restriction of sucrose export from leaves will lead to a decreased expression of genes encoding proteins involved in photosynthesis, such as RBCS, ATP-δ and CAB (Sheen 1990, Krapp and Stitt 1995). This effect can be mimicked by applying low concentrations of 2-DOG (Jang and Sheen 1994). 2-DOG is phosphorylated by hexokinases to form 2-deoxyglucose-6-phosphate, which is poorly metabolized (Klein and Stitt 1998). Applications of 2-DOG can be used tentatively to differentiate between hexokinase-dependent and hexokinase-independent sugar sensing. In principle, 2-DOG might also operate as a P scavenger. However, the level of 2-DOG used for the experiment was only 0.9 mM, and did not result in a marked reduction in P in the incubated leaf segments (Table 2). Any gene induction or repression by 2-DOG is therefore more likely to be caused by the signalling function of 2-DOG. Expression of CAB is regulated by hexokinase-dependent signalling (Jang et al. 1997, Xiao et al. 2000), and was used as a control to verify signalling by 2-DOG. Incubation with 2-DOG gave a clear decrease of CAB (Fig. 3). When P was added, the repression of CAB by 2-DOG was comparable to the effect of sucrose. Thus, 2-DOG had the expected signalling function.

In contrast to sucrose, 2-DOG did not induce the expression of β-AMY and CHS (Fig. 3). In leaf segments, which were not supplied with P, 2-DOG also did not induce any of the P-starvation-inducible genes, and the expression of β-AMY, CHS and IPSI was even clearly reduced by addition of 2-DOG. Opposed to this, sucrose in the absence of P induced the expression particularly of IPSI, RNSI, β-AMY and CHS. The lack of induction by 2-DOG suggests that the
studied P-starvation-inducible genes are all responding to sugars via a hexokinase-independent pathway. Two other genes, AGPS and SPS, were included to represent key enzymes in starch- and sucrose biosynthesis, respectively. Both genes were weakly induced by sucrose, but this was independent from P level in the leaf segments (Figs 2,3), and a slight but consistent induction by low P could be due to P-starvation-induced sugar accumulation.

These different response patterns to sucrose and 2-DOG suggest that different signal transduction pathways are involved and imply that P-signalling interacts synergistically with a hexokinase-independent pathway of sugar signalling. This is in accordance with results of Xiao et al. (2000) and Sheen et al. (1999), who described β-AMY and CHS as genes whose regulation is independent from hexokinase, whereas CAB was characterized as hexokinase-dependent (Xiao et al. 2000).

During incubation with 2-DOG without P in the incubation solution, we observed a slight increase in P_i concentration of the leaf tissue, and this may have triggered the decrease of transcript level observed for several of the investigated genes.

**Gene expression in the gin2-1 mutant**

To further differentiate between different types of sugar sensing, the glucose insensitive mutant gin2-1 was studied. This mutant has no detectable HXK1 protein, which operates as a glucose sensor (Moore et al. 2003). When intact plants were grown on different P regimes, β-AMY, CHS and the P-regulated genes all responded to P starvation in both wild-type and gin2-1 plants (Fig. 4). This excludes that HXK1-mediated signalling is required for the observed response of these genes to P_i and sucrose. In intact wild-type plants, CAB transcript decreased in response to P deficiency (Fig. 4), presumably due to the accumulation of soluble sugars in the P-starved leaves (data not shown). In gin2-1 plants no decrease in CAB transcript was observed in response to P starvation, confirming the hexokinase dependence of this gene regulation. Incubation of gin2-1 and wild-type leaf segments further substantiated that P starvation- and sugar-induced expression of β-AMY, CHS, IPS1 and RNS1 did not depend on a functional HXK1 protein (Fig. 5A). In wild-type plants, the experiments with isolated leaf segments further showed that glucose feeding resulted in repression of the gene coding for CAB. In gin2-1 mutant this repression of CAB by glucose was not observed (Fig. 5B). Repression of CAB is known as a hexokinase-dependent response, and our data verify that this response pathway was not operative in the mutant.

In conclusion, our data show P-starvation-induced gene expression, both for genes already known to be P regulated and for β-AMY and CHS, which are sugar-regulated via the hexokinase-independent pathway. Here, we provide evidence that hexokinase-independent sugar sensing is dependent on the P status of the tissue. Our data indicate that sugar sensing is intensified by P starvation, whereas supplying P_i to intact plants or via incubation of leaf segments reverses both the effect of P starvation and sugar-induced gene expression. It can also be concluded that P sensing of IPS1, RNS1 and ACP5 is not depending on HXK1 signalling. We therefore suggest that cellular P level or P turnover is a key factor in defining hexokinase-independent sugar sensing, and that sugar accumulation intensifies P starvation signalling.
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