Involvement of HLS1 in Sugar and Auxin Signaling in Arabidopsis Leaves

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Sugar regulates a variety of genes and controls plant growth and development similarly to phytohormones. As part of a screen for Arabidopsis mutants with defects in sugar-responsive gene expression, we identified a loss-of-function mutation in the HOOKLESS1 (HLS1) gene. HLS1 was originally identified to regulate apical hook formation of dark-grown seedlings (Lehman et al., 1996, Cell 85: 183–194). In hls1, sugar-induced gene expression in excised leaf petioles was more sensitive to exogenous sucrose than that in the wild type.

Exogenous IAA partially repressed sugar-induced gene expression and concomitantly activated some auxin response genes such as AUR3 encoding GH3-like protein. The repression and the induction of gene expression by auxin were attenuated and enhanced, respectively, by the hls1 mutation. These results suggest that HLS1 plays a negative role in sugar and auxin signaling. Because AUR3 GH3-like protein conjugates free IAA to amino acids (Staswick et al., 2002, Plant Cell 14: 1405–1415; Staswick et al., 2005, Plant Cell 17: 616–627), enhanced expression of GH3-like genes would result in a decrease in the free IAA level. Indeed, hls1 leaves accumulated a reduced level of free IAA, suggesting that HLS1 may be involved in negative feedback regulation of IAA homeostasis through the control of GH3-like genes. We discuss the possible mechanisms by which HLS1 is involved in auxin signaling for sugar- and auxin-responsive gene expression and in IAA homeostasis.

Keywords: Arabidopsis — Auxin — HOOKLESS1 — Sugar signaling.

Abbreviations: ARF, auxin response transcription factors; AuxRE, auxin response element; HKX, hexokinase; Suc, sucrose.

Introduction

In many plant species, sugar produced in source leaves is transported via the phloem to sinks such as roots, fruits and seeds or tubers. Sugar is an essential energy source and provides carbon needed for plant growth and development. In addition, sugar has hormone-like functions in regulating a variety of genes that control growth and development, e.g. seed germination, post-germinative growth, floral transition, leaf senescence and development of storage organs such as seeds or tubers (Bernier et al. 1993, Koch 1996, Jackson 1999, Smeekens 2000, Rolland et al. 2002, Borisjuk et al. 2004, Gibson 2005, Wingler et al. 2006).

Studies carried out over the past 10 years have begun to reveal the molecular mechanisms underlying sugar signaling in higher plants. Genetic screens for Arabidopsis mutants have provided powerful tools to identify genes involved in sugar signaling. A number of Arabidopsis mutants have been isolated based on the negative effects of sugar on seed germination and post-germinative growth. Many of the genes identified act in ethylene and ABA pathways, indicating a complex genetic interactions between ethylene, ABA and sugar signaling (Gazzarrini and McCourt 2001, Rolland et al. 2002, Rook and Bevan 2003, Gibson 2005). Genetic screens based on the expression of sugar-responsive endogenous genes or reporter transgenes have also been performed, and mutants with altered expression of sugar-responsive genes have been isolated (Rolland et al. 2002, Rook and Bevan 2003, Yoine et al. 2006).

Here, we report an unusual sugar response2 (uns2) mutation in Arabidopsis that causes altered expression of sugar-responsive genes. uns2 is a new allele of the hookless1 (hls1) mutation that causes defects in apical hook formation in dark-grown seedlings (Lehman et al. 1996). Apical hook formation, i.e. differential growth of hypocotyls in dark-grown seedlings, which is regulated by ethylene and light, coincides with the unequal distribution of auxin that stimulates cell elongation in hypocotyls (Schwark and Schierle 1992). Auxin is also known to play a pivotal role in many aspects of plant growth and development such as cell cycle control, apical dominance, the differentiation of root or vascular cells, and cell elongation during gravitropic and phototropic responses (Estelle and Klee 1994, Chen et al. 1999, Vanneste et al. 2005, Woodward and Bartel 2005). In dark-grown hls1 seedlings, patterns of auxin response gene expression were altered (Lehman et al. 1996), suggesting a possible involvement of HLS1 in auxin...
transport or auxin signaling. Li et al. (2004) identified suppressor mutations of \( hls1 \) that were allelic with loss-of-function mutations of the auxin response transcription factor gene, \( ARF2 \). These results showed that ethylene- and light-regulated differential cell elongation in hypocotyls of dark-grown seedlings was mediated by regulation of \( ARF2 \) in a \( HLS1 \)-dependent manner.

Here, we show that \( hls1 \) mutants exhibit greater sensitivity to exogenous IAA as judged by the expression of a selection of auxin response genes in leaves as compared with the wild type, suggesting that \( HLS1 \) plays a negative role in IAA-inducible gene expression. In addition, sugar-responsive gene expression in \( hls1 \) was more sensitive to exogenous sucrose (Suc) than the wild type. In this paper, we discuss the complex mechanisms by which auxin regulates sugar-responsive genes auxin homeostasis. Furthermore, this paper discusses the involvement of \( HLS1 \) in these processes.

**Results**

**hls1 mutations cause defects in sugar-responsive gene expression**

In order to identify genes that are involved in sugar signaling, we screened our activation-tagged mutant populations (Suzuki et al. 2004) and isolated putative mutants with altered levels of amylase activity and/or anthocyanin accumulation in excised leaf petioles that were supplied with Suc through the cut edges (Mita et al. 1995). One of these mutants was named unusual sugar response2 (\( uns2 \)). \( uns2 \) showed increased levels of amylase activity and anthocyanin accumulation in Suc-supplied leaf petiole as compared with the wild type (Fig. 1), implying a hypersensitivity to exogenous Suc in \( uns2 \). We further discovered that the left border of T-DNA was inserted \(~650 \text{bp downstream from the stop codon of} \ HLS1 \) on chromosome 4 and that \( uns2 \) genomic DNA sequence flanking the right border was identical to that of \( At4g38540 \), a gene located \(~360 \text{kb downstream from} \ HLS1 \), suggesting that a large genomic rearrangement occurs near \( HLS1 \) upon T-DNA insertion that caused a loss-of-function mutation in the \( HLS1 \) gene (data not shown). Indeed, \( uns2 \) seedlings grown in the dark did not form an apical hook, similar to \( hls1-1 \) (Supplementary Fig. S1). Furthermore, strong alleles of \( hls1 \) mutants such as \( hls1-1, hls1-7 \) and \( hls1-9 \) in the Columbia ecotype also showed increases in amylase activity and anthocyanin accumulation in Suc-supplied excised leaf petioles as compared with wild-type plants (Supplementary Fig. S2). Crossing a male \( uns2 \) with a female \( hls1-1 \) and \( hls1-7 \) did not complement the mutations, indicating that \( uns2 \) is an allele of the \( hls1 \) mutants (data not shown).

In order to investigate further the effects of the \( hls1 \) mutation on sugar-responsive gene expression, we analyzed sugar-induced and sugar-repressed genes in Suc-supplied leaf petioles. Expression of \( At\beta\text{-Amy} \) encoding cytosolic \( \beta \)-amylase and \( CHS \) encoding chalcone synthase, a rate-limiting enzyme for anthocyanin biosynthesis, was induced 20-fold or more by Suc in a day (Mita et al. 1995, Mita et al. 1997), whereas expression of \( APS \) encoding the small subunit of ADP-glucose pyrophosphorylase, a key enzyme for starch biosynthesis, was increased by Suc to a much lower extent than \( At\beta\text{-Amy} \) and \( CHS \) (Sokolov et al. 1998). Fig. 2 shows that \( At\beta\text{-Amy} \) and \( CHS \) transcripts in the wild type were induced by Suc in a dose-dependent manner. In support of the results in Fig. 1, their transcripts levels were 10–20 times higher in leaf-petioles treated with 5% Suc than in those not treated with Suc. In \( hls1 \) mutants, \( At\beta\text{-Amy} \) and \( CHS \) transcripts increased in a dose-dependent manner, but transcript levels were 3–6 times higher than those in the wild type. The \( APS \) transcript increased in a dose-dependent manner as well, but to a lesser extent in both wild type and \( hls1 \) mutants as compared with \( At\beta\text{-Amy} \) and \( CHS \) (Fig. 2). The \( APS \) transcript level was only twice as high in leaf-petioles treated with 5% Suc than in those without Suc in the wild type. In \( hls1 \) mutants, transcript levels were only 1.6- to 1.8-fold higher than in the wild type.

We used a photosynthetic gene, \( RBCS \) encoding the Rubisco small subunit, as representative of sugar-repressed genes, because it is subject to negative feedback regulation by sugars (Sheen 1990). In control (0% Suc) leaves, the \( RBCS \) transcript was present at equal levels in \( hls1 \) and the wild type (Fig. 2). Following treatment with Suc, \( RBCS \)
negatively regulate sugar-responsive gene expression by sensitive to exogenous Suc, suggesting that HLS1 caused expression of all sugar-responsive genes to be more than in the wild type (Fig. 2). Thus, the hls1 transcript levels decreased in a dose-dependent manner in real-time PCR and normalized relative to the total RNA. Relative levels of each transcript were determined by treated with 0–5% Suc for 24 h and harvested for the isolation of ion. Leaf-petioles excised from 3-week-old plants on 2% Suc were Effects of the hls1 mutation on sugar-responsive gene expression. Leaf-petioles excised from 3-week-old plants on 2% Suc were treated with 0–5% Suc for 24 h and harvested for the isolation of total RNA. Relative levels of each transcript were determined by real-time PCR and normalized relative to the ACT2 transcript level. For Atf1-Amy, CHS and APS, and for RBCS and HLS1, values for the wild type treated with 5% Suc and with 0% Suc were set at 1, respectively. All values are averages determined in three independent experiments. Error bars indicate one SD of the mean. n.d., not determined.

transcript levels decreased in a dose-dependent manner in hls1, but the decreases were more pronounced in hls1 than in the wild type (Fig. 2). Thus, the hls1 mutation caused expression of all sugar-responsive genes to be more sensitive to exogenous Suc, suggesting that HLS1 may negatively regulate sugar-responsive gene expression by affecting a common part of the signaling pathways for both sugar-induced and sugar-repressed gene expression.

HLS1 expression in vegetative and reproductive tissues was much lower (20-fold less) than that in etiolated seedlings in the dark (Lehman et al. 1996). hls1-1 and hls1-7 genes have base substitution mutations in the transcribed region (Lehman et al. 1996), and hls1-1 and hls1-7 transcripts were detected by Northern blot hybridization at a size that is identical to that of the wild type (data not shown). Fig. 2 shows that the levels of hls1-1 and hls1-7 transcripts were higher than that of the wild type, suggesting that HLS1 is subject to negative feedback regulation. A decrease in HLS1/hls1 transcripts by the presence of Suc was observed only in hls1 mutants, indicating that HLS1 is a conditional sugar-repressed gene only when Suc sensitivity is high as a result of the hls1 mutation. However, sugar repression of HLS1 was less than that for RBCS.

One explanation for the effects of the hls1 mutation on sugar-responsive gene expression is that hls1 cells incorporate more sugars than wild-type cells. We tested this possibility with plants grown on different concentration of Suc (Table 1). In both the wild type and hls1, the accumulation of soluble sugar and starch was increased by Suc in a dose-dependent manner, but hls1 mutants accumulated less total carbohydrate (soluble sugar and starch) than the wild type. For example, the starch level was higher in hls1 mutants grown on 5% Suc than in the wild type, possibly due to enhanced expression of APS. However, the total soluble sugar level was much lower in hls1 mutants than in the wild type, causing total carbohydrate levels to be lower in the mutant. These results do not indicate that hls1 cells are capable of incorporating more sugars.

Effects of the hls1 mutation on auxin-responsive gene expression

Altered expression of sugar-responsive genes by the hls1 mutation (Fig. 2) and the regulation of ARF2 by HLS1 during apical hook formation (Li et al. 2004) may suggest that sugar-responsive gene expression is affected by auxin. In order to examine this possibility, we analyzed the effects of exogenous IAA on sugar-responsive gene expression. We used hls1-7 for further analyses because hls1-7 plants more closely resembled wild-type plants than hls1-1 plants with respect to size (data not shown). Since sugar treatment of excised leaf-petioles has been performed for 24–48 h (Mita et al. 1997, Masaki et al. 2005, Yoine et al. 2006), we supplied exogenous IAA to excised leaf petioles for 24 h. Fig. 3 shows the effects of exogenous IAA on sugar-responsive gene expression in the wild type and hls1-7. In the wild type, IAA partially repressed sugar-induced accumulation of Atf1-Amy, CHS and APS transcripts.

![Graphs showing effects of hls1 mutation on sugar-responsive gene expression](https://academic.oup.com/pcp/article-abstract/47/12/1603/2277553)
Following induction by Suc, the ratios of increased \( \text{At}^{-}\text{Amy}, \text{CHS} \) and \( \text{APS} \) transcripts in the presence and absence of IAA were 0.30, 0.65 and 0.79, respectively [values were determined from relative values in Fig. 3 by the formula: ratios = (5\% Suc/IAA − 0\% Suc/IAA)/(5\% Suc − 0\% Suc)]. In \( \text{hls}1^{-7} \) mutants, IAA partially repressed induction of \( \text{At}^{-}\text{Amy}, \text{CHS} \) and \( \text{APS} \) transcripts by Suc. The ratios of \( \text{At}^{-}\text{Amy}, \text{CHS} \) and \( \text{APS} \) transcripts following Suc treatment in the presence and absence of IAA were 0.50, 0.70 and 0.87, respectively. Thus, the inhibitory effect of IAA on sugar-induced gene expression occurred to a lesser extent in \( \text{hls}1 \) mutants as compared with the wild type, although the \( \text{hls}1 \) mutation only partially relieved IAA repression of Suc-induced gene expression. The effect of the \( \text{hls}1 \) mutation was the least pronounced with the \( \text{APS} \) transcript, which was similar to the effects of the \( \text{hls}1 \) mutation on the Suc-increased \( \text{APS} \) transcript. These results suggest that \( \text{HLS}1 \) may be partially involved in the negative effects of IAA on sugar-responsive gene expression.

To test the involvement of \( \text{HLS}1 \) in auxin signaling, we examined several early auxin response genes, \( \text{AUXIN \ UPREGULATED1 (AUR1)} \) encoding SAUR-AC1-like protein, \( \text{AUR3} \) encoding GH3-like protein, and \( \text{IAA2} \) in addition to \( \text{HAT2} \) encoding a protein of the HD-Zip II subfamily which was also activated rapidly by exogenous auxin (Sawa et al. 2002). \( \text{AUR1} \) and \( \text{AUR3} \) transcripts were highly induced by IAA in both the wild type and \( \text{hls}1 \), but transcript levels were 2-fold higher in \( \text{hls}1^{-7} \) as compared with the wild type (Fig. 4). In contrast, \( \text{HAT2} \) and \( \text{IAA2} \) transcripts increased to a lesser extent than those of \( \text{AUR1} \) and \( \text{AUR3} \) in the wild type (Fig. 4). In \( \text{hls}1^{-7} \), \( \text{HAT2} \) transcripts were several-fold higher than those in the wild type in the absence and presence of IAA, whereas increases in \( \text{IAA2} \) transcripts by IAA were very weakly affected by the \( \text{hls}1 \) mutation. These results indicate that \( \text{HLS}1 \) is involved in auxin-regulated expression of some, but not all, auxin response genes. In the presence of Suc, the levels of \( \text{AUR3} \) transcript, but not of the other three transcripts, was clearly affected by the \( \text{hls}1 \) mutation.

On the other hand, application of exogenous IAA caused decreases in \( \text{RBCS} \) transcript to some extent in both the wild type and \( \text{hls}1 \), but decreases in the transcript appeared to be more significant in \( \text{hls}1 \) than in the wild type (Fig. 3). The negative effects of exogenous IAA on photosynthetic gene expression confirm previous observations that auxin repressed photosynthetic gene expression in \textit{Arabidopsis} cotyledons of dark-grown seedlings (Gil et al. 2001). Negative effects of IAA on the levels of \( \text{HLS}1/\text{hls}1 \) transcripts were observed only in \( \text{hls}1 \) mutants, which were similar to the negative effects of Suc on the \( \text{hls}1 \) transcript (Fig. 3).

### Involvement of \( \text{HLS}1 \) in auxin homeostasis

Auxin-inducible expression of \( \text{AUR3} \) encoding GH3-like protein is up-regulated by the \( \text{hls}1 \) mutation (Fig. 4). Even in the absence of exogenous IAA, \( \text{AUR3} \) transcripts were approximately four times higher in \( \text{hls}1 \) than in the wild type, with relative values representing \( \text{AUR3} \) transcripts in the absence or presence of Suc in Fig. 4 being 0.011 and 0.049, or 0.020 and 0.077 in the wild type and \( \text{hls}1^{-7} \), respectively. Staswick et al. (2002, 2005) reported that the \( \text{AUR3} \) protein and six other GH3 homologs exhibited IAA adenylation activity, an initial step in the conjugation of free IAA to amino acids.

### Table 1  Carbohydrate accumulation (mg g fresh weight) in \( \text{hls}1 \) leaves

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Suc in medium</th>
<th>Soluble sugar (A)</th>
<th>Starch (B)</th>
<th>Total carbohydrate (A+B)</th>
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<tbody>
<tr>
<td><strong>Wild type</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1.7%</td>
<td>1.72</td>
<td>0.28</td>
<td>1.99</td>
<td></td>
</tr>
<tr>
<td>3.4%</td>
<td>4.01</td>
<td>0.58</td>
<td>4.59</td>
<td></td>
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<tr>
<td>5.1%</td>
<td>8.46</td>
<td>1.16</td>
<td>9.62</td>
<td></td>
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<tr>
<td><strong>hls1-1</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>1.7%</td>
<td>1.25</td>
<td>0.17</td>
<td>1.41</td>
<td></td>
</tr>
<tr>
<td>3.4%</td>
<td>3.00</td>
<td>0.43</td>
<td>3.42</td>
<td></td>
</tr>
<tr>
<td>5.1%</td>
<td>5.53</td>
<td>2.41</td>
<td>7.94</td>
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<tr>
<td><strong>Wild type</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>1.7%</td>
<td>1.43</td>
<td>0.28</td>
<td>1.70</td>
<td></td>
</tr>
<tr>
<td>3.4%</td>
<td>2.25</td>
<td>0.51</td>
<td>2.76</td>
<td></td>
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<tr>
<td>5.1%</td>
<td>5.35</td>
<td>0.76</td>
<td>6.11</td>
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<td><strong>hls1-1</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1.7%</td>
<td>1.05</td>
<td>0.22</td>
<td>1.27</td>
<td></td>
</tr>
<tr>
<td>3.4%</td>
<td>1.73</td>
<td>0.36</td>
<td>2.09</td>
<td></td>
</tr>
<tr>
<td>5.1%</td>
<td>2.59</td>
<td>1.65</td>
<td>4.24</td>
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</tr>
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</table>

Wild type and \( \text{hls}1^{-1} \) were grown on 1.7, 3.4 and 5.1\% Suc for 3 weeks. Mature leaves were immediately harvested to determine soluble sugar (glucose, fructose and Suc) and starch accumulation.
suggesting its role in a negative feedback regulation of auxin levels. Similarly, activation of GH3-like genes by an elevated level of free IAA may cause a decrease in free IAA levels because GH3 proteins cause conjugation to IAA-amino acid. In order to test the possibility that the free IAA level is altered by the hls1 mutation, we measured the free IAA level in hls1-7. Table 2 shows that the free IAA level in hls1-7 leaves was approximately 67% of that in the wild type. The lower free IAA level in hls1 relative to the wild type may explain why mutant plants exhibit low auxin phenotypes such as decreased apical dominance (Lehman et al. 1996) and small rosette leaves (Table 2) (Ouyang et al. 2000).

Discussion

HLS1 regulates sugar-responsive gene expression

HLS1 was originally identified as a gene that regulates differential growth of hypocotyls in dark-grown seedlings...
Table 2  

<table>
<thead>
<tr>
<th>hls1 leaves contained reduced levels of free IAA</th>
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<tr>
<td>Free IAA (ng g⁻¹ fresh weight)</td>
</tr>
<tr>
<td>Wild type</td>
</tr>
<tr>
<td>hls1-7</td>
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<td>hls1-1</td>
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Plants were grown on 3% Suc for 3 weeks, and mature leaves were used to determine levels of free IAA. Values ± SD for free IAA levels are the average determined in five independent experiments. The mean values ± SD for total weights of the third to sixth leaves per plant were determined in one of the experiments. Differences between values for the wild type and each hls1 mutant are significant at the <0.005 level. Numbers in parentheses indicate the number of plants used to measure leaf weight. n.d.: not determined.

(Lehman et al. 1996, Li et al. 2004). Here we show that loss-of-function hls1 mutants are hypersensitive to Suc for the expression of sugar-responsive genes in mature leaves (Figs. 1, 2). How does HLS1 regulate the expression of sugar-responsive genes? One possibility was that the hls1 mutation affects the uptake of sugars into cells. However, determination of glucose, fructose, Suc and starch levels in hls1 cells did not accumulate sugars more actively than wild-type (Table 1), suggesting that the hls1 cells did not accumulate sugars more actively than wild-type cells (Table 1), suggesting that the hls1 mutation affects a sugar signaling pathway. We show here that both sugar induction of Atβ-Amy, CHS and APS expression and sugar repression of RBCS expression were enhanced by the hls1 mutation (Fig. 2), suggesting that HLS1 may control a common step in the sugar signaling pathways for sugar-induced genes and sugar-repressed genes (Fig. 5).

Jang and Sheen (1997) proposed that there are at least three sugar signaling pathways: (i) a hexokinase (HXK)-dependent pathway in which HXK acts as a sugar sensor; (ii) a glycolysis-dependent pathway where certain metabolites downstream of hexose phosphorylation could serve as signals; and (iii) an HXK-independent pathway possibly involving a sugar receptor on the plasma membrane. HXK is a branch point of the HXK-dependent pathway and the glycolysis-dependent pathway. Sugar-repressed genes, such as RBCS and CAB, are known to be regulated under the control of the HXK-dependent pathway (Jang and Sheen 1994, Jang et al. 1997). Sugar-induced expression of Atβ-Amy, CHS and APS genes is under the control of the glycolysis-dependent pathway (Ohoto et al. in preparation). Therefore, although more direct evidence is needed to elucidate how HLS1 controls sugar-responsive gene expression at the molecular level, one possible mechanism is that HLS1 may control HXK activity, a common step of the HXK-dependent pathway and the glycolysis-dependent pathway.

HLS1 negatively regulates auxin-responsive gene expression

The hls1 mutation enhances auxin-induced expression of several auxin response genes such as AUR1 encoding SAUR-AC1-like protein, AUR3 encoding GH3-like protein and HAT2 encoding a protein of the HD-Zip II subfamily, but not IAA2 (Fig. 4). These findings indicate a negative role for HLS1 in auxin-induced expression of some auxin response genes (Fig. 5).

Expression of early auxin response genes is regulated positively and negatively by auxin response transcription factors (ARFs) that bind to auxin response elements (AuxREs) in the upstream regions of auxin response genes (Umasov et al. 1999a, Ulmasov et al. 1999b, Hagen and Guilfoyle 2002, Liscum and Reed 2002). ARF5–ARF8 activate and ARF1–ARF4 and ARF9 repress the activity of auxin-responsive synthetic promoters containing AuxREs (Tiwari et al. 2003). ARF1–ARF4 and ARF6–ARF10 are uniformly expressed in cultured cells, roots, rosette leaves, cauline leaves and flowers, and ARFs transcripts (ARF1, ARF2 and ARF4–ARF8) were unchanged in response to exogenous auxin (Umasov et al. 1999a, Hagen and Guilfoyle 2002).

Li et al. (2004) proposed that ARF2 negatively regulates apical hook formation in a HLS1-dependent manner. Consistent with this interpretation, results obtained in mature leaves suggest that ARF activators or repressors may require HLS1 to negatively regulate AUR1, AUR3 and HAT2 in a HLS1-dependent manner. For example, ARF activators that are negatively regulated by HLS1 may regulate AUR1, AUR3 and HAT2. In this model, HLS1 decreases ARF protein levels by decreasing the stability of ARF activators or increasing the stability of Aux/IAA proteins that repress ARF activators.
(Hagen and Guilfoyle 2002). Alternatively, HLS1 may increase ARF repressors that regulate AUR1, AUR3 and HAT2. On the other hand, it is not clear why the hls1 mutation did not affect IAA-induced IAA2 expression significantly. The reason may be due to the difference in tissue-specific expression patterns of HLS1 and IAA2, since IAA2 showed tissue-preferential expression in leaves (Marchant et al. 2002). However, cell- or tissue-specific expression of HLS1 in leaves has not been reported, yet.

The hls1 mutation causes altered expression of sugar-responsive genes (Figs. 1, 2), which also implies that sugar-responsive gene expression is regulated by HLS1 through its role in auxin signaling. HLS1 encodes a protein homologous to N-acetyltransferase (Lehman et al. 1996). N-Acetyltransferases transfer the acetyl group from acetyl coenzyme A to the amino group of proteins, peptides and small molecules. N-terminal acetylation of proteins is known to affect protein function and stability (Kendall et al. 1990). Li et al. (2004) proposed that HLS1 may acetylate ARF2 or other components to regulate the stability of ARF2. Since as many as 50–80% of eukaryotic proteins are N-terminally acetylated (Driessen et al. 1985), it may be possible that HLS1 has a wide spectrum of substrates, including ARF(s), to regulate sugar signaling. Our findings that exogenous IAA partially repressed sugar-induced gene expression and that the IAA repression was less pronounced in hls1 than in the wild type (Fig. 3) may suggest that the negative effect of IAA on sugar signaling is mediated at least in part by ARF(s) in a HLS1-dependent manner.

Role of HLS1 in IAA homeostasis

The activities of group II GH3-like proteins from Arabidopsis including AUR3 that conjugate free IAA with amino acid (Staswick et al. 2002, Staswick et al. 2005) suggest a negative feedback regulation of auxin levels. Activation of GH3-like genes by elevated levels of free IAA increases production of IAA-amino acids, resulting in a decrease in the effective IAA concentration. Both an enhancement of AUR3 expression (Fig. 4) and reduction in the free IAA level (Table 2) in hls1 mutant leaves suggest an involvement of HLS1 in the negative feedback regulation of free IAA level through its role in controlling GH3-like genes (Fig. 5). Tian et al. (2004) reported that overexpression of ARF8 increased gene expression for group II GH3-like proteins and decreased the free IAA level to 80% of that in the wild type in light-grown Arabidopsis seedlings. Thus, auxin levels are regulated at least in part through the activities of genes encoding GH3-like proteins that are in turn regulated by ARF activators and repressors, and by HLS1.

Materials and Methods

Plant materials and growth conditions

hls1-1, hls1-7 and hls1-9 in the Columbia ecotype were obtained from the Arabidopsis Biological Resources Stock Center (Ohio State University, Columbus, OH, USA). uns2 in the Wassilewskija (Ws) ecotype was isolated from a population of plants mutagenized with T-DNA (Suzuki et al. 2004). uns2 seeds are immediately available upon request. Sterilized seeds were imbibed, then treated in the dark at 4°C for 4 d before sowing. Plants cultured in vitro were grown on medium that contained 2% (w/v) Suc, unless otherwise indicated. 0.3% (w/v) Gellan Gum, 2.5 mM MES-KOH (pH 5.7), 1× MS salts and B5 vitamins at 22°C under continuous light. The light intensity during plant growth was 50 μmol m−2 s−1. For apical hook analyses, seeds sown on medium that contained 2% (w/v) Suc, 1.2% agarose, 1× MS salts and B5 vitamins were illuminated for 12 h to accelerate germination. Plates were subsequently placed vertically in the dark for 4 d.

Treatment of excised leaf-petioles with Suc, and determinations of amylase activity and anthocyanin

For experiments using leaf-petioles treated with Suc, the third to sixth rosette leaves were excised with a sharp razor blade from 3-week-old plants grown on 2% Suc, unless otherwise indicated. The cut edges of petioles were immersed in a sterile solution of sugar and incubated at 22°C under continuous light. To determine levels of amylase activity and anthocyanin, tissues from 10–15 plants were combined before the extraction procedures. Protein extractions and the determination of both protein levels and total amylase activities were performed as described previously (Mita et al. 1995). One unit of total amylase activity was defined as the amount of enzymes that released 1 μmol of reducing sugar per min under the assay conditions. Extraction and determination of anthocyanin were carried out as described previously (Mita et al. 1997).

Quantification of free IAA, soluble sugars and starch

Plants were grown on 3% Suc for 3 weeks, and mature leaves at the third to sixth leaf position were used to determine free IAA, soluble sugar and starch levels. Quantification of the free IAA level was performed as described previously (Woodward et al. 2005). Determination of soluble sugars and starch was performed as described by Mita et al. (1997).

RNA preparation and expression analysis

Total RNA isolation and Northern blot hybridization were performed as described previously (Mita et al. 1995). For quantitative analyses of transcript levels by real-time PCR, total RNA was treated with DNase I and subsequently purified with an RNeasy RNA purification column (Qiagen, Hilden, Germany). First strand cDNA was synthesized from 2.5 μg of total RNA using oligo(dT)20 primers using SUPERSCRIPT III (Invitrogen, Carlsbad, CA, USA) and diluted with 10 μl of water. Real-time PCR was performed in duplicate on a 25 μl reaction mixture containing 5 μl of diluted cDNA solution, 12.5 μl of a Cybergreen dye set (Bio-Rad, Hercules, CA, USA), and 0.5 μl of each primer (final concentration of 200 nM). PCR was initiated with denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 30 s. The comparative threshold cycle method was used to determine the relative mRNA levels. ACT2 was used as an internal reference, and transcripts levels
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were presented as values relative to the control treatment. The following primer sets were used: ACT2 [At3g18780, GI (GenBank Gene Info Identifier number): 1669386], 5'-CTG TTGACTACGAGCAGGATGGA-3' and 5'-GACTTCTGG GCATCTGAATCTCTCA-3'; APS [At5g48300, GI: 1575753], 5'-GTCAGATCATACACAGGCACACG-3' and 5'-GGGAT TAAGGGCTGTATGGTAAACC-3'; Athy-Amy [At4g15210, GI: 166601], 5'-CACTGAGATTGTGCCGTTGAA-3' and 5'-CCAGGAAGGATGGAACCTCGG-3'; AUR1 [At2g18010, GI: 20197595/ T27K22.12], 5'-GAAAGATGATCTTCCTCAAGACGTGC-3' and 5'-GGGACGAATTGTTGGAACCTCGG-3'; AUR2 [At4g37390, GI: 4468881/F6G17.40], 5'-GAGGCTCACACTTATCATGGAGCTG-3' and 5'-CTTAAACGGCACCATTCTCGGTTG-3'; C5 [At2g13930, GI: 166669], 5'-GGAGGAAAGTACGTAAGGATGTTG-3' and 5'-AGGGAACTGGTCTGTTGATAAGG-3'; HAT2 [At5g47370, GI: 527626], 5'-AGAGGCTATGAGGCTTGACACCCT-3' and 5'-CCTGTGATTGGTGATGGTATGGTACCC-3'; HSL1 [At4g37580, GI: 1277089], 5'-TCTACTATTCCACACTACCTCCTCC-3' and 5'-CCCTTACACCACCGCTATGTTTG-3'; IAA2 [At3g23030, GI: 2598931], 5'-GCA ATGGCGTACGAGAAGATTCAAC-3' and 5'-CATACGAGTTCTCCTCAAATAGACG-3'; RBCS [At5g38410, At5g38420, At5g38430, GI: 2264320/MXI10.13–MXI10.15], 5'-GGTTGAAGAGTCAGGATGACC-3' and 5'-GGGCTTGTAGGCAATGAACACTGG-3'.

Supplementary material
Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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References

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