Signaling from an Altered Cell Wall to the Nucleus Mediates Sugar-Responsive Growth and Development in Arabidopsis thaliana

Yunhai Li, Caroline Smith, Fiona Corke, Leiying Zheng, Zara Merali, Peter Ryden, Paul Derbyshire, Keith Waldron, and Michael W. Bevan

INTRODUCTION

 Sugars such as glucose function as signal molecules that regulate gene expression, growth, and development in plants, animals, and yeast. To understand the molecular mechanisms of sugar responses, we isolated and characterized an Arabidopsis thaliana mutant, high sugar response8 (hsr8), which enhances sugar-responsive growth and gene expression. Light-grown hsr8 plants exhibited increased starch and anthocyanin and reduced chlorophyll content in response to glucose treatment. Dark-grown hsr8 seedlings showed glucose-hypersensitive hypocotyl elongation and development. The HSR8 gene, isolated using map-based cloning, was allelic to the MURUS4 (MUR4) gene involved in arabinose synthesis. Dark-grown mur1 and mur3 seedlings also exhibited similar sugar responses to hsr8/mur4. The sugar-hypersensitive phenotypes of hsr8/mur4, mur1, and mur3 were rescued by boric acid, suggesting that alterations in the cell wall cause hypersensitive sugar-responsive phenotypic. Genetic analysis showed that sugar-hypersensitive responses in hsr8 mutants were suppressed by pleiotropic regulatory locus1 (prl1), indicating that nucleus-localized PRL1 is required for enhanced sugar responses in hsr8 mutant plants. Microarray analysis revealed that the expression of many cell wall-related and sugar-responsive genes was altered in mur4-1, and the expression of a significant proportion of these genes was restored to wild-type levels in the mur4-1 prl1 double mutant. These findings reveal a pathway that signals changes in the cell wall through PRL1 to altered gene expression and sugar-responsive metabolic, growth, and developmental changes.

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hypersensitivity to sugar and several hormones (Nemeth et al., 1998). G protein–coupled sugar signaling mechanisms were recently identified in Saccharomyces cerevisiae and in Arabidopsis (Lemaire et al., 2004; Huang et al., 2006). Mutant screens have also identified several other genes potentially involved in sugar signaling, such as low-β-amylose1 (Yoine et al., 2006) and impaired sucrose induction1 (Rook et al., 2001, 2006). The abscisic acid biosynthetic mutant aba2 and the abscisic acid response mutant abi4 (for ABA-insensitive4) have been consistently isolated in screens for reduced responses of seedlings to high levels of glucose or sucrose (Arenas-Huertero et al., 2000; Huijser et al., 2000; Labey et al., 2000; Rook et al., 2001; Cheng et al., 2002), demonstrating interactions between sugar-mediated and abscisic acid–mediated signaling pathways. In addition, the ethylene overproduction (eto1) and ethylene constitutive signaling (ctr1) mutants are glucose-insensitive (Zhou et al., 1998; Cheng et al., 2002). Consistent with this, several ethylene-insensitive mutants display glucose oversensitivity (Zhou et al., 1998; Leon and Sheen, 2003; Yanagisawa et al., 2003), indicating an overlap between sugar and ethylene signaling.

To further understand the molecular mechanisms involved in sugar responses and carbohydrate resource allocation, we have isolated Arabidopsis high sugar response (hsr) mutants that show enhanced luciferase activity and altered sugar responses using transgenic lines containing a luciferase reporter gene driven by the highly sugar-inducible promoter of the ApL3 gene (Baier et al., 2004). Here, we describe the HSR8 gene, which encodes UDP-D-xylose-4-epimerase, which is involved in cell wall arabino-nose biosynthesis and is allelic to MURUS4 (MUR4) (Reiter et al., 1997; Burget and Reiter, 1999; Burget et al., 2003). We present evidence that cell wall changes caused by mutations in HSR8/ MUR4 activate a sugar-responsive pathway requiring PRL1 that mediates gene expression, cell division, and cell expansion.

RESULTS

Identification and Genetic Characterization of the hsr8 Mutant

A transgenic Arabidopsis line, A3L3 (Columbia [Col-0] background), expressing firefly luciferase cDNA under the control of the sugar-inducible ApL3 promoter (Baier et al., 2004), exhibited very low luciferase activity on sucrose- or glucose-free medium and was inducible to high levels in an almost linear response on medium containing between 1 and 3% (w/v) sucrose or glucose (Baier et al., 2004). The hsr8-1 mutant exhibiting relatively high luciferase activity was isolated from ethyl methanesulfate–mutagenized M2 A3L3 seedlings grown on medium containing 1% (w/v) glucose (Figures 1A and 1B). There was no significant difference in luciferase levels between hsr8-1 and wild type seedlings grown on glucose-free medium or medium containing 1% mannitol (data not shown), demonstrating that the hsr8-1 mutant did not have a general increase in luciferase activity and exhibited increased sugar responsiveness. The expression of both the endogenous ApL3 gene and another sugar-responsive gene encoding Arabidopsis β-amylose (Mita et al., 1995) was also increased significantly in hsr8 in response to 1% (w/v) glucose compared with the parental line (A3L3) (Figure 1C). Genetic analysis showed that all of the F1 plants from crosses between A3L3 and hsr8-1 had the wild-type phenotype and that the F2 population showed a segregation ratio of three wild type to one mutant, indicating that hsr8-1 is a single recessive mutant.

hsr8 Plants Have Altered Starch, Chlorophyll, and Anthocyanin Levels

The ApL3 gene encodes one of four large subunits of ADP-pyrophosphorylase (AGPase), which catalyzes the first committed step in starch synthesis. Starch levels were increased significantly in the hsr8-1 mutant compared with the parental line A3L3 (Table 1), consistent with increased expression of ApL3. Anthocyanins also accumulated to higher levels in hsr8-1 (Martin et al., 2002) compared with the parental line A3L3 (Table 1). High levels of exogenous glucose and sucrose antagonize the light-dependent induction of photogene expression (Krapp et al., 1993; Martin et al., 2002). The chlorophyll content of the hsr8-1 mutant was
lower than that of the parental line A3L3 (Table 1), whereas there was no significant difference in response to 3% mannitol (data not shown), suggesting that hsrl further represses chlorophyll synthesis on high-sugar medium. These results indicated that the hsrl mutation enhances several glucose-mediated metabolic responses.

### hsrl Seedlings Exhibit Glucose-Hypersensitive Dark Development and Hypocotyl Elongation

Dark-grown Arabidopsis seedlings develop leaf- and flower-like organs when exogenous sugars are supplied directly to the developing shoot meristem (Roldan et al., 1999). Seedlings of the A3L3 parental line grown on medium containing 0.05% (w/v) glucose for 3 weeks did not develop beyond a slight opening of the cotyledonary petals and expansion of the cotyledon (Figure 2A). At 0.5% (w/v) glucose, the cotyledonary petals were fully expanded and true leaves had just started to develop (Figure 2B), and at 1.0% (w/v) glucose, the first pair of true leaves had developed and a clear internode was apparent (Figure 2C). Propidium iodide staining revealed an increase in leaf primordia size and cell number in 8-d dark-grown A3L3 seedlings in response to increasing concentrations of glucose (Figures 2G to 2I). These results show that dark development is a progressive response to low levels of exogenous glucose. Dark development was then compared in 14-d-old dark-grown A3L3 and hsrl seedlings grown on medium containing 1% glucose. A3L3 seedlings had just started to develop the true leaves in these conditions (Figures 2D and 5A), while at this stage most hsrl seedlings had fully expanded cotyledonary petals, the first true leaves had formed, and an internode was visible (Figures 2D and 5A).

This enhanced dark development in hsrl was also observed when seedlings were grown on medium containing increasing concentrations of glucose for 8 d (Figures 2J to 2L). The increased dark development in the hsrl mutant was not a result of earlier germination (data not shown) or an osmotic effect, because seedlings never developed beyond a slight opening of the cotyledonary petals and expansion of the cotyledon on medium without sugar (Figure 2E) or containing 1% mannitol (Figure 2F).

In dark-grown A3L3 seedlings, hypocotyl length increased in response to 0.05 and 0.5% glucose and elongation was progressively inhibited at glucose concentrations between 1 and 3% (Figure 3A). Hypocotyl elongation in hsrl seedlings was similar to that of wild-type seedlings at 0.05 or low glucose levels (Figure 3A) but became progressively shorter and thinner than in A3L3 (Figures 2D and 3A to 3D) at higher glucose levels. Thus, hsrl seedlings displayed glucose-hypersensitive inhibition of hypocotyl elongation. hsrl hypocotyl epidermal cells were significantly shorter and fatter than wild-type cells (Figures 3B and 3C). There were no significant differences in the number of epidermal cells of hypocotyls between wild-type and hsrl seedlings (data not shown), showing that the inhibition of hypocotyl elongation by glucose in hsrl was largely attributable to reduced hypocotyl cell elongation rather than reduced cell division.

Ethylene inhibits the elongation of dark-grown hypocotyls. The reduction in length of dark-grown hypocotyls in response to 1-aminocyclopropane-carboxylic acid (ACC; an ethylene precursor) was not significantly different between A3L3 and the hsrl mutant (Figure 3E), indicating normal ethylene response in the hsrl mutant. The ethylene-resistant mutant ein2 was not responsive to ACC (Alonso et al., 1999), as expected. Therefore, hsrl does not display altered ethylene responses previously associated with sugar response mutants (Yanagisawa et al., 2003).

### hsrl Has Normal Sugar Levels and Uptake

To rule out the possibility that increased cellular levels of sugars contribute to enhanced responses of hsrl plants to exogenous glucose, sucrose, fructose, and total sugar levels were measured in 7-d-old seedlings of the hsrl mutant and the parental line A3L3. No significant differences were seen (see Supplemental Figure 1A online). We tested whether altered glucose uptake contributes to the enhanced glucose responses observed in the hsrl mutant. The uptake of [14C]glucose into 7-d-old seedlings of the hsrl mutant, its parental line A3L3, a transgenic line expressing the glucose transporter STP1 under the control of the 35S promoter (Baier et al., 2004), and its parental Wassilewskija line was measured. There were no detectable differences in 14C uptake between the A3L3 parental line and the hsrl mutant (see Supplemental Figure 1B online). The line expressing 35S:STP1 showed greatly elevated levels of [14C]glucose uptake, as expected. We concluded that neither altered intracellular sugar levels nor altered glucose uptake accounts for the enhanced glucose responses seen in the hsrl mutant.

### Positional Cloning and Expression Patterns of HSR8

The HSR8 gene was identified by map-based cloning in an F2 population of a cross between hsrl and Landsberg erecta. The HSR8 gene was mapped into a 48-kb interval between markers T518-908 and T518-740 (Figure 4A) on chromosome 1. DNA sequencing revealed that hsrl has a G-to-A transition in gene At1g30620, leading to a change from Arg-105 (CGG) to Gln-105 (CAG) (Figure 4B). At1g30620 is MUR4, encoding UDP-α-xyllose-4-epimerase (Reiter et al., 1997; Burget and Reiter, 1999; Burget et al., 2003). The derived cleaved-amplified polymorphic sequence (dCAPS) marker At1g30620cap1 was developed based on this mutation in hsrl, and it cosegregated with the hsrl phenotypes (Figures 4A and 4C). A plasmid containing the entire At1g30620 open reading frame, the 2.6-kb promoter sequence, and the 1.5-kb downstream sequence was introduced into the hsrl, mur4-1, and mur4-3 mutants. Nearly all transgenic lines exhibited complementation of hsrl phenotypes (Figures 1A, 1B,
and 2D). Therefore, At1g30620 is the HSR8 gene. AT-DNA insertion into the 3’ untranslated region of At1g30620 (Salk_010548) was called hsr8-2 (Figure 4B), and the original ethyl methanesulfate–induced allele is henceforth called hsr8-1. All mutants (hsr8-2, mur4-1, mur4-3, and mur4-4) in the At1g30620 gene show the hsr8-1 dark development phenotypes (Figure 5A). F1 progeny of crosses of the five lines (hsr8-1, hsr8-2, mur4-1, mur4-3, and mur4-4) all exhibited a similar dark development phenotype, showing that these lines were allelic (data not shown).

HSR8 transcripts were detected in various tissues by RT-PCR analysis, including roots, stems, leaves, and flowers (Figure 4D), consistent with previous RNA gel blot analysis (Burget et al., 2003). Figure 4E shows that HSR8 expression in light-grown seedlings is elevated in response to glucose, consistent with

Figure 2. Glucose-Hypersensitive Dark Development in the hsr8-1 Mutant.

(A) to (C) Dark development of Col-0 seedlings grown on medium containing 0.05% glucose (A), 0.5% glucose (B), and 1% glucose (C) for 21 d.

(D) to (F) Fourteen-day-old dark-grown seedlings of the wild type (right), hsr8-1 (middle), and hsr8-1com (left) grown on MS medium (E) and MS medium containing 1% glucose (D) and 1% mannitol (F).

(G) to (I) The leaf primordium of 8-d-old dark-grown seedlings of A3L3 grown on MS medium (G) and MS medium containing 0.5% glucose (H) and 1% glucose (I).

(J) to (L) The leaf primordium of 8-d-old dark-grown hsr8-1 seedlings on MS medium with no glucose (J), 0.5% glucose (K), and 1% glucose (L). Bars = 1 mm in (A) to (C), 0.5 cm in (D), 0.2 cm in (E) and (F), and 0.1 μm in (G) to (L).
previous microarray data (Li et al., 2006). The tissue-specific expression patterns of HSR8 were assessed using histochemical assay of β-glucuronidase (GUS) activity of transgenic plants containing a HSR8 promoter:GUS fusion (HSR8pro:GUS). High levels of GUS activity were detected in the shoot and root apices and hypocotyls of both light- and dark-grown seedlings (Figures 4F to 4J). These expression patterns and induction by glucose are consistent with the dependence of glucose-responsive hypocotyl elongation and shoot apical development on HSR8 function in many actively growing tissues (Figure 2).

Altered Sugar Responses in hsr8 Are Rescued by Arabinose

Mutations in MUR4 lead to a reduction in l-arabinose levels in most organs and affect arabinose-containing pectic cell wall polysaccharides and arabinoxylan proteins (Reiter et al., 1997; Burget and Reiter, 1999; Burget et al., 2003). Cell wall monosaccharide composition in the parental line A3L3, hsr8-1, and mur4-1 was determined by gas chromatography of alditol acetate derivatives. Table 2 shows that the amounts of arabinose in hsr8-1 and mur4-1 were reduced to 54.9 and 50.2% of A3L3 levels, respectively, consistent with previously reported results (Reiter et al., 1997; Burget and Reiter, 1999; Burget et al., 2003). Feeding l-arabinose to mur4 plants restored cell wall monosaccharide composition to wild-type levels (Burget and Reiter, 1999), showing that l-arabinose can be used directly by plants through a salvage pathway as a source of arabinosyl units for polymer synthesis. Treatment with 1% glucose and 30 mM arabinose fully restored the enhanced dark development and glucose-hypersensitive hypocotyl elongation phenotypes of hsr8-1 and mur4 seedlings to that of wild-type seedlings (Figures 5B to 5D).

l-Arabinose is found mainly in the arabinogalactan side chains of pectins, glucuronoxylans, Hyp-rich glycoproteins, and cell wall arabinoxylan proteins (AGPs) (Carpita and Gibeaut, 1993). Mutations in the ARAD1 gene (At2g35100) cause a specific decrease in arabinoxylan side chains of rhamnogalacturonan I (RG-I), a major component of pectins (Harholt et al., 2006). To test whether specific changes in RG-I cause sugar responses in hsr8/mur4, we tested the dark development responses of the arad1-2 mutant. Figures 5F and 5G show no significant differences in dark development phenotype between the wild type and arad1-2, suggesting that alterations in arabinan side chains of RG-I did not significantly affect the increased dark development phenotype in the hsr8/mur4 mutant. mur4 mutations reduce the arabinose content of both cell wall polysaccharides and AGPs.
Figure 4. Map-Based Cloning and Expression Patterns of the HSR8 Gene.

(A) Fine-mapping of the HSR8 locus. The HSR8 locus was mapped to chromosome 1 (Chr1) between markers F1K23 and F17F8. The HSR8 locus was further refined to a 48-kb genomic DNA region between CAPS markers T518-908 and T518-740 and cosegregated with dCAPS marker At1g30620d-CAPS1. The numerals at bottom indicate the number of recombinants identified from F2 plants.

(B) HSR8 gene structure, showing the mutated sites of the two hsr8 alleles. The start codon (ATG) and the stop codon (TGA) are indicated. Closed boxes indicate the coding sequences, and lines between boxes indicate introns. The mutation site in hsr8-1 and the T-DNA insertion site in hsr8-2 also are shown.

(C) The mutation in hsr8-1 was measured with the dCAPS marker At1g30620d-CAPS1.

(D) RT-PCR analysis of HSR8/MUR4 gene expression. Total RNA was isolated from roots, stems, leaves, and flowers.

(E) Expression of HSR8 in response to glucose after 6 h of treatment.

(F) to (J) Histochemical analysis of GUS activity in a HSR8pro:GUS seedling (F), hypocotyl and shoot apices (G), a primary root (H), a lateral root (I), and a dark-grown seedling (J).
AGPs have been implicated in many aspects of cell development, including cell proliferation, cell expansion, organ extension, apoptosis, germination, and somatic embryogenesis (Willats and Knox, 1996; Majewska-Sawka and Nothnagel, 2000; Gaspar et al., 2001; Lee et al., 2005). We tested whether altered AGP function could account for altered sugar responses in hsr8-1 using β-D-glucosyl Yariv (βGlcY) (Yariv et al., 1962, 1967) to disrupt AGP function (Willats and Knox, 1996). Roots of wild-type seedlings grown for 14 d in 50 mM βGlcY were significantly shorter than those of untreated seedlings, while roots of hsr8-1 showed increased dark development compared with the wild type.

Figure 5. Dark Development Phenotypes of Cell Wall Biosynthetic Mutants.

(A) Dark development phenotypes of 14-d-old wild-type Col-0, mur4-1, and hsr8-1 alleles grown on MS medium supplemented with 1% glucose. All hsr8 alleles exhibit increased dark development compared with the wild type.

(B) Dark development phenotypes of 14-d-old wild-type Col-0 and hsr8-1 alleles grown on MS medium supplemented with 1% glucose and 30 mM L-arabinose. The increased dark development phenotypes of hsr8 mutants in response to glucose were restored to wild-type levels by exogenous L-arabinose.

(C) Hypocotyl lengths of 14-d-old dark-grown seedlings of the wild type and hsr8-1 mutants grown on MS medium supplemented with 1% glucose. The reduced hypocotyl elongation of hsr8 mutants was rescued by L-arabinose.

(D) Hypocotyl lengths of 14-d-old dark-grown seedlings of the wild type and hsr8 mutants grown on MS medium supplemented with 1% glucose and 30 mM L-arabinose. The increased dark development phenotypes of hsr8 mutants in response to glucose were restored to wild-type levels by exogenous L-arabinose.

(F) and (G) Dark development phenotypes of 14-d-old wild type Col-0 (F) and arad1-2 (G) alleles grown on MS medium supplemented with 1% glucose.

(H) Hypocotyl lengths of 14-d-old dark-grown seedlings of Col-0, mur4-1, and mur3-1 grown on MS medium supplemented with different glucose concentrations.

Error bars represent s.d (n > 30). Bars = 0.5 mm in (E) and 1 mm in (A), (B), (F), and (G).

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the growth of roots of seedlings grown in the same concentration of β-0-mannosyl Yariv (βManY), which does not bind to AGPs, showed no significant difference compared with that of untreated seedlings (see Supplemental Figures 2E and 2G online). In addition, reduction in root growth was proportional to concentrations of βGlcY supplied in the medium (see Supplemental Figure 2G online). This indicated that AGP function was altered by βGlcY. However, sugar-responsive phenotypes such as dark development and hypocotyl elongation were not affected significantly by βGlcY (see Supplemental Figures 2A to 2D and 2F online), suggesting that altered AGP function did not significantly affect the enhanced sugar responses in hsr8-1.

### Dark-Grown mur1 and mur3 Seedlings Exhibit Similar Sugar Responses to hsr8/mur4

We investigated whether other cell wall biosynthetic mutants also affect dark development. mur1-1 and mur2-1 have decreased cell wall fucose (Bonin et al., 1997; Vanzin et al., 2002), mur3-1 has altered xyloglucan structure (Madson et al., 2003), mur5-1, mur6-1, and mur7-1 have decreased cell wall arabinose (Reiter et al., 1997), mur4-1 has reduced cell wall rhamnose (Reiter et al., 1997), mur9-1 has reduced cell wall xylose and fucose (Reiter et al., 1997), radial swelling phenotype1 (rsw1) and isoxaben resistance2 (irx2) have reduced cellulose synthesis (Arioli et al., 1998; Fagard et al., 2000; Desprez et al., 2002), and irx4 has 50% less lignin than wild-type plants (Jones et al., 2001). mur2-1, mur5-1, mur6-1, mur7-1, mur8-1, mur9-1, rsw1, irx2, and irx4 seedlings showed similar glucose-responsive dark development to the wild type. By contrast, mur1-1 (in At3g51160) and mur3-1 (in At2g03220) seedlings had similar levels of enhanced dark development in response to glucose as the hsr8-1 mutant (Figure 5E) and similar glucose-hypersensitive reduction in hypocotyl elongation (Figures 5E and 5H). These data showed that several different cell wall changes, including reduced arabinose and fucose content and altered xyloglucan structure, can lead to altered glucose-responsive growth and development. Furthermore, the strong alleles mur4-1, mur4-3, and mur4-4, which have less cell wall arabinose than the weaker alleles mur4-2, mur5-1, mur6-1, and mur7-1 (Reiter et al., 1997), had a greater extent of glucose-responsive dark development and hypocotyl elongation than the weaker allele (Figures 5A and 5C; see Supplemental Figure 3 online), suggesting that only major changes in the cell wall may lead to sugar-hypersensitive development and hypocotyl elongation.

### Glucose-Responsive Growth and Development in hsr8 Plants Is Suppressed by prl1

Mutations in PRL1 (At4g15900) lead to transcriptional derepression of glucose-responsive genes and increase the sensitivity of plants to growth hormones, including cytokinin, ethylene, abscisic acid, and auxin (Nemeth et al., 1998). Since the Arabidopsis prl1 mutant shows hypersensitivity to sugars (Nemeth et al., 1998), we tested the dark development responses of prl1 seedlings in response to glucose. Dark development (Figures 6A and 6C) and the pattern of hypocotyl elongation responses (Figure 6E) were essentially similar in prl1 and wild-type Col-0 seedlings, although hypocotyl length was slightly and equally reduced in prl1 compared with Col-0. This suggested that the sugar-responsive pathways controlling dark development and hypocotyl elongation in wild-type seedlings have little or no dependence on PRL1. To investigate whether PRL1 is involved in hsr8/mur4-mediated sugar responses, dark development and hypocotyl elongation phenotypes were assessed in a mur4-1 prl1 double mutant and compared with those of its parents. The mur4-1 prl1 double mutant exhibited reduced dark development (Figure 6D) compared with mur4-1 (Figure 6B) and similar to that seen in prl1 (Figure 6C). The double mutant also displayed similar glucose-responsive

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**Table 2.** Comparison of Cell Wall Monosaccharide Composition in A3L3 and the hsr8-1, mur4-1, prl1-3, and mur4-1 prl1-3 Mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Arabinose</th>
<th>Rhamnose</th>
<th>Fucose</th>
<th>Xylose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Glucose</th>
</tr>
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<tbody>
<tr>
<td>A3L3</td>
<td>17.28 ± 0.31</td>
<td>12.33 ± 0.10</td>
<td>3.51 ± 0.07</td>
<td>39.38 ± 0.12</td>
<td>17.35 ± 0.08</td>
<td>27.47 ± 0.43</td>
<td>173.68 ± 5.11</td>
</tr>
<tr>
<td>hsr8-1</td>
<td>9.49 ± 0.36</td>
<td>12.58 ± 0.14</td>
<td>3.27 ± 0.03</td>
<td>39.27 ± 0.45</td>
<td>17.76 ± 0.27</td>
<td>30.11 ± 1.01</td>
<td>194.21 ± 3.28</td>
</tr>
<tr>
<td>mur4-1</td>
<td>8.68 ± 0.33</td>
<td>12.57 ± 0.36</td>
<td>3.52 ± 0.00</td>
<td>37.47 ± 0.33</td>
<td>19.21 ± 0.08</td>
<td>27.62 ± 0.16</td>
<td>195.56 ± 2.50</td>
</tr>
<tr>
<td>prl1</td>
<td>14.81 ± 0.03</td>
<td>12.49 ± 0.41</td>
<td>3.84 ± 0.14</td>
<td>36.34 ± 0.83</td>
<td>17.51 ± 0.38</td>
<td>25.35 ± 0.11</td>
<td>174.63 ± 1.82</td>
</tr>
<tr>
<td>mur4-1 prl1</td>
<td>8.53 ± 0.03</td>
<td>12.90 ± 0.06</td>
<td>3.16 ± 0.01</td>
<td>37.58 ± 0.88</td>
<td>18.62 ± 0.42</td>
<td>29.27 ± 0.15</td>
<td>188.66 ± 0.83</td>
</tr>
</tbody>
</table>

The sugar content of cell walls are means ± SE of three independent assays. Each wall component was calculated as milligrams per gram of total cell wall residues.
hypocotyl growth to prl1 (Figure 6E), indicating that prl1 suppressed the hsr8/mur4 glucose-hypersensitive dark development and hypocotyl elongation phenotypes. Therefore, the enhanced sugar responses seen in hsr8/mur4 seedlings require PRL1 function. Furthermore, light-grown mur4-1 prl1 double mutant seedlings had similar phenotypes to prl1 (Figures 6F and 6G), and the expression patterns of the ApL3 and At β-amylase genes in prl1 (Figure 6H) suggested that prl1 is also epistatic to hsr8/mur4 in the light. Analysis of cell wall monosaccharide composition revealed that the mur4-1 prl1 double mutant had similar reduced cell wall arabinose levels as mur4-1 (Table 2), indicating that the suppression of hsr8/mur4 growth and developmental phenotypes by prl1 is not due to an alteration of cell wall arabinose content seen in the mur4-1 mutant. Analysis of mur4-1 gin2-1 and hsr8-1 abi4-1 double mutants indicated that GIN2 and ABI4, which have well-established roles in sugar signaling (Rolland et al., 2006), were not required for the dark development phenotype in hsr8/mur4 seedlings (data not shown).

Gene Expression Profiles in mur4-1, prl1, and mur4-1 prl1

Genome-wide gene expression analysis using ATH1 Arabidopsis whole genome microarray chips was conducted on RNA isolated from seedlings of Col-0, mur4-1, prl1, and mur4-1 prl1 6-d-old dark-grown seedlings. At this early stage of dark development, all seedlings showed a similar degree of growth and development. Compared with wild-type Col-0, expression levels of 92 genes were increased and those of 97 genes were reduced in mur4-1. Approximately one-third (30) of the genes with increased expression in mur4-1 encoded enzymes involved in cell wall metabolism, such as xyloglucan endotransglycosylase, pectin acetyxyesterase, and glycosyl hydrolase, proteins associated with

Figure 6. The Glucose-Responsive Phenotypes of Dark-Grown mur4-1 Seedlings Are Suppressed by prl1.

(A) to (D) Fourteen-day-old dark-grown seedlings of the wild type (A), mur4-1 (B), prl1 (C), and mur4-1 prl1 (D) grown on MS medium supplemented with 1% glucose.

(E) Hypocotyl lengths of 14-d-old dark-grown seedlings of the wild type, prl1, mur4-1, and mur4-1 prl1 grown on MS medium supplemented with different glucose concentrations. Error bars represent SD (n > 30).

(F) and (G) Constant light–grown seedlings of the wild type, mur4-1, prl1, and mur4-1 prl1 grown on MS medium supplemented with 1% glucose (F) and 3% glucose (G).

(H) RT-PCR analysis of transcript levels in Col-0, mur4-1, prl1, and mur4-1 prl1. RT-PCR was performed on first-strand cDNA made from 9-d-old seedlings grown in constant light on medium containing 1% glucose. cDNA was standardized by reference to an actin standard. Bars = 2 mm in (A) to (D) and 1 cm in (F) and (G).
structural constituents of the cell wall (e.g., Pro-rich extensin proteins), enzymes involved in primary metabolism, transcription factors, and regulatory proteins (see Supplemental Table 1 online). Consistent with the sugar-hypersensitive phenotypes of mur4-1, the expression levels of several classes of sugar-regulated genes (Li et al., 2006) were also increased in mur4-1, such as lipid transfer and seed storage proteins, enzymes of carbohydrate metabolism, transcription factors, and regulatory proteins. The main class of genes with reduced expression in mur4-1 (10%) were also those involved in cell wall–related functions, such as AGP14 and AGP22. A significant proportion were also involved in disease and stress responses (e.g., RD22), transcription, and regulatory processes (see Supplemental Table 1 online).

Compared with wild-type Col-0, expression levels of 74 genes were increased and those of 112 genes were repressed in prl1 (see Supplemental Table 2 online). Consistent with PRL1 function in derepressing sugar-repressed genes (Nemeth et al., 1998), the expression of a set of light-activated and sugar-repressed genes, such as genes encoding photosystem subunits (e.g., psbA photosystem II protein and photosystem II G protein), and α/β binding proteins, such as LHC8B3 and LHC8B6, was increased in prl1. The expression of genes involved in sugar metabolism and transport was also upregulated in prl1, including genes encoding a glucose-6-phosphate/phosphate translocator, glycerol-3-phosphate transporter, pyruvate kinase, and 2-isopropylmalate synthase2, consistent with the accumulation of free sugars in prl1 (Nemeth et al., 1998). Among genes with reduced expression in prl1 were those involved in stress responses, such as RD21A, RD29B, RAB18, low-temperature-induced LTI3, and drought-induced D21, consistent with altered abscisic acid responses reported in prl1 (Nemeth et al., 1998). Other major classes of genes differentially regulated in prl1 were those encoding proteins and enzymes involved in cell wall modification, transcription factors, and protein kinases.

To gain insight into the mechanisms by which prl1 may suppress the sugar-responsive phenotypes of mur4-1, we classified the genes with increased and reduced expression in mur4-1 into different clusters using the quality threshold method (Li et al., 2006) according to their expression profiles in Col-0, mur4-1, prl1, and the mur4-1 prl1 double mutant (Figures 7A and 7B). These expression profiles were confirmed by quantitative RT-PCR analysis of eight selected genes (see Supplemental Figure 4 online). In the 92 genes upregulated in mur4-1, the expression of 31 genes in cluster 2 and cluster 6 was suppressed to levels seen in the wild type in mur4-1 prl1 (see Supplemental Table 3 online). A significant proportion of these genes (nine encoded proteins associated with cell wall cell metabolism and modification, including xyloglucan endotransglycosylase, putative pectinesterases, and Pro-rich extensin proteins. Other classes of genes encoding lipid transfer proteins, storage proteins, and regulatory proteins, such as nonsymbiotic hemoglobin 1 (GLB1) and PSK5, were also suppressed to levels seen in Col-0. Plants overexpressing GLB1 have considerably faster growth of both roots and shoots compared with control plants (Hunt et al., 2002), suggesting that GLB1 may be required for enhanced dark development in mur4-1. PSK-α, a peptide growth factor, has several biological activities, including promoting plant cell proliferation (Yang et al., 2001). In the set of 97 genes downregulated in mur4-1, the expression of 41 genes in cluster 1, cluster 3, and cluster 5 was restored to wild-type levels in mur4-1 prl1 (see Supplemental Table 3 online). Genes encoding stress-responsive proteins (RD22) and disease-responsive proteins, cell wall–related proteins, ubiquitin-protein ligases, and protein kinase (GPK1) formed a major group. Genes encoding components of protein degradation pathways are repressed by glucose, whereas the expression of genes involved in protein biosynthesis is upregulated by glucose (Li et al., 2006). Consistent with the sugar-hypersensitive phenotypes seen in mur4-1, the expression of two ubiquitin-protein ligases was repressed in mur4-1 (see Supplemental Table 1 online). Their expression was restored to the wild-type level in mur4-1 prl1 (see Supplemental Table 3 online), suggesting that PRL1 mediates the expression of protein degradation pathways activated in mur4-1.

DISCUSSION

Cell Division and Cell Elongation Responses to Sugars Are Altered in the hsr8-1 Mutant

Sugars modulate multiple plant growth, development, gene expression, and metabolic responses (Rolland et al., 2006). To identify genes involved in these responses, we have isolated hsr mutants exhibiting increased glucose-responsive expression of the ApL3 gene encoding a large subunit of AGPase, the first step of starch synthesis (Baier et al., 2004). In addition to increased ApL3 expression, the hsr8-1 mutant exhibited a range of hypersensitive sugar-responsive phenotypes, including increased starch and anthocyanin levels, reduced chlorophyll levels (Table 1), glucose-responsive hypocotyl elongation, and dark development, compared with the parental line (Figures 1 and 3). These phenotypes, which are similar to those found in other hsr mutants (Baier et al., 2004), revealed that mutations in the HSR8 gene enhance a wide range of responses to physiologically relevant sugar levels. During dark development, cell numbers in the shoot apex increase progressively in response to glucose (Figures 2G to 2L). The elevated cell division in response to low glucose levels seen in the hsr8-1 mutant suggests that this mutant affects links between sugar availability and cell proliferation. In cultured Arabidopsis cells, sucrose depletion arrests cells at the G1-to-S transition (Riou-Khamlichi et al., 2000) due to depleted CYCD3;1 levels (Menges et al., 2006), suggesting a potential mechanism to explain this phenotype. The increased hypocotyl cell elongation in response to low glucose concentrations and increased inhibition of hypocotyl elongation at higher glucose concentrations (Figure 3A) observed in the hsr8-1 mutant show that cell elongation regulation by sugars is also altered in the hsr8-1 mutant. These glucose-responsive cell division and cell elongation responses will be useful phenotypes in genetic screens for identifying mutants affecting sugar-dependent responses.

The hsr8 Mutation Establishes a Link between Cell Wall Changes and Sugar-Responsive Growth and Development

We demonstrated that HSR8 is At1g30620, previously identified as MUR4 encoding UDP-α-xylene-4-epimerase, which catalyzes
the first step of arabinose synthesis in the Golgi (Reiter et al., 1997; Burget and Reiter, 1999; Burget et al., 2003). Like hsr8-1, mur4 mutations do not exhibit light-grown phenotypes (Reiter et al., 1997; Burget and Reiter, 1999; Burget et al., 2003); consequently, their sugar-dependent growth and development phenotypes have not been recognized previously. The glucose-hypersensitive growth and developmental phenotypes caused by mutations in HSR8/MUR4 were abrogated by growth on low levels of arabinose, further confirming the identity of the HSR8 gene and revealing a link between arabinose biosynthesis and sugar-responsive growth and development. Among a variety of other mutants known to affect cell wall composition, the mur1-1 and mur3-1 mutations also exhibited hypersensitive sugar-responsive growth and development. mur1-1 is a mutation in a gene encoding GDP-β-mannose-4,6-dehydratase, which catalyzes the first step in fucose synthesis (Bonin et al., 1997), and mur3-1, a mutation in a xyloglucan-specific galactosyltransferase, alters xyloglucan structure (Madson et al., 2003).

A wide range of cell wall polysaccharides are affected by the mur1, mur3, and mur4 mutations. It is conceivable that changes in several cell wall components, or a single component affected by all three mutants, may lead to altered sugar-responsive growth. Mutations in MUR4 cause decreased arabinose in pectins, xylans, xyloglucans, and AGPs (Burget and Reiter, 1999). Arabinosylated glycans such as RG-I are believed to play important roles in plant development (Burget and Reiter, 1999), and AGPs have been assigned various roles in plant development, including embryogenesis, cell–cell interactions, fertilization, cell

Figure 7. Gene Expression Profiles in mur4-1, prl1, and mur4-1 prl1.

(A) Upregulated genes in mur4-1 were classified into six clusters according to their expression profiles in the wild type, mur4-1, prl1, and mur4-1 prl1. Cluster numbers and mutant alleles are indicated as follows: C, Col-0; m, mur4-1; p, prl1; mp, mur4-1 prl1.

(B) Downregulated genes in mur4-1 were classified into six clusters according to their expression profiles in the wild type, mur4-1, prl1, and mur4-1 prl1. Cluster numbers and mutant alleles are indicated as in (A).

(C) Model of cell wall signaling based on mutant and gene expression analysis.
proliferation, and cell expansion (Gaspar et al., 2001; Lee et al., 2005). Altered dark development responses were not seen in the arad1-2 mutation, which specifically decreases the levels of arabinan side chains of RG-I (Harholt et al., 2006). Furthermore, disruption of AGP function with βGlCT reagent also had no significant effect on dark development responses. This raises the possibility that changes in other arabinoxylan-containing moieties may lead to altered sugar-responsive growth and developmental responses. It is also feasible that changes in specific arabinoxylan-containing moieties are insufficient to activate sugar responses and that overall reduction ofcell wall arabinose levels leads to altered sugar responses. This is consistent with the observation that only large changes in the cell wall lead to sugar hypersensitivity. For example, the strong alleles mur4-1, mur4-3, and mur4-4, which have less cell wall arabinose than the weaker alleles mu r4-2, mu r5-1, mu r6-1, and mu r7-1 (Reiter et al., 1997), had a greater extent of glucose-responsive dark development and hypocotyl elongation than the weaker alleles (Figures 5A and 5C; see Supplemental Figure 3 online).

The mur1 and mur3 mutations led to reduced cell wall fucose levels (Reiter et al., 1997). In mur3-1 plants, a fucosylated side chain in xylan glucans is absent, implicating xylan glucans in sugar responses (Madsen et al., 2003). However, mu r2-1 plants, which are deficient in a xylan glucan-specific fucosyl transferase that also affects xylan glucans, did not exhibit altered sugar-responsive dark development and growth phenotypes, suggesting that only some types of xylan glucans may modulate sugar responses. The structure and function of RG-II are known to be altered by the mur1 mutation (O’Neill et al., 2001). Treatment of mur1-1 plants with low concentrations of borate rescued their growth defects and resulted in an increase in the extent of borate cross-linking of RG-II (O’Neill et al., 2001), indicating that the dwarf phenotype and brittle cell walls of mur1 plants were consequences of reduced cross-linking of RG-II by borate diesters. Treatment of hsr8-1, mur4-1, mur3-1, and mur1-1 seedlings with 1% glucose and 2 mM boric acid also reduced the extent of sugar-responsive dark development to that of wild-type seedlings, and the severely reduced hypocotyl elongation was also reversed to that seen in the wild type (Figures 5E and 5I). This observation suggested that RG-II may be a common factor in altered sugar responses. However, borate is known to cross-link a variety of cell wall components in addition to RG-II (Blevins and Lukaszewski, 1998), including pectic moieties that are known to be altered in mur4 (Burget et al., 2003) and xylan glucans known to be altered in mur3 (Blevins and Lukaszewski, 1998; Madson et al., 2003); therefore, borate responsiveness does not provide conclusive evidence for a sole role for RG-II in sugar responses. Furthermore, mur3 has specific effects on the structure of xylan glucans and is not likely to alter RG-II function directly (Madson et al., 2003). Thus, the present level of analysis indicates that alterations in several cell wall components, including RG-II, a specific class of xylan glucans, and either a subset of arabinoxylan-containing cell wall moieties or large-scale reductions in cell wall arabinose, activate sugar responses. Other mutations that lead to cell wall changes also have major effects on growth and defense responses. The cev1 and ell1 mutations in Ce SA3 (encoding a cellulose synthase subunit) exhibit constitutive expression of ethylene- and jasmonate-responsive stress genes, enhanced resistance to fungal pathogens, and elevated lignin levels (Ellis et al., 2002; Cano-Delgado et al., 2003). Reduced callose synthase levels lead to enhanced salicylic acid–dependent disease resistance (Nishimura et al., 2003). Mutations in the RH1 gene encoding a UDP-rhamnose synthase suppressed the root hair mutant lrx1, which encodes an extracellular Leu-rich repeat extensin protein (Diet et al., 2006). Collectively, those studies and this report illustrate that properties of the plant cell wall are actively monitored during growth and development and signaled to multiple cellular responses. Such mechanisms have been proposed to monitor the performance and alteration of the cell wall (Pilling and Hotte, 2003; Somerville et al., 2004). The recent discovery of a membrane-bound receptor kinase that mediates hypocotyl elongation in response to reduced cellulose synthesis reveals one mechanism linking cell wall changes to cellular functions (Hernéty et al., 2007).

**PRL1 Reveals a Potential Mechanism Linking the Cell Wall with Sugar-Responsive Phenotypes**

Mutations in the PRL1 gene suppressed the glucose-hypersensitive growth and developmental phenotypes caused by mutations in the HSR8/MUR4 gene (Figure 6) without altering cell wall monosaccharide composition. This genetic analysis suggests that changes in the cell wall are signaled via the nucleus by a mechanism involving PRL1 in sugar-responsive processes (Nemeth et al., 1998).

The prl1 mutation causes a range of phenotypes, including hypersensitive responses to glucose and sucrose, cytokinins, auxin, ethylene, and abscisic acid (Nemeth et al., 1998). PRL1 encodes a nucleus-located WD repeat protein that interacts with and inhibits the activity of the Arabidopsis SnRK1 proteins AKN10 and AKN11, which are structurally and functionally related to yeast SNF1 and mammalian AMPK regulatory kinases (Bhalerao et al., 1999). This mechanism may alter carbohydrate-responsive growth and storage processes, consistent with their regulatory function in carbon metabolism in plants, animals, and yeast (Gancedo, 1998; Halford and Hardie, 1998; Carlson, 1999) and in glucose-dependent growth in the moss Physcomitrella patens (Thelander et al., 2004).

To further understand how prl1 suppresses hsr8/mur4-activated sugar responses, we performed whole genome array analysis using wild-type, mur4-1, prl1, and mur4-1 prl1 seedlings. Expression of light-activated and sugar-repressed genes was upregulated in prl1, supporting previous analyses of the expression of individual genes in prl1 seedlings (Nemeth et al., 1998). Furthermore, the expression of known sugar-inducible genes was also upregulated in prl1, consistent with sugar-hypersensitive phenotypes of this mutant (Nemeth et al., 1998).

The expression of a significant proportion of mur4-1-regulated genes was dependent upon PRL1. We classified mur4-1-regulated genes into different groups according to their expression profiles in the wild type, mur4-1, prl1, and mur4-1 prl1 to identify genes whose expression levels were restored to that of the wild type in the mur4-1 prl1 double mutant. The expression levels of three sets of genes whose functions are relevant to altered dark development and hypocotyl elongation phenotypes seen in mur4-1/hsr8-1 fell into this class. Expression of a large set of genes
involved in cell wall metabolism and modification was restored to wild-type levels in the double mutant, suggesting that PRL1 is required for the regulation of mur4-activated transcriptional responses leading to compensatory cell wall changes. As proposed above, changes in the cell wall trigger these transcriptional responses. The increased expression of several growth regulatory genes such as GLB1 (Hunt et al., 2002) and At PSK5 (Yang et al., 2001) in mur4-1 was repressed to wild-type levels in mur4-1 prl1. The increased expression of these genes in mur4-1 may contribute to altered dark development and hypocotyl elongation. The expression of two ubiquitin ligase genes repressed in mur4-1 was restored to wild-type levels in the mur4-1 prl1 double mutant, suggesting that PRL1 regulates a protein degradation pathway involved in the regulation of mur4-1-activated responses. Finally, the expression of genes involved in stress and disease responses was restored to wild-type levels, suggesting that PRL1 is involved in the regulation of stress responses caused by cell wall defects in mur4-1.

Figure 7C shows a model of PRL1 function in the regulation of hsr8-1/mur4-1-activated sugar responses that is based on the sugar-hypersensitive growth and developmental phenotypes of hsr8-1/mur4-1 and an analysis of gene expression profiles. In this model, changes in the cell wall (in this case caused by mutants affecting polysaccharide synthesis) signal to PRL1 function in the nucleus, which then coordinates the expression of hormone- and stress-related genes (Nemeth et al., 1998) and genes related to cell wall modification and growth, leading to altered sugar-dependent growth and developmental responses. The regulatory pathway described in this report may contribute to the dynamic regulation of cell wall structure and function during growth and development and integrate metabolic regulation with the synthesis of new cell wall polysaccharides through PRL1 regulation of the SnRK1 proteins AKB10 and AKB11 (Bhalerao et al., 2004). hsr8-1 seedlings showing higher luminescence than the parental line grown on the same plate were selected. hsr8-2 (SALK_010548) and mur4 were obtained from the Nottingham Arabidopsis Stock Centre. SALK_010548 T-DNA insertion in the HSR8 gene was confirmed by PCR and sequencing with primers SALK_010548LP (5′-TTTCTCCAGAT-CAAAGGAC-3′), SALK_010548RP (5′-TGGAATACATTTTGCCTTATG-ATC-3′), and LBa1 (5′-TGTTCACTAGTGGCCATCG-3′).

RNA Isolation, RT-PCR, and Quantitative Real-Time RT-PCR Analysis

Total RNA was extracted from Arabidopsis seedlings, roots, stems, leaves, and flowers using the RNeasy plant mini kit (Qiagen). RT-PCR analysis was performed as described (Li et al., 2006). cDNA samples were standardized on actin transcript amount using the primers AtactIF and AtactIR (Baier et al., 2004). The primers used for RT-PCR are as follows: AT5G09810-ACTIN (AtactIF, 5′-GAGAGATGACTGATGATC-3′, and AtactR, 5′-ATCTTTCTCGATATCGAC-3′); AT4G39210-ApL3 (ApL3F, 5′-CGTTCTGAAATCATGCAACC-3′, and ApL3R, 5′-GACATTCTCTGATTTGTTAGTCTCG-3′); AT4G15210-β-Amylase (β-AmyIF, 5′-CCGGAAGAGGGAGAGTTTTTC-3′, and β-AmyR, 5′-AATCTCTATGCCTACCTTCG-3′); and HSR8 (HSR8RTF, 5′-AACAACCTACATGCTCGCG-3′, and HSR8RTR, 5′-ATACAAATGTTTCGTCCTTC-3′).

Quantitative real-time RT-PCR analysis was performed with an Opticon 2 DNA engine (MJ Research) using the SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich). TUB6 mRNA was used as an internal control, and relative amounts of mRNA were calculated using the comparative threshold cycle method. The primers used are described in Supplemental Table 5 online.

Propidium Iodide Staining

Samples were submerged in 10 mL of 37% formalin, 5 mL of propionic acid, and 70 mL of 100% ethanol overnight, dehydrated in 85, 95, and 100% ethanol, stained in propidium iodide solution (5 μg/mL) for 1 d, rinsed with 0.1 M L-Arg, pH 8.0, dehydrated in an ethanol series (15, 30, 50, 70, 85, 95, and 100%), cleared through an ethanol:xylene series (75:25%, 50:50%, 25:75%, and 100% xylene), and then viewed.

Carbohydrate, Chlorophyll, Anthocyanin, and Cell Wall Analyses

Carbohydrate, chlorophyll, and anthocyanin analyses were performed as described (Arnon, 1949; Galtier et al., 1995; Baier et al., 2004). Glucose, fructose, and sucrose were measured sequentially in cleared supernatants of K2CO3-neutralized HClO4 extracts of ground, frozen plant material (Galtier et al., 1995). Glucose uptake measurement was performed as described (Baier et al., 2004). For cell wall analysis, plant material was harvested and ground into a fine powder with a combination of liquid N2 and dry ice. For monosaccharide analysis, ground material was resuspended in 0.1 M MOPS/NaOH, pH 7.0, and centrifuged for 10 min at 15,000 rpm at 4°C. The pellet was washed twice with 0.25% extraction buffer, incubated for 1 h at 40°C in phenol:acetic acid:water (2:1:1, w/v/v), and centrifuged. The supernatant was discarded, and the pellet was washed twice with water and then freeze-dried. The neutral sugar content was determined after dispersion in 72% (w/v) H2SO4 for 3 h at 20°C, dilution to 1 M, and hydrolysis at 100°C for 2.5 h (Selvendran et al., 1979). The alditol acetates were prepared and analyzed by gas chromatography (Blakeney et al., 1983).

Scanning Electron Microscopy

Seedlings grown for 4 d in the dark were frozen in nitrogen slush at −190°C. Ice was sublimed at −90°C, and the specimen was sputter-coated and
examined on an XL 30 FEG (Philips) cryo-electron microscope fitted with a cold stage.

**Mapping of HSR8 and Complementation Tests**

The HSR8 gene was mapped in the F2 population of crosses to Arabidopsis ecotype Landsberg erecta using simple sequence length polymorphism (Bell and Ecker, 1994) and CAPS (Konieczny and Ausubel, 1993) markers. To fine-map the HSR8 locus, new molecular markers were developed according to public databases (see Supplemental Table 4 online). A genomic DNA fragment containing the entire HSR8 coding region, the 2.6-kb upstream sequence, and the 1.5-kb downstream sequence was inserted into the binary vector pGreen to generate the transformation plasmid pGHSR8com for complementation. The plasmid pGHSR8com was introduced into the hsr8-1, mur4-1, and mur4-3 mutants using Agrobacterium tumefaciens GV3101, and transformants were selected on β-phosphonothricin medium. The HSR8 promoter: GUS construct (HSR8prom-GUS) was made using a PCR-based Gateway system. The promoter-specific primers for the HSR8 gene were TOPAT1G30620PROM-F (5'-CACCTGAGCAGCGAGGTTG-3') and TOPAT1G30620PROM-R (5'-TTGATTCACTTCAGCTGGCG-3'). PCR products were subcloned into the pENTR/D-TDO vector (Invitrogen) using TOPO enzyme and sequenced. The HSR8 promoter was then subcloned into Gateway binary vector (pGWB3) containing the GUS reporter gene.

**RNA Preparation, cRNA Synthesis, and Microarray Hybridization**

Total RNA was extracted from 6-d-old dark-grown seedlings of Col-0, mur4-1, prl1, and mur4-1 prl1 using the RNeasy plant mini kit (Qiagen). Affymetrix AxiomChip array expression profiling was performed at the Nottingham Arabidopsis Stock Centre according to Affymetrix Expression Analysis Technical Manual II (http://www.affymetrix.com/support/technical/manuals.affx). Two independent biological replicates were conducted. Microarray data analysis and clustering were performed as described (Li et al., 2006). Rank products (Breitling et al., 2004) were compared with the rank products of 10,000 random permutations of the same data to assign E-values. To correct for the multiple testing problem, we used the false discovery rate (Storey, 2003), in which the E-value of each gene was divided by its position in the list of changed transcripts. A false discovery rate < 0.05 means that only 5% or fewer genes up to this position are expected to be observed by chance (false positive) and the remaining 95% are true positives.

**Accession Number**

Microarray data from this article can be found in the ArrayExpress data library under accession number E-NASC-78.

**Supplemental Data**

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

**Supplemental Figure 1.** Carbohydrate Levels and Uptake in the hsr8-1 Mutant.

**Supplemental Figure 2.** Dark Development and Hypocotyl Elongation of hsr8 Are Not Affected by pGfGfY.

**Supplemental Figure 3.** Dark Development Phenotypes of the Wild Type and mur4-2.

**Supplemental Figure 4.** Quantitative Real-Time RT-PCR Analysis.

**Supplemental Table 1.** Analysis of Gene Expression Changes in mur4-1 using Affymetrix ATH1 Microarrays.

**Supplemental Table 2.** Analysis of Gene Expression Changes in prl1 using Affymetrix ATH1 Microarrays.

**Supplemental Table 3.** Cluster Analysis of Differentially Regulated Genes in mur4-1 and prl1.

**Supplemental Table 4.** PCR-Based Molecular Markers.

**Supplemental Table 5.** Primers Used in Quantitative Real-Time RT-PCR.

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