FINAL REPORT: DE-FG02-03ER15417

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TITLE: "Characterization of *sugar-insensitive* (*sis*) Mutants of Arabidopsis"

SUMMARY OF PROGRESS

- Two new loci involved in sugar response have been identified. Mutations in *HAC1*, which is predicted to encode a histone acetyl-transferase, confer a *sugar-insensitive* (*sis*) phenotype whereas mutations in *SOV1*, which is predicted to encode a previously uncharacterized protein phosphatase 2c, confer a *sugar oversensitive* (*sov*) phenotype.
- An extensive characterization of sugar and phytohormone responses has been completed for the previously identified *sis3-1* mutant.
- The *SIS3* gene has been identified using a map-based approach and is predicted to encode a protein with ubiquitin E3 ligase activity.
- Biochemical analyses of the SIS3 protein indicate that, as predicted by its sequence, it has ubiquitin E3 ligase activity.
- Characterization of *sis7* indicates that, like *sis3* but unlike many other sugar-response mutants, *sis7* exhibits a wild type or near wild-type response to ABA and the GA biosynthesis inhibitor paclobutrazol.
- The *SIS7* gene has been cloned using a map-based cloning approach.
- GeneChip experiments have been used to identify genes that are altered in expression in germinating seeds of the *sis2, sis4, sis5, sis6* and *sis7* mutants.
- Characterization of sorbitol (osmotic control), 3-OMG, mannose, Glc and Suc-regulated gene expression has resulted in identification of genes most likely to be regulated via different sugar-response pathways.
- Characterization of Glc, ABA, ethylene and GA-regulated gene expression in wild-type germinating seeds indicates a particularly strong relationship between Glc and ABA regulated gene expression.
- Comparison of *sis2, sis6* and *sis7* gene expression patterns with genes regulated by different sugars and sugar analogs has allowed identification of the groups of genes (e.g. Glc regulated, mannose regulated, etc.) that are most likely to be altered in expression levels by these different mutations. Interestingly, *sis7* affects expression of genes apparently regulated via different sugar-response pathways, including a potentially Suc-specific pathway.
- Analysis *of sis2, sis6* and *sis7* gene expression patterns suggests that SIS2 and SIS6 are themselves positively regulated by sugars whereas SIS7 is most likely to be negatively regulated.

- Cross comparison of *sis2, sis6* and *sis7* gene expression patterns has revealed a significant correlation between *sis2* and *sis6* affected genes and between *sis6* and *sis7* affected genes, but only a weak correlation between *sis2* and *sis7* affected genes.

Publications that acknowledge DOE funding

Gibson SI (2004) Sugar and phytohormone response pathways: navigating a signaling network. J. Exp. Bot. **55:** 253-264

Gibson SI (2005) Control of plant development and gene expression by sugar signaling. Curr. Opin. Plant Biol. **8:** 93-102

Huang Y, Li CY, Biddle KD, Gibson SI (2008) Identification, cloning and characterization of *sis7* and *sis10* sugar-insensitive mutants of *Arabidopsis*. BMC Plant Biol. **8:** 104

Huang Y, Li CY, Park S, Gibson SI (2009) SIS8, a putative mitogen-activated protein kinase kinase kinase, regulates sugar resistant seedling development in Arabidopsis. under revision

Huang Y, Li CY, Pattison DL, Gray W, Park S, Gibson SI (2009) SUGAR INSENSITIVE3, a RING E3 ligase, is a new player in plant sugar response. under revision

Characterization of Previously Identified *sugar-insensitive* (*sis*) Mutants of Arabidopsis

High concentrations of either Suc or Glc inhibit very early seedling development of Arabidopsis (Gibson and Kincaid, 1995). When seeds are sown on minimal media supplemented with 0.3 M Glc or Suc, most of the seeds germinate, but only 0-5% develop relatively normal shoot systems with expanded cotyledons and true leaves. Media-shift experiments indicate that Arabidopsis is sensitive to sugar-mediated inhibition of early seedling development during only the first ~ 40 hours after the start of imbibition (Gibson et al., 2001). In addition, biochemical analyses indicate that high sugar concentrations inhibit metabolism of seed storage lipids (Martin et al., 2002, To et al., 2002) and development or replication of mature chloroplasts (To et al., 2003).

The finding that Glc and Suc can act as negative regulators of seedling development made possible an exceptionally efficient screen for identifying mutants defective in sugar response. In brief, mutagenized seeds were sown on solid minimal media containing ~ 0.3 M Glc or Suc. After 12-14 days, seedlings that had developed relatively normal shoot systems were transferred to soil, grown to maturity and re-assayed in the next generation. To date, four independent mutant screens have been conducted, using EMS-mutagenized, T-DNA tagged and activation tagged seed populations. These screens have resulted in the identification of 28 *sugar-insensitive* (*sis*) mutants, that fall into at least 7 complementation groups. Later efforts focused on characterization of a subset of these mutants, described below.

Characterization of sugar sensitivity in *sis3, sis6* and *sis7*. The *sis3, sis6* and *sis7* mutants are resistant to the inhibitory effects of both Glc and Suc on early seedling development but display a wild-type response to equi-molar concentrations of sorbitol (Figure 1). Characterization at higher sorbitol levels reveals that these mutants are somewhat resistant to greater than equi-molar sorbitol concentrations. However, *sis3* and *sis7* (*sis6* has not been extensively analyzed) are also somewhat resistant to the inhibitory effects of mannose on seed germination (data not shown). Mannose is a Glc analog that is poorly metabolized and that inhibits seed germination through a hexokinase-mediated pathway (Pego et al., 1999) at concentrations that are too low (~ 1-5 mM) to exert an osmotic effect, indicating that the effects of the *sis3* and *sis7* mutations cannot be explained solely by alterations in osmo-tolerance.

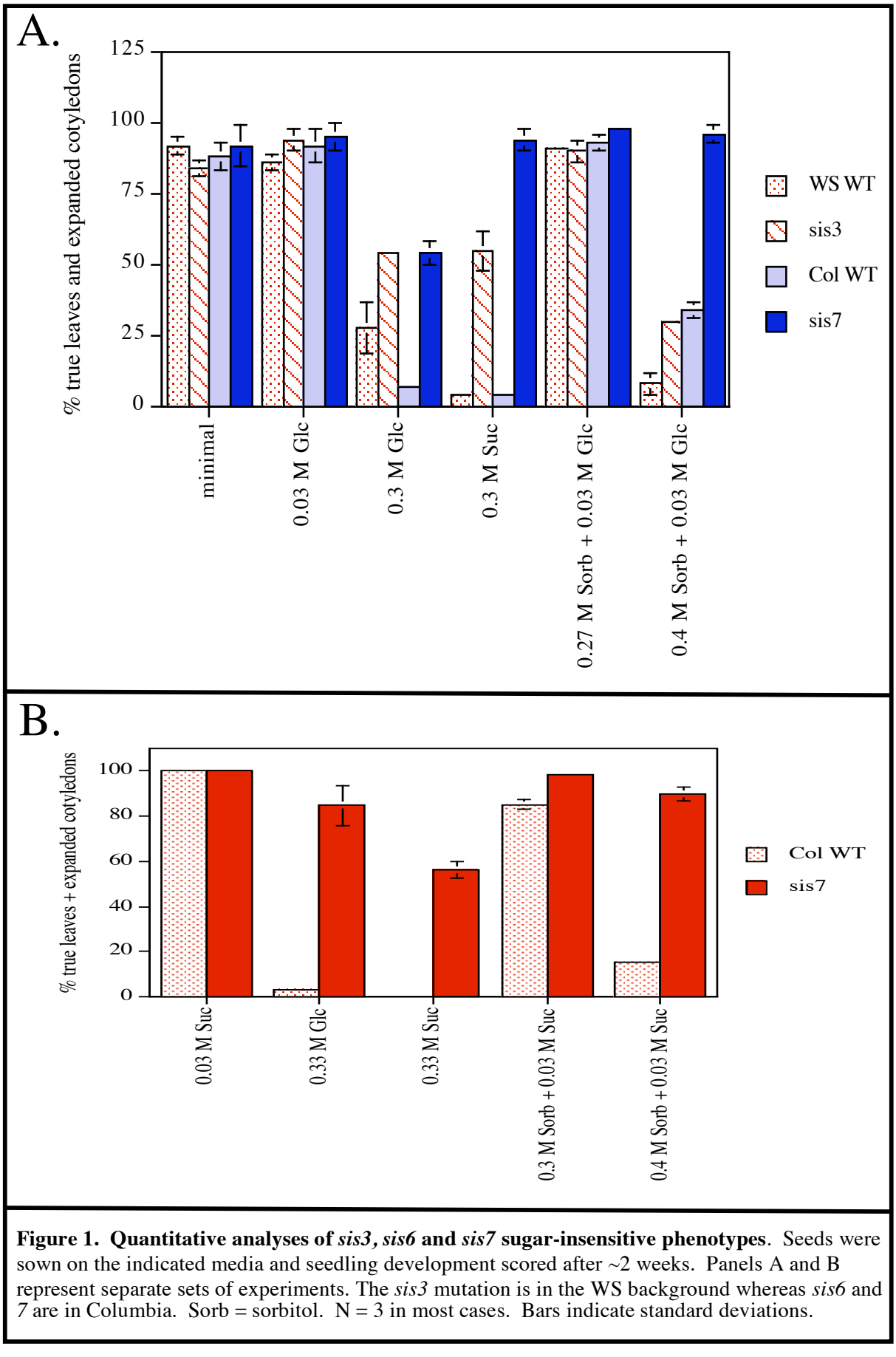


Figure 1. Quantitative analyses of *sis3, sis6* and *sis7* sugar-insensitive phenotypes. Seeds were sown on the indicated media and seedling development scored after ~2 weeks. Panels A and B represent separate sets of experiments. The *sis3* mutation is in the WS background whereas *sis6* and *7* are in Columbia. Sorb = sorbitol. N = 3 in most cases. Bars indicate standard deviations.

Characterization of phytohormone response in the *sis2*, *sis3*, *sis6* and *sis7* mutants. As many previously characterized sugar-response mutants have been found also to be defective in response to ABA, ethylene (Gibson, 2004) or the GA biosynthesis inhibitor paclobutrazol (Sommerlad and Gibson, unpublished results), it was of interest to characterize the response of the *sis* mutants to different phytohormones. The *sis3* mutant has been characterized particularly extensively and found to exhibit a wild type or near wild-type response in all phytohormone metabolic and response assays conducted to date. These assays have included: root elongation on epibrassinolide, methyljasmonate, auxin, cytokinin, salicylic acid and ABA; hypocotyl elongation on GA and AVG (an inhibitor of ethylene biosynthesis) and seed germination on ABA and paclobutrazol. Although *sis3* exhibits a slightly increased ability to germinate on both ABA (data not shown) and paclobutrazol (Figure 2), these effects are minor compared to known ABA insensitive mutants, such as *abi4*/*sis5* (Finkelstein, 1994), or known paclobutrazol-resistant mutants, such as *spy3* (Jacobsen and Olszewski, 1993), suggesting that these could be indirect effects or secondary consequences of these mutations. For example, mutations in *CTR1*, which acts in ethylene response (Kieber et al., 1993), cause stronger ABA insensitive (data not shown) and paclobutrazol-resistant phenotypes (Gibson et al., 2001) than mutations in *SIS3*. The *sis2, sis6* and *sis7* mutants exhibit near wild-type responses to ABA (data not shown). The *sis7* mutant also exhibits a wild-type response to paclobutrazol (Figure 2). In contrast, *sis2* and *sis6* exhibit significant resistance to the inhibitory effects of paclobutrazol on seed germination (data not shown).

Identification of genes altered in expression in *sis2*, *sis4-1/aba2-3*, *sis5-3/abi4-103*, *sis6* and *sis7*. As a broad method to screen for defects in sugar response, Affymetrix ATH1 GeneChips that contain information from ~ 24,000 genes were used to examine transcript levels in several of the *sis* mutants (Table 1, each GeneChip represents an independent biological replicate). Mutant and wild-type seeds were sown on nytex screens on solid minimal media (Kranz and Kirchheim, 1987). After 20 hour at 21°C and ~60 μmol photons m^{-2} s^{-1} continuous light, the nytex screens and seeds were transferred to minimal media supplemented with 0.1 M Glc or sorbitol. Seeds were harvested and frozen after an additional 12.6 hours under the same growth conditions. A concentration of 0.1 M Glc or sorbitol was chosen as this concentration has been shown to be sufficient to cause significant alterations in gene expression without exerting the severe developmental effects seen at higher sugar concentrations (Laby et al., 2000, Gibson et al., 2001). Seeds were first incubated on minimal media for 20 hours prior to induction to minimize differences in developmental stage between Glc and sorbitol treated seeds, as sugars have been shown to delay seed germination (To et al., 2002, Price et al., 2003). A total growth time of 32.6 hours was chosen to fit within a critical developmental window during which wild-type germinating seeds are sensitive to high concentrations of exogenous Suc or Glc (Gibson et al., 2001). At the end of the 32.6 hour total growth period, only 0-5% of the seeds had germinated. Seeds harvested from a minimum of three Petri plates were used to isolate RNA samples that were then used for GeneChip analyses.

Wild type and *sis* transcript profiles were compared for samples grown on 0.1 M Glc (Table 2). *P* value cutoffs of < 0.05 and < 0.2 were chosen to identify those genes that are "highly" or "moderately" likely really to differ in transcript levels. In contrast, the *p* value cutoff of > 0.8 was chosen to identify those genes that are least likely really to differ in transcript levels. As no other labs have published data from the same developmental stage and sugar treatments characterized here, comparison of these results with results of other labs is currently not practical. In addition, space constraints prevent a detailed description of all of the results. However, several of the genes that exhibit altered transcript levels in one or more of the *sis* mutants are of particular interest as they suggest some possible biological effects of these mutations. For example, transcripts of genes encoding a seed storage protein and an oleosin are present at 15 and 2 fold higher levels, respectively, in *sis7* than wild type, suggesting SIS7 may affect production of seed storage proteins and lipids. Similarly, two genes involved in photosynthesis are expressed at 2 fold lower levels in *sis7*, suggesting SIS7 affects photosynthesis.

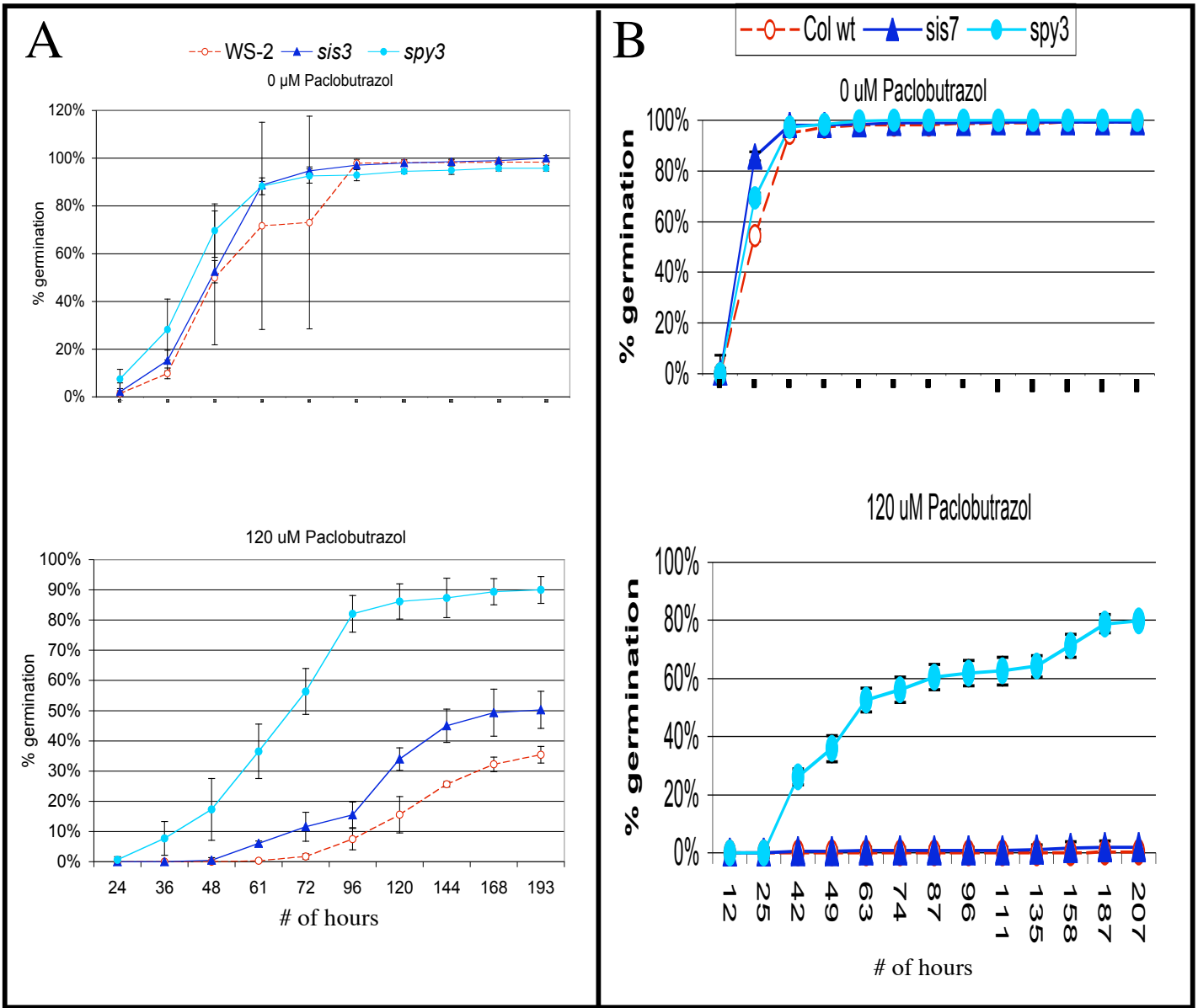


Figure 2. The *sis3* and *sis7* mutants exhibit wild-type or near wild-type responses to paclobutrazol. Mutant and wild-type seeds were sown on paclobutrazol, an inhibitor of GA biosynthesis. Seed germination was scored at regular intervals. A known paclobutrazol-resistant mutant, *spy3*, was included as a positive control. N = 3 in most cases. Bars indicate standard deviations.

Additional results from these experiments are discussed below in "Network analyses".

Identification of Components of Sugar-Response Pathways

Identification of *SIS3, SIS6* and *SIS7.* Few components of sugar-response pathways have been identified, making identification of additional components a high priority. The *sis6-1* mutant was isolated from an activation tagged population (LeClere and Bartel, 2001) which has mutations resulting from

Table 1. Summary of GeneChip experiments completed.

Genotype	Inducing Agent	Induction Period	# GeneChips Completed
Wild type	No agent (minimal media)	3 hrs	2
Wild type	100 mM sorbitol	3 hrs	2
Wild type	0.5 mM mannose	3 hrs	3
Wild type	100 mM glucose	3 hrs	3
Wild type	100 mM sucrose	3 hrs	3
Wild type	10 μM ABA	3 hrs	1
Wild type	50 μM ACC	3 hrs	2
Wild type	10 μM GA3	3 hrs	2
Wild type	No agent (minimal media)	12.6 hrs	4
Wild type	100 mM sorbitol	12.6 hrs	5
Wild type	100 mM 3-OMG	12.6 hrs	3
Wild type	0.5 mM mannose	12.6 hrs	3
Wild type	100 mM glucose	12.6 hrs	5
Wild type	100 mM sucrose	12.6 hrs	3
Wild type	10 μM ABA	12.6 hrs	4
Wild type	50 μM ACC	12.6 hrs	4
Wild type	10 μM GA3	12.6 hrs	3
sis2-1	100 mM sorbitol	12.6 hrs	4
sis2-1	100 mM glucose	12.6 hrs	3
sis4-1/aba2-3	100 mM glucose	12.6 hrs	4
sis5-3/abi4-103, gl1	100 mM glucose	12.6 hrs	3
sis6-1	100 mM sorbitol	12.6 hrs	4
sis6-1	100 mM glucose	12.6 hrs	3
sis7-1	100 mM sorbitol	12.6 hrs	3
sis7-1	100 mM glucose	12.6 hrs	3

Table 2. GeneChip analyses of *sis2, sis6* and *sis7*. "Present" refers to the number of probe sets that exhibit an average Affymetrix present rating of greater than "moderate" in at least one of the two comparison groups. "Down $p < 0.05$" refers to probe sets where the signal intensities of the two comparison groups also vary with a p value of less than 0.05 and that are lower in the first than in the second comparison group. "Down, $p > 0.05$ and > 1.5X" refers to probe sets where the average signal intensities also differ by > than 1.5 fold between the two comparison groups.

	# Probe Sets:							
Comparison Groups	Present	Down, $p < 0.05$	Down, $p < 0.2$	$p > 0.8$	Up, $p < 0.2$	Up, $p < 0.05$	Down, $p < 0.05$ and > 1.5X	Up, $p < 0.05$ and > 1.5X
sis2/Glc vs. WT/Glc	11,938	475	1,575	1,991	1,529	475	213	299
sis6/Glc vs. WT/Glc	11,411	313	1,160	2,379	1,360	463	110	235
sis7/Glc vs. WT/Glc	11,091	690	1,921	1,740	1,531	500	389	245

overexpression or co-expression of Arabidopsis cDNAs cloned behind the CaMV 35 S promoter or by T-DNA insertion. Several lines of evidence suggest that the *sis6-1* phenotype is due to overexpression of the gene carried on the T-DNA insert. First, characterization of the mutant revealed that it has a dominant phenotype, consistent with an effect due to gene overexpression or co-suppression, and GeneChip analyses revealed that this gene is expressed at > 5 fold higher levels in *sis6-1* than in wild-type. Finally, genetic analyses indicate that the *sis* phenotype co-segregates with the T-DNA insert. The putative *SIS6* gene is predicted to encode a small protein with no significant sequence similarity to proteins of known biochemical function. Genes with similar DNA sequences are found in other plant species, but not in non-plant species, suggesting this gene may be plant specific.

The *sis3-1* and *sis7-1* mutants exhibit recessive phenotypes and were identified from T-DNA mutagenized populations, but neither mutation is linked to a T-DNA insert. Therefore, a map-based approach was used to identify these genes.

Identification of *HAC1* and *SOV1*. As reverse genetics approaches can provide an efficient means of identifying some of the genes involved in certain processes, a reverse genetics approach was initiated for the identification of additional components of sugar-response pathways. This approach is based on the rationale that some genes involved in sugar response are likely to be themselves sugar regulated and that some of these genes are likely to encode factors with activities commonly associated with response pathways (e.g. protein kinases, protein phosphatases and transcription factors). Therefore, Affymetrix GeneChips were used to identify genes that are Glc and/or Suc regulated in wild-type germinating seeds. Seeds were treated as described above for characterization of the *sis* mutants. Results from Glc or Suc treated seeds were then compared with results from sorbitol (= osmotic control) treated seeds. Of ~ 24,000 genes represented on the GeneChips, ~ 11,000 are expressed in germinating seeds. Of these, 2,394 genes exhibit significant ($p < 0.05$ in a Student's T-test) alterations in signal intensities when Glc or Suc replicates are compared with sorbitol replicates. 1,258 of these genes also exhibit a >1.5 fold average difference in signal intensity. 189 of these genes have been designated as "target" genes, based on being predicted to encode proteins with functions often associated with response pathways.

To test which of the 189 target genes affect sugar response, T-DNA insertion lines were obtained from the ABRC. To date, plants that are homozygous for 220 T-DNA insertion events in a total of 134 target genes have been identified. Although phenotypic characterization of these lines has only recently begun, two new groups of sugar-response mutants have already been identified, demonstrating the utility of this approach. Three independent mutations in a gene (*HAC1*) predicted to encode a protein homologous to CREB-binding proteins, which act as co-activators of transcription and exhibit histone acetyl-transferase activity, confer weak, but consistent, *sis* phenotypes (data not shown). In addition, two independent mutations in a previously uncharacterized gene predicted to encode a protein phosphatase 2c confer sugar oversensitive (*sov*) phenotypes (Figures 3 and 4). Further characterization of the mutants identified using this reverse genetics approach will be supported by funding from the Consortium for Plant Biotechnology Research.

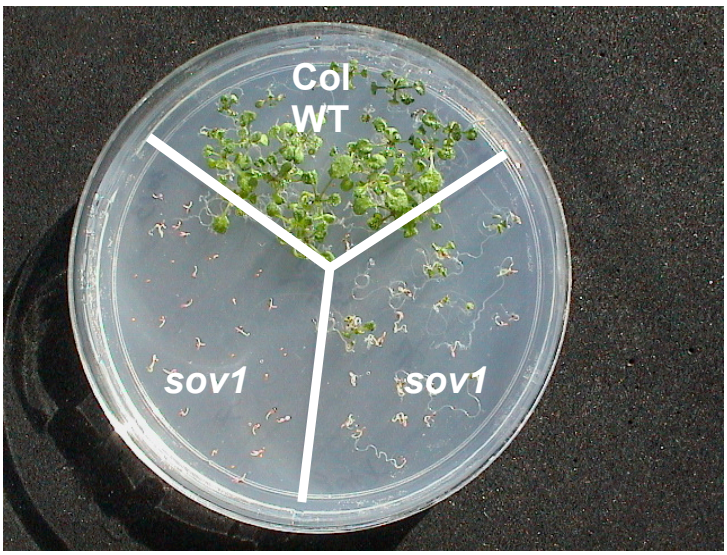


Figure 3. The *sov1* mutants are sugar oversensitive. Mutant and wild-type seeds were sown on 0.23 M Glc and photographed after 14 days under continuous light.

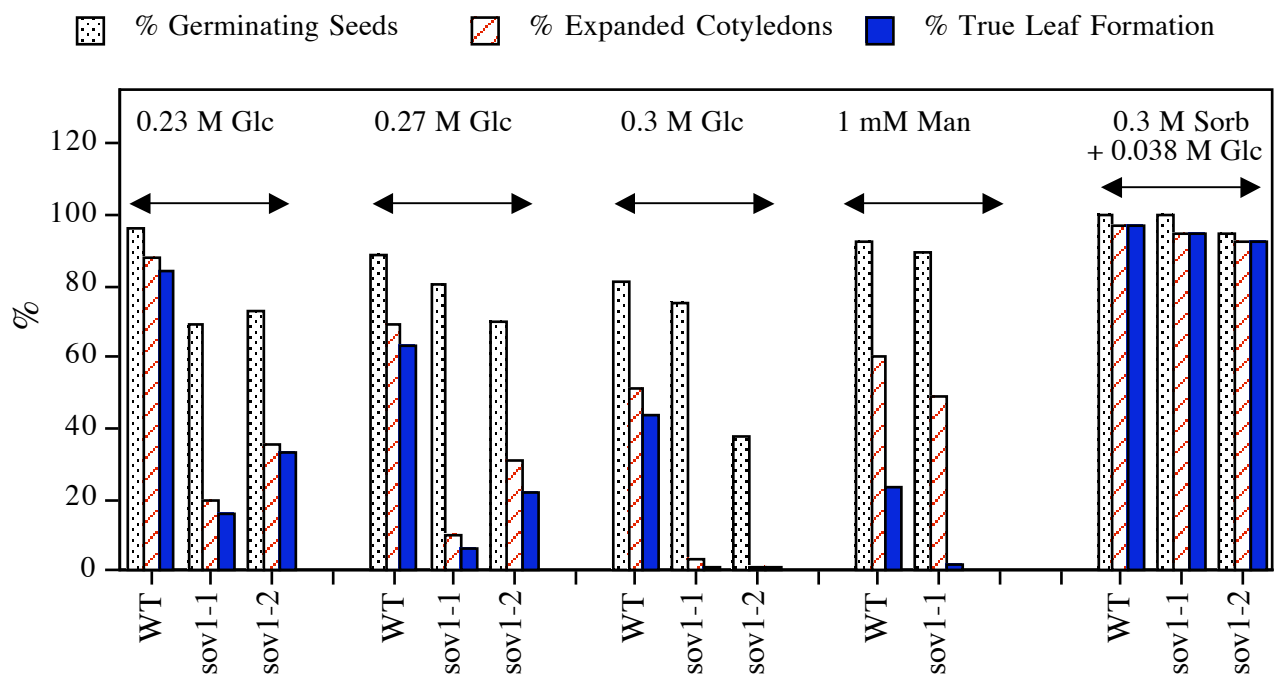


Figure 4. Quantitative analysis of *sov1* phenotype. Seeds were sown on the indicated media and seed germination, cotyledon expansion and true leaf formation scored after ~ 2 weeks. Man = mannose.

Network Analyses

Soluble sugars, such as Glc or Suc, are believed to act via multiple sugar-response pathways (Gibson, 2000, Smeekens, 2000, Xiao et al., 2000). In addition, sugar-response pathways "interact" with multiple other response pathways, such as those for phytohormones. Characterization of these signaling "networks" represents a complex problem that needs to be addressed using multiple approaches. In addition to identifying and characterizing mutants that are altered in one or more response, as described above, transcriptional profiling experiments can provide useful information for network analyses. For example, transcriptional profiling experiments have allowed development of models for the plant-pathogen response network (Glazebrook et al., 2003, Katagiri and Glazebrook, 2003).

Identification of genes likely to be regulated via different sugar-response pathways. Soluble sugars are believed to act via multiple sugar-response pathways, including hexokinase-independent, hexokinase-dependent and Suc-specific pathways (Gibson, 2000, Smeekens, 2000, Xiao et al., 2000). To identify genes most likely to be regulated via different sugar-response pathways, Affymetrix GeneChips were used to perform transcriptional profiling of wild-type germinating seeds treated with different sugars and sugar analogs (Table 1). Sugar treatments were as described above. Mannose was used at a concentration of only 0.5 mM as mannose is 100-200X more potent an inhibitor of seed germination and early seedling development than Glc. Comparison of seeds treated with 100 mM Glc or Suc with seeds treated with an equi-molar concentration of sorbitol as an osmotic control reveals that several hundred genes are significantly regulated by Glc and/or Suc (Table 3). Comparison of 3-OMG and sorbitol treated seeds identifies those genes that are the most likely candidates for genes regulated via a hexokinase-independent pathway. Comparison of 0.5 mM mannose vs. minimal and 3-OMG vs. sorbitol samples identifies genes that are the most likely to be regulated via a hexokinase-dependent pathway. Finally, comparison of Glc and Suc regulated genes allows identification of genes that represent the best

Table 3. Analyses of sugar and phytohormone affected gene expression in wild-type plants.

	# Probe Sets:							
Comparison Groups (all WT, 12.6 hr treatments)	Present	Down, $p < 0.05$	Down, $p < 0.2$	$p > 0.8$	Up, $p < 0.2$	Up, $p < 0.05$	Down, $p < 0.05$ and > 1.5X	Up, $p < 0.05$ and > 1.5X
Sorbitol vs. minimal	10,877	103	683	2,500	627	82	17	21
3-OMG vs. sorbitol	10,620	279	1,096	1,999	970	224	165	27
Mannose vs. minimal	11,147	583	1,740	1,533	2,038	712	266	356
Glucose vs. sorbitol	11,192	561	1,427	1,916	1,709	626	289	229
Sucrose vs. sorbitol	11,034	497	1,541	1,619	1,863	669	302	323
Sucrose vs. glucose	11,213	415	1,366	1,959	1,409	410	201	177
ABA vs. minimal	10,742	1,093	2,275	1,329	1,985	894	736	534
ACC vs. minimal	11,066	260	991	2,142	1,280	376	103	187
GA3 vs. minimal	11,171	207	934	2,610	766	167	27	36

candidates to be regulated via a Suc-specific pathway. Alternatively, these genes could be regulated via a Glc-specific pathway, although most Glc-regulated genes will also appear to be Suc regulated as feeding with Suc will tend to raise intracellular Glc (and Glc metabolite) as well as Suc levels. Of course, the reverse is also true, as feeding with Suc will tend to raise intracellular levels of both Suc and Glc. However, the identification of genes that are, in fact, regulated by Suc feeding but not by Glc feeding (Chiou and Bush, 1998) suggests that a Suc sensor may be located at or near the plasma membrane and may sense Suc as it enters the cell. Also, further analysis of Glc, Suc and sorbitol expression patterns should allow a more exact identification of genes regulated through a Suc-specific pathway.

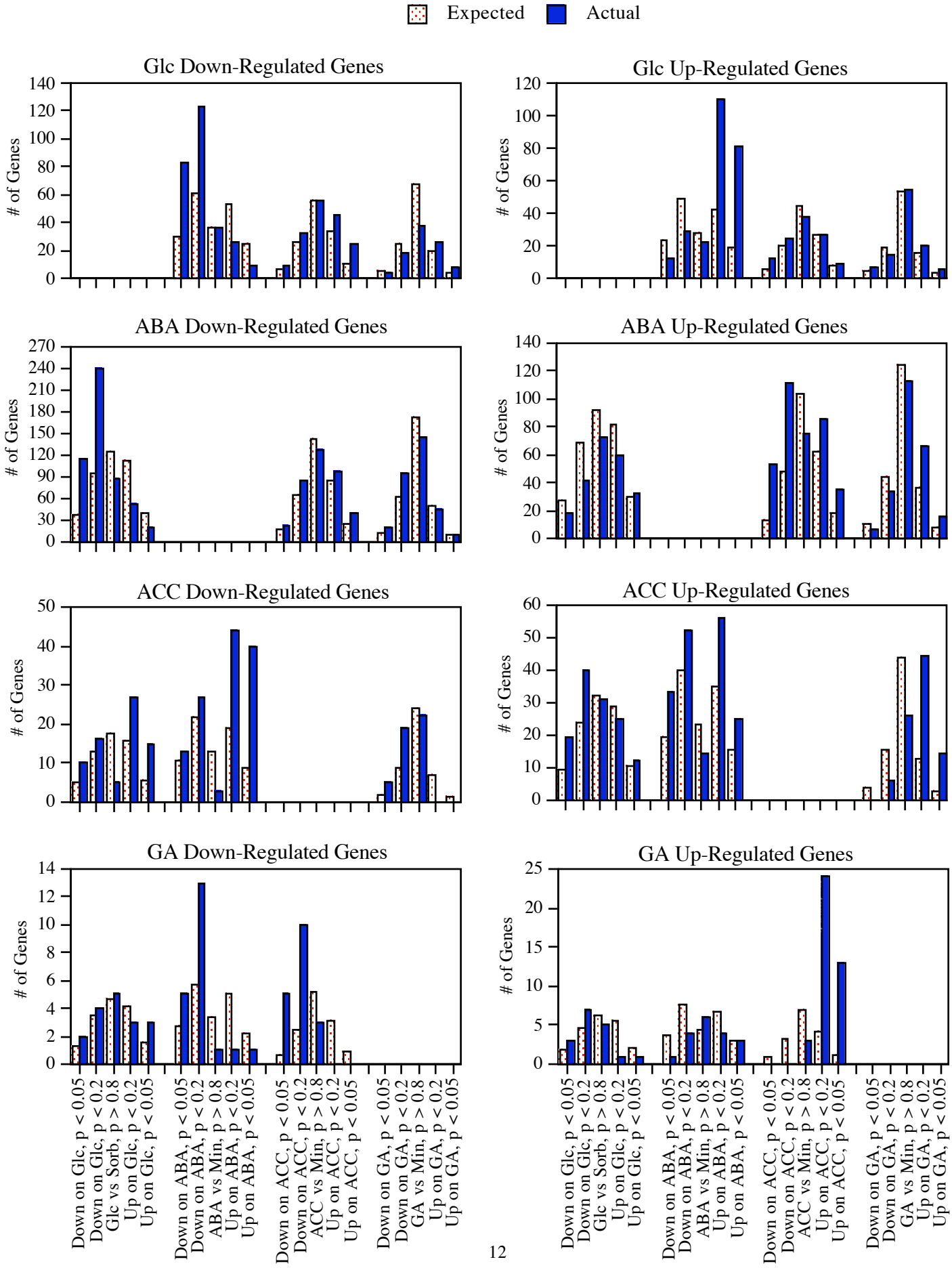
Characterization of sugar versus phytohormone regulated gene expression. Results from our lab and others indicate that many mutations that affect sugar response also affect phytohormone response. For example, some mutations that confer a *sis* phenotype also confer ABA (Laby et al., 2000) and/or paclobutrazol resistant (Sommerlad and Gibson, unpublished results) seed germination phenotypes or altered response to ethylene (Gibson et al., 2001). Therefore, a more complete understanding of sugar response will require additional information regarding the "relationships" between these different pathways. A variety of biochemical, genetic, genomic and molecular approaches will be needed to address this area. For example, comparison of genes that are regulated in response to different stimuli will allow determination of the numbers of genes that are regulated by different combinations of stimuli. In addition, these analyses will indicate whether genes that are regulated in response to multiple stimuli tend to be regulated in the same or opposing directions by those stimuli. A strong correlation between genes that are regulated by one stimulus being also regulated in the same direction by a different stimulus provides evidence that these stimuli may act to regulate a significant number of the same or similar processes in a common manner. In contrast, a finding that the group of genes that are up regulated by one stimulus disproportionately includes genes that are also down regulated by a different stimulus suggests

that the two stimuli act to regulate a significant number of the same or similar processes, but do so in an opposing manner (i.e. one may stimulate the process whereas the other may inhibit it).

To allow analyses of the types described above, GeneChip experiments were conducted on wild-type germinating seeds treated with 10 μM ABA, 50 μM 1-aminocyclopropane-1-carboxylic acid (ACC, a precursor for ethylene biosynthesis) or 10 μM gibberellic acid (GA3). These experiments were conducted in parallel with the experiments examining sugar-regulated gene expression described above, to allow cross comparison of the results. Comparison of Glc-regulated genes with ABA, ACC and GA regulated genes reveals that genes that are Glc regulated exhibit a very significant tendency also to be ABA regulated, but only a slight or no tendency also to be regulated by ACC or GA (Figure 5). In addition, a disproportionately large number of genes that are down regulated on Glc are also down regulated on ABA. Similarly, genes that are up regulated by Glc are significantly more likely to be up regulated by ABA than are random groups of genes expressed in germinating seeds. These results suggests that Glc and ABA regulate a number of the same or similar processes during seed germination and are consistent with results indicating that Glc can affect ABA levels in germinating seeds (Price et al., 2003). Although not a focus of this study, the results from these experiments also allow cross comparisons between the three phytohormones tested. Surprisingly, there appears to be a somewhat stronger correlation between GA and ACC regulated genes than between GA and ABA.

Characterization of the effects of the *sis2*, *sis6* and *sis7* mutations on expression of genes likely regulated via different sugar-response pathways. As stated above, plants are believed to have several sugar-response pathways that together comprise a sugar-response network. To determine which response pathways are most likely to be affected by different *sis* mutations, lists of genes that are mis-regulated in different mutant backgrounds were compared with groups of genes that are regulated in response to different sugars and sugar analogs in wild-type germinating seeds. To identify genes that are mis-regulated, GeneChip results from *sis* seeds germinating in the presence of 0.1 M Glc were compared with the results of germinating wild-type seeds grown on the same media under identical conditions. Note that Glc-feeding may directly affect expression not only of Glc-regulated genes, but also of 3-OMG and mannose-regulated genes. In addition, Glc-feeding may raise intracellular Suc concentrations, affecting the expression of genes that are affected by Suc feeding, although not directly affecting the expression of any genes regulated via a Suc-specific pathway. Finally, although Glc-feeding is not expected to have a significant effect on the expression of genes regulated via a Suc-specific pathway, endogenous Suc levels may affect the expression of genes regulated via a Suc-specific pathway, potentially allowing detection of differences in the expression levels of some Suc-specific genes between the mutant and wild-type samples. By comparing mutant and wild-type results, groups of genes that are down or up in particular mutants relative to wild type were identified. Note that, as the *sis2-1* and *sis7-1* mutations are likely to represent loss of function mutations, genes that are down in *sis2* or *sis7* relative to wild type are genes that are positively regulated by wild-type SIS2 or SIS7. In contrast, as *sis6-1* is likely to represent a gain of function mutation caused by overexpression of the putative *SIS6* gene, genes that are down in *sis6* are genes that are negatively regulated by wild-type SIS6.

Figure 5. Comparison of Glc, ABA, ACC and GA regulated gene expression (see next page). "Glc Down-Regulated Genes" are defined as those genes corresponding to probe sets that are "present" with an average Affymetrix present rating of greater than moderate for at least one of the two comparison groups (wild type on 0.1 M Glc vs. wild type on 0.1 M sorbitol) and where the signal intensities of the two comparison groups vary with a p value of < 0.05 and are at least 1.5X lower on Glc than on sorbitol. ABA, ACC and GA Up and Down lists were generated in a similar manner. The numbers of genes on each list can be found in the two columns on the right side of Table 3. These lists of genes were analyzed to determine how many genes on each list are also affected by other treatments. For example, genes that are on the Glc Down list were analyzed to determine how many are also down on ABA vs. minimal with a p value of < 0.05 (but with no minimum fold difference in signal intensities). For comparison purposes the numbers of genes on each list that would be expected to be regulated in response to a second treatment if everything was random are also indicated. For example, 1,093 out of 10,742 expressed genes have a lower average signal intensity on ABA than minimal and differ in signal intensities with $p < 0.05$. Given that there are 289 genes on the Glc Down list, if there is no correlation between Glc and ABA regulated gene expression one would expect 1,093/10,742 X 289 = 29 genes on the Glc Down list also to be down on ABA with $p < 0.05$. Expected = # of genes that would be expected to be in each category if expression patterns were random. Actual = # of genes actually found in each of the indicated categories.


Expected
Actual
Glc Down-Regulated Genes
Glc Up-Regulated Genes
ABA Down-Regulated Genes
ABA Up-Regulated Genes
ACC Down-Regulated Genes
ACC Up-Regulated Genes
GA Down-Regulated Genes
GA Up-Regulated Genes
# of Genes
Down on Glc, p < 0.05
Down on Glc, p < 0.2
Glc vs Sorb, p > 0.8
Up on Glc, p < 0.2
Up on Glc, p < 0.05
Down on ABA, p < 0.05
Down on ABA, p < 0.2
ABA vs Min, p > 0.8
Up on ABA, p < 0.2
Up on ABA, p < 0.05
Down on ACC, p < 0.05
Down on ACC, p < 0.2
ACC vs Min, p > 0.8
Up on ACC, p < 0.2
Up on ACC, p < 0.05
Down on GA, p < 0.05
Down on GA, p < 0.2
GA vs Min, p > 0.8
Up on GA, p < 0.2
Up on GA, p < 0.05

The results of these analyses indicate that the list of genes that exhibit lower transcript levels in *sis2* relative to wild type (= "Genes Down in *sis2*") disproportionately includes genes that are up regulated by Glc in wild type (data not shown). This result suggests that Glc acts as a positive regulator of SIS2 and that genes that are expressed at lower levels in a *sis2* loss of function mutant include genes that are normally positively regulated by SIS2 in response to SIS2 activation by Glc. In other words, Glc positively regulates SIS2 which then, directly or indirectly, positively regulates a significant number of Glc-induced genes. Interestingly, the group of genes that is down in *sis2* does not include a disproportionate, or significantly greater than expected if random, number of genes that are up-regulated by 3-OMG or mannose, although this group does contain a somewhat decreased number of genes that are down-regulated by 3-OMG or mannose. These results suggest that SIS2 typically, but not necessarily solely, up-regulates Glc-induced genes (either directly or indirectly) via a pathway that requires metabolism. In contrast, the group of genes that is up in *sis2* includes disproportionate numbers of genes that are down regulated in wild type by both Glc and mannose. This result suggests that SIS2 may down regulate a significant number of Glc-repressed genes via a pathway that requires hexokinase activity but not significant further metabolism. The group of genes that is down in *sis2* also includes a disproportionate number of genes that are Suc induced and, conversely, the group of genes that is up in *sis2* includes a disproportionate number of genes that are Suc repressed. However, neither group contains a disproportionate number of genes that are expressed at significantly different levels between wild-type seeds treated with Glc versus Suc. These results suggest that SIS2 affects the expression of a significant number of genes that may be altered in expression in response to Suc feeding, but affects the expression of few if any genes that are regulated via a Suc-specific pathway.

Similar types of conclusions may be drawn from the analyses of the effects of the *sis6* and *sis7* mutations (data not shown). Although space limitations do not permit a detailed description of the results of these analyses, a few points are worthy of special attention. First, as the available evidence suggests the *sis6-1* phenotype is due to overexpression of *SIS6*, genes that are down in *sis6-1* represent genes that are likely to be negatively regulated by wild-type SIS6. The results therefore suggest that Glc activates SIS6, which then represses expression (directly or indirectly) of some Glc-repressed genes and induces expression of some Glc-induced genes. In contrast, the finding that the group of genes that is down in *sis7* disproportionately includes genes that are down regulated by different sugars and sugar analogs suggests that SIS7 activity is repressed by sugars. A particularly interesting finding is that both the genes that are down in *sis7* as well as those that are up in *sis7* disproportionately include genes that are the best candidates to be regulated in wild type via a Suc-specific pathway. This result suggests that SIS7 may play a role in a Suc-specific pathway, in addition to affecting Glc-responsive gene expression.

Comparison of *sis2, sis6* and *sis7* gene expression patterns. Groups of genes that are expressed at lower or higher levels in a particular mutant relative to wild type can also be analyzed to determine whether they include disproportionate numbers of genes that are highly or moderately likely also to be altered in expression in a different mutant background. This type of analysis can provide information regarding which loci are most likely to be involved in the regulation of similar groups of genes and whether those loci are likely to affect gene expression in the same or opposite directions. Comparisons of groups of genes affected by the *sis2, sis6* and *sis7* mutations suggest that *sis2* and *sis6* affect significant numbers of the same genes, but that SIS2 and SIS6 affect expression of these genes in opposite fashions (Figure 6). Similarly, these results suggest a disproportionate number of the genes whose expression is affected by *sis6* are also affected by *sis7*, and that SIS6 and SIS7 affect these genes in opposite fashions. In contrast, there is little apparent correlation between groups of genes affected by the *sis2* mutation and those affected by *sis7*. This result suggests that SIS2 and SIS7 may play relatively distinct roles in sugar response, although it should not be interpreted as indicating that no genes are regulated by both factors, but simply that this is not a common occurrence.

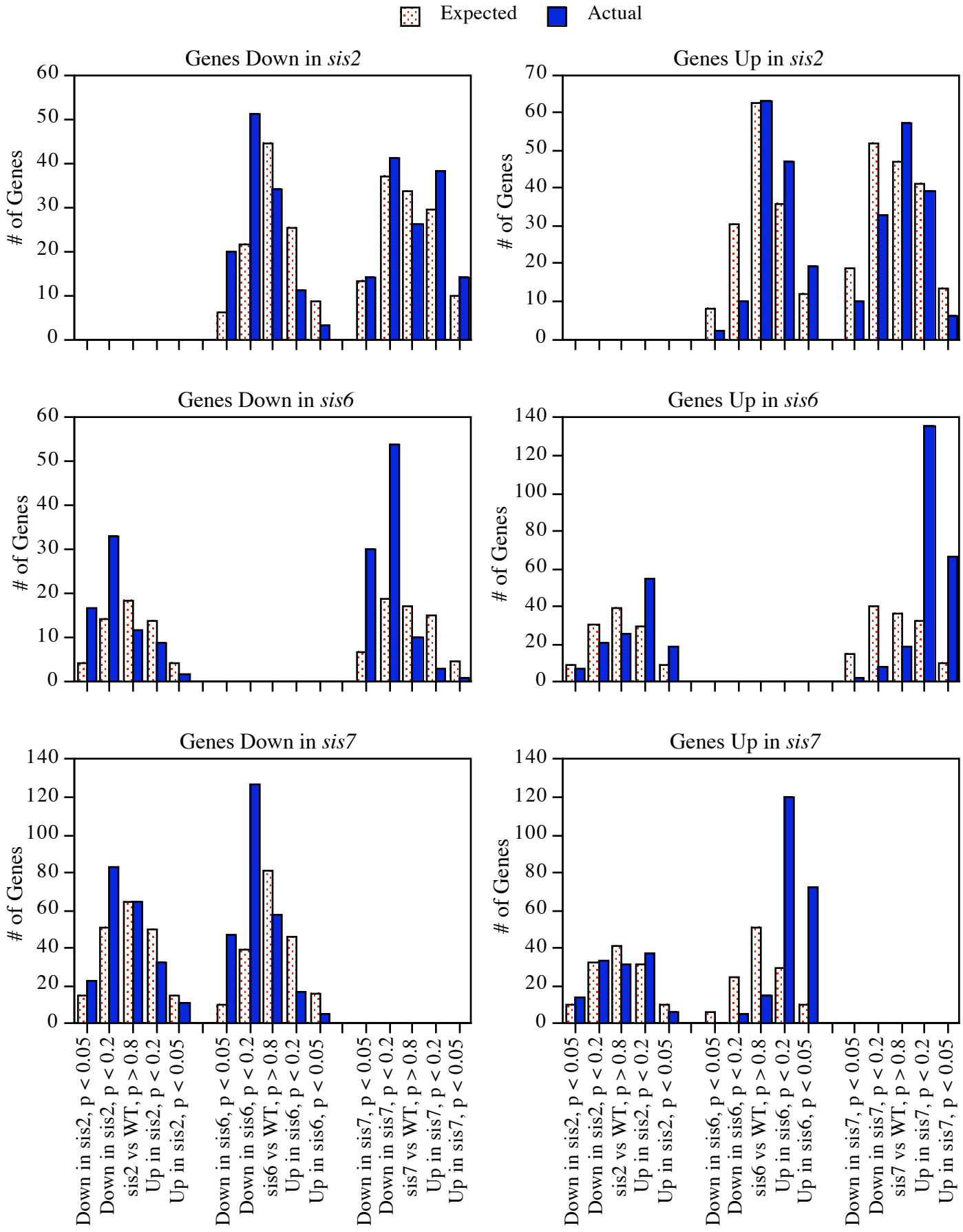


Figure 6. Comparison of *sis* transcriptional profiling experiments.

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