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The Arabidopsis sugar-insensitive2 (sis2) mutant displays a novel combination of altered sugar and phytohormone responses

by

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ABSTRACT

The Arabidopsis sugar-insensitive2 (sis2) Mutant Displays a Novel Combination of Altered Sugar and Phytohormone Responses

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Sugars are thought to affect many plant developmental processes; however, little is known about the mechanisms involved. Ultimately, gaining an understanding of these mechanisms may help improve crop yield. As a first step, this work characterized the Arabidopsis sugar-insensitive2 (sis2) mutant. Previous work shows that sis2, unlike wild type, develops a substantial shoot system on high concentrations of glucose, sucrose, and sorbitol; in addition, the sis2 mutant germinates on paclobutrazol, a gibberellin biosynthesis inhibitor. This thesis demonstrates that sis2 exhibits resistance to the effects of gibberellin and brassinosteroid biosynthesis inhibitors during germination, but not during later developmental stages. In addition, sis2 is resistant to the combined inhibitory effects of glucose and paclobutrazol on germination. These results suggest a novel connection between phytohormone and sugar response pathways.
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LIST OF ABBREVIATIONS

3-OMG, 3-0-methyl glucose

ABA, abscisic acid

ACC, 1-aminocyclopropane-1-carboxylic acid

Anc, ancymidol

AVG, aminoethoxyvinylglycine

Brz, brassinazole

Col, Columbia ecotype

EBR, 24-epibrassinolide

EMS, ethyl methane sulfonate

fwt, fresh weight

GA, gibberellic acid (gibberellin)

Glc, glucose

h, hours

IAA, indole-3-acetic acid

kb, kilobases

Man, mannose

Pac, paclobutrazol

SD, standard deviation

sis, sugar-insensitive

Sorb, sorbitol
Suc, sucrose

WT, wild type
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INTRODUCTION and BACKGROUND

Sugar levels are postulated to affect many plant developmental processes; however, little is known about the mechanisms by which plants sense and respond to sugar. The work described in this thesis takes a first step towards understanding these mechanisms. This work uses the plant model system Arabidopsis thaliana and the molecular genetic techniques of characterizing and mapping a sugar-sensing/response mutant, sugar-insensitive2 (sis2). The sis2 mutant was isolated in our lab on the basis of its resistance to high levels of exogenous glucose. Previous work shows that unlike wild type, the sis2 mutant is able to form a substantial shoot system on high concentrations of exogenous glucose, sucrose, and sorbitol, and also displays resistance to mannose. Previous work also shows that the sis2 mutant is resistant to the inhibitory effects of paclobutrazol, a gibberellin biosynthesis inhibitor, on germination. This work examines other sugar responses of sis2 and looks at the response of sis2 to all classical phytohormones. Results of this work show that the sis2 mutant is insensitive to the inhibitory effects of glucose on germination, which cannot be accounted for simply by precocious germination. The sis2 mutant may have wild-type endogenous sugar levels, although this is difficult to determine.
Interestingly, the *sis2* mutant demonstrates novel phytohormone responses. The *sis2* mutant is resistant to the inhibitory effects of gibberellin and brassinosteroid biosynthesis inhibitors during germination, but shows little to no resistance to these inhibitors during later developmental stages. The *sis2* mutant is also resistant to the combined negative effects of glucose and paclobutrazol on germination. The finding that glucose greatly exacerbates the negative effects of paclobutrazol on seed germination is also a result of this work. These results suggest a novel connection between phytohormone and sugar response pathways.

SIGNIFICANCE

The way that plants sense and respond to stimuli is fascinating. Plants integrate signals from the environment with internal signals and moderate the plant's growth accordingly. Plants cannot move or walk about like animals in order to avoid an unpleasant situation or to find a better home. Instead, plants adjust their responses to enable them to survive and develop in their current environment. Such responses are often mediated by phytohormones, most of which have multiple functions. For instance, the phytohormone gibberellin promotes seed germination, stem elongation, flowering,
trichome (leaf hair) formation, and mobilization of seed storage reserves (Arteca, 1996). In addition, the effects of different phytohormones can partially overlap. For example, gibberellin (Karssen et al., 1989), ethylene (Ghassemian et al., 2000; Karssen et al., 1989), and brassinosteroids (Steber and McCourt, 2000) promote germination. However, ethylene also promotes fruit ripening, a process in which gibberellin and brassinosteroids have comparatively little or no effect (Howell, 1998). The pathways of phytohormone signal transduction are complex and may interact with signaling pathways of other phytohormones (Beaudoin et al., 2000; Ghassemian et al., 2000) or other signal-inducing substances such as sugars (Arenas-Huertero et al., 2000; Gibson et al., 2001; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001; Zhou et al., 1998).

Recent evidence indicates that messages can be relayed by sugars acting as signaling molecules. More specifically, sugars activate or repress signal transduction pathways that alter gene expression (reviewed in Gibson 2000; Gibson and Graham, 1999; Koch, 1996; Sheen et al., 1999; Smeekens, 1998; Smeekens, 2000; Yu, 1999). Sugar levels are thought to play a role in the regulation of many plant developmental processes, including germination (Pego et al., 1999; Thomas and Rodriguez, 1994), flowering (Bernier et al.,
1993; Corbesier et al., 1998; Roldán et al., 1999), tuber formation (Müller-Röber et al., 1992), and source/sink interactions (Krapp et al., 1993). Sugar response pathways have also been shown to interact with light (Dijkwel et al., 1997), oxygen (Zeng et al., 1998), nitrogen (Klein et al., 2000; Lam et al., 1994; Nielsen et al., 1998; Stitt, 1999), and phosphate (Nielsen et al., 1998) sensing/response pathways.

The mechanisms of sugar sensing and sugar signal transduction remain largely unknown. What little is currently known about sugar-sensing/response mechanisms is presented below in the "Background" section. Ultimately, gaining an understanding about sugar-sensing/response mechanisms may enable the improvement of crop yields by directing more photosynthate to edible portions of the plant. This, in turn, may help to address the problem of world hunger.
BACKGROUND

This background section presents a broad overview of current knowledge pertaining to the field of research of this thesis — plant sugar sensing and response. Much of what we know about plant sugar sensing and response mechanisms are based on similar studies in yeast and bacteria. Therefore, sugar-sensing/response mechanisms in yeast, bacteria, and plants are described in this background section. Background information is presented on the plant model system *Arabidopsis thaliana*, on which this work is based.

In this section, following the background description of the plant model system *Arabidopsis thaliana*, the method of sugar transport is described. Next, the effects of sugars as signaling molecules are delineated, including the effects of sugars on developmental processes and on gene expression. Then, the few known components of sugar-sensing/response pathways are described, including hexokinase, SNF1, and cAMP. Then, sugar-sensing/response mutants isolated in Arabidopsis are listed. Also, phytohormone defects in sugar-sensing/response mutants of Arabidopsis are described. Finally, the known connections between phytohormone response and sugar response are presented.
Arabidopsis thaliana as a model system

The plant model system Arabidopsis thaliana is the subject of all of the experiments described in this thesis. The common name used by scientists for this species is Arabidopsis. The general public may know this plant as thale cress. It is a weed that grows naturally in cool climates, such as in Wisconsin (USA), Germany, and Australia. Arabidopsis belongs to the mustard family, Brassicaceae. Its characteristics include perfect flowers (containing both a pistil and six stamens) with four petals that enclose the reproductive organs so that the flowers self-fertilize naturally. Out-crosses can be performed in the laboratory. Each flower gives rise to a silique containing approximately twenty seeds. A single plant has over one hundred siliques, yielding thousands of seeds. The seeds are very small, weighing only 20 µg each. Arabidopsis seeds are oilseeds, meaning that energy is stored in the form of lipids, a compact storage form of energy. In contrast, cereal grains (such as wheat, barley, oat, rye) store energy in the form of starch, a bulky storage form of energy. One thousand Arabidopsis seeds can be sown on media in a petri dish. Arabidopsis seedlings can grow on media or in soil. Arabidopsis plants produce rosette leaves in a circular spiral pattern until they bolt (a bud appears in the center of the rosette); once they bolt, they do not produce any more rosette leaves.
Hence, the number of rosette leaves (after bolting) can be used as a reliable measure of developmental time to flowering. Time to flowering can also be measured according to the calendar, by the number of days until bolting. Arabidopsis flowers quickly (four weeks after germination) under a photoperiod of 16 h light/8 h dark or under continuous light. Arabidopsis flowers much later (three months after germination) under an 8 h light/16 h dark photoperiod. A complete life cycle takes six weeks when grown under continuous light. An Arabidopsis plant flowers only once, then dies.

Arabidopsis is a useful organism for laboratory study. It has a small genome size of 120,000 kb, containing 26,000 genes (reviewed in Walbot, 2000). The genome size of cereal plant species ranges from 420,000 kb (rice) to 4.8 million kb (barley) to 160 million kb (wheat, reviewed in Adam, 2000). The Arabidopsis genome was recently completely sequenced (Arabidopsis Genome Initiative, 2000). Arabidopsis is diploid, unlike most plants, which are polyploid. This makes it easier to manipulate Arabidopsis genetically. New genes can be introduced into Arabidopsis plants easily by a technique called transformation, mediated by a bacterium (*Agrobacterium tumefaciens*) with a natural ability to insert its plasmid containing transfer-DNA (T-DNA) into a plant at a
wounded site (for review, see Azpiroz-Leehan and Feldmann, 1997). Point mutations can be introduced in Arabidopsis upon exposure to X-rays or chemicals such as ethyl methane sulfonate (EMS). For example, the *sis2* mutation was created by EMS mutagenesis.

Sugar transport

Both yeast and plants possess sugar transporters, which possess structural and functional homology with each other. Generally, sugar transporters facilitate the uptake of sugars into cells for use or storage. Yeast possess only monosaccharide transporters, whereas plants have monosaccharide transporters and disaccharide transporters.

*Yeast monosaccharide transporters.* Yeast (*Saccharomyces cerevisiae*) have monosaccharide transporters which import hexoses from the growth medium. Although yeast lack disaccharide transporters, they utilize exogenous sucrose by cleaving it with extracellular invertase and importing the resulting glucose and fructose through hexose transporters (reviewed in Lalonde et al., 1999). Structurally, yeast monosaccharide transporters have twelve putative membrane-spanning domains (reviewed in Lalonde et al., 1999).
Yeast also have two interesting hexose transporter-like transmembrane proteins that function in hexose sensing, rather than in transport. These sensors, Snf3 (low level glucose sensor) and Rgt2 (high level glucose sensor), sense the external glucose concentration via their transmembrane domain (Özcan et al., 1998). Snf3 and Rgt2 themselves do not transport glucose. Rather, Snf3 and Rgt2 transmit the sugar signal through their unique cytoplasmic C-terminal tail (Özcan et al., 1998). This signal induces transcription of yeast Hexose Transporter (Hxt) genes (Özcan et al., 1996); these Hxt transporters mediate glucose uptake (Özcan et al., 1998). Upon glucose uptake, cytosolic glucose levels are sensed by hexokinase PII (Hxk2), according to some models (reviewed in Lalonde et al., 1999). Glucose signaling then causes transcriptional repression of genes encoding enzymes necessary for alternate sugar utilization (reviewed in Lalonde et al., 1999); this process is called glucose repression.

**Plant monosaccharide transporters.** Monosaccharide transporters in plants are similar structurally and functionally to those in yeast. Arabidopsis has in excess of twenty-six genes belonging to the monosaccharide transporter family (reviewed in Lalonde et al., 1999). Expression patterns of these genes suggest that they function in hexose uptake in sink tissues (reviewed in
Lalonde et al., 1999). Similar to the process in yeast, sucrose can be taken up indirectly by sink cells (such as root cells) after being cleaved by extracellular invertase; the resulting glucose and fructose are imported into the sink cell by monosaccharide transporters (reviewed in Lalonde et al., 1999).

*Plant disaccharide transporters.* Sucrose transporters in plants are very important because sucrose is the main soluble component of phloem sap and the major transported form of sugar in plants (reviewed in Lemoine, 2000). Sucrose is transported long distances via phloem from source organs such as leaves, where it is synthesized, to sink organs such as roots or flowers, where it is stored as starch or metabolized for energy (reviewed in Lemoine, 2000). Sucrose transport begins when sucrose is loaded against its concentration gradient from leaf mesophyll cells where it is made (via photosynthesis), into sieve elements that comprise the tube in which phloem sap is carried (Figure 1). The high level of sucrose in the sieve tube exerts great osmotic pressure, which causes a lot of water to follow it into the tube. The great influx of water exerts hydrostatic pressure, forcing the phloem (carrying sucrose) to flow downward in the sieve tube. Finally, sucrose is unloaded into sink cells (Figure 1; reviewed in Lalonde et al., 1999; Lemoine, 2000).
**Figure 1.** Sucrose is transported from its source (i.e., leaf mesophyll cell) to its sink (i.e., root cell) via the sieve tube.
The transport of sucrose from cell to cell, on its way from source to sink, can occur in either of two ways: across the plasma membrane (apoplastic transport), or through plasmodesmata connecting the cytoplasm of two adjacent cells (symplastic transport). Apoplastic transport requires energy and must be mediated by sucrose transporters. Apoplastic transport is postulated to occur at every step of the sucrose transport pathway (reviewed in Lalonde et al., 1999; Lemoine, 2000).

All plant sucrose transporters, usually named SUT or SUC, are apoplastic, energy-dependent, and function as proton symporters (reviewed in Lalonde et al., 1999). Six sucrose transporters have been identified in Arabidopsis: AtSUC1, AtSUC2, AtSUT2, AtSUT4, AtSUT5, and AtSUT8 (reviewed in Lalonde et al., 1999). Like monosaccharide transporters in yeast and in plants, sucrose transporters have twelve membrane-spanning domains (reviewed in Lalonde et al., 1999). The affinity of sucrose transporters for sucrose is 0.2-2 mM (reviewed in Lemoine, 2000). Sucrose transporter activity is regulated by sucrose at several levels: transcription (Chiou and Bush, 1998), protein turnover (Chiou and Bush, 1998), and protein phosphorylation (Roblin et al., 1998).

Mutations in the Arabidopsis SUC2 sucrose transporter cause seedling growth arrest when grown on media lacking sucrose.
Mutant *suc2* seedlings germinate on minimal media but have pale, translucent cotyledons, and no true leaves (Gottwald et al., 2000). Growth arrest can be partially rescued if *suc2* seedlings are transplanted to media containing 1% sucrose (Gottwald et al., 2000). In contrast, when germinated on 1% sucrose media, *suc2* seedlings appear wild-type (Gottwald et al., 2000). During adult developmental stages, mutations in SUC2 cause growth retardation and loss of fertility. Mutant rosette leaves are small, juvenile, and dark green due to anthocyanin accumulation. Flowers occasionally develop on *suc2* plants, but they never produce viable seed. In addition, sucrose is not transported efficiently out of *suc2* leaves (Gottwald et al., 2000). This suggests that SUC2 is required for apoplastic phloem loading, loading sugar into the sieve tube (Gottwald et al., 2000).

Snf3 and Rgt2 homologs have been identified in some plant species. The sucrose transporter-like protein SUT2 in tomato (and its homolog in Arabidopsis) has a long cytoplasmic C-terminus like Snf3 and Rgt2. SUT2 may be involved in controlling expression and activity of other sucrose transporters. Evidence supporting this hypothesis is that SUT2 colocalizes with the high-affinity sucrose transporter SUT1 and with the low-affinity sucrose transporter SUT4 (Barker et al., 2000). Some researchers postulate that SUT2 may
act to control sucrose flux across the plasma membrane of sieve elements (Barker et al., 2000).

**Developmental processes affected by sugar**

As stated previously, sugar levels are thought to play a role in the regulation of many plant developmental processes, including germination (Pego et al., 1999; Thomas and Rodriguez, 1994), flowering (Bernier et al., 1993; Corbesier et al., 1998; Roldán et al., 1999), tuber formation (Müller-Röber et al., 1992), and source/sink interactions (Krapp et al., 1993). The effects of sugars on these processes are described in the following paragraphs.

**Germination.** Results from this work show that near-physiological levels of glucose delay germination in a concentration-dependent manner (see "Phenotypic characterization of the sis2 mutant" chapter). Glucose and sucrose also inhibit the breakdown of starch storage reserves important for germination of barley grains (reviewed in Thomas and Rodriguez, 1994). Mannose, in particular, has a very potent inhibitory effect on germination. As little as 15 mM mannose completely inhibits germination of Arabidopsis seeds (Pego et al., 1999). There are two possible reasons why mannose exerts a severe effect. First, mannose could be metabolized into something toxic. The problem with this
explanation is that the metabolism of mannose varies between plant species. Mannose is metabolized efficiently in celery (Stoop and Pharr, 1993; Williamson et al., 1995) but slowly or poorly metabolized in many other species (reviewed in Herold and Lewis, 1977). Little is known about mannose metabolism in Arabidopsis. The second possible reason for its severe effect is that mannose-6-phosphate may tie up inorganic phosphate and cause ATP depletion (reviewed in Herold and Lewis, 1977; Sheu-Hwa et al., 1975) — however, other studies contradict this possibility (Pego et al., 1999).

**Flowering.** Sugars also play a role in flowering. Plants do not normally flower when grown in constant darkness. However, when Arabidopsis is grown in darkness on vertically-placed agar plates containing sucrose, the plants flower (Roldán et al., 1999). The crucial factor is making the sucrose available to the shoot apex. In addition, starch mobilization and the export of carbohydrates from leaves induces flowering (Corbesier et al., 1998). Also, the accumulation of sucrose in the shoot apical meristem (SAM) has been postulated to induce flowering. This accumulation occurs prior to the time when metabolites are needed for energy by the SAM to undergo flowering. Such evidence supports the idea that the presence of sucrose in the SAM has a signaling function (Bernier et al., 1993).
**Tuber formation.** Tuber formation is strongly affected by sugars. Inhibition of ADP-glucose pyrophosphorylase, an enzyme required for the synthesis of starch from glucose, causes potato tubers to store high levels of sugars but little starch. The sugar-storing tubers are smaller in size and more numerous than starch-storing tubers (Müller-Röber et al., 1992).

**Source/sink interactions.** Sugars also mediate source/sink interactions. Glucose represses expression of photosynthetic genes in leaves of many plant species (Krapp et al., 1993). When sink tissues (i.e., flowers and roots) cannot absorb the photosynthate as quickly as it is synthesized, excess sugar accumulates in source tissue (i.e., leaves) and may be responsible for the signal that represses photosynthetic genes (Krapp et al., 1993).

**Sugar-regulated genes**

Over forty sugar-regulated plant genes have been identified (reviewed in Koch, 1996; Thomas et al., 1995). A few of these genes, and the ways in which sugars regulate their expression, are described in the following paragraphs.

**ATB2.** Expression of ATB2, encoding an Arabidopsis leucine zipper transcription factor, is repressed at the translational level by sucrose. This regulation involves the ATB2 leader sequence;
deletion of the leader results in a loss of sucrose repression (Rook et al., 1998).

*cab* and *rbcS*. Transcription of the photosynthetic genes *chlorophyll a/b binding protein (cab)*, and *rbcS* encoding the small subunit of Rubisco, are repressed by exogenous glucose and sucrose (Sheen, 1990). One study found that chlorophyll levels decrease when detached spinach leaves are supplied with glucose through the transpiration stream (Krapp et al., 1991). However, in a later study, chlorophyll levels increase with increasing levels of exogenous sugar, when the sugar is applied to whole Arabidopsis plants (Ohto et al., 2001). Chlorophyll levels in the *sis2* mutant follow the latter scenario, as shown below under "Previous work on the *sis2* mutant".

*Chalcone synthase*. Chalcone synthase (CHS) is one of the key enzymes involved in biosynthesis of anthocyanin, a purple pigment that gives color to flower petals and seed coats. In petunia, *CHS-A* expression increases with increasing concentrations of exogenous sucrose, glucose, or fructose (but not mannitol), suggesting that *CHS-A* is a sugar-regulated gene (Tsukaya et al., 1991). The upregulation of *CHS-A* causes an increase in anthocyanin biosynthesis, and therefore leads to anthocyanin accumulation (Rook et al., 1998). Thus, with higher sugar concentrations, anthocyanin levels increase.
**ADP-glucose pyrophosphorylase.** ADP-glucose pyrophosphorylase (AGPase), an enzyme required for the synthesis of starch from glucose, is comprised of two large subunits and two small subunits. Arabidopsis genes encoding AGPase subunits include *ApS* (small subunit), *ApL1*, *ApL2* and *ApL3* (large subunits, Villand et al., 1993). Expression of several AGPase subunits are induced by sugars (Bae and Liu, 1997; Müller-Röber et al., 1990; Sokolov et al., 1998).

Other sugar-regulated genes include plastocyanin, the expression of which depends on both light and sucrose (Dijkstra et al., 1997); ß-amylase (Laby et al., 2001; Mita et al., 1997a; Mita et al., 1997b); and patatin (Martin et al., 1997). Arabidopsis mutants defective in regulation of these genes are described below under "Sugar-sensing/response mutants".

**Sugar signaling pathway components**

Very few of the components of sugar-sensing/response pathways have been identified to date. The few known components of sugar-sensing/response pathways, including hexokinase and SNF1/SNF1-related kinases in yeast and plants, and cAMP in bacteria and yeast, are described in the following paragraphs.
**Hexokinase.** Hexokinase is postulated to be one of the key sugar sensors and signal transmitters in plants (Graham et al., 1994; Jang et al., 1997; Jang and Sheen, 1994). Studies in yeast were the first to suggest that hexokinase has a sugar signaling function — glucose repression — distinct from its metabolic function (Entian, 1980; Ma et al., 1989; Rose et al., 1991). The term "glucose repression" signifies the signaling process by which glucose represses transcription of genes encoding enzymes that metabolize non-glucose carbon sources, such as galactose (Gancedo, 1998). Several plant studies also suggest that hexokinase mediates glucose repression (Jang et al., 1997; Jang and Sheen, 1994). However, other studies provide conflicting evidence regarding the nature of hexokinase as a sugar sensor and signal transducer. The two sides of the debate are described in the following two paragraphs.

Evidence for hexokinase as a sugar sensor includes the following: 1) Glucose analogs that can be phosphorylated by hexokinase (namely, mannose and 2-deoxyglucose) severely repress glucose-repressed genes, whereas glucose analogs that cannot be phosphorylated by hexokinase (namely, 6-deoxyglucose and 3-O-methyl glucose) do not affect expression of glucose-repressed genes in maize protoplasts (Jang and Sheen, 1994). 2) Overexpression of the Arabidopsis **HXK1** (*AtHXK1*) and **HXK2** genes in Arabidopsis
seedlings confers hypersensitivity to glucose; conversely, anti-sense expression of \textit{AtHXX1} and \textit{AtHXX2} in seedlings confers hyposensitivity to glucose (Jang et al., 1997). 3) Overexpression of the putative yeast sugar sensor \textit{YHxx2} in Arabidopsis confers increased catalytic Hxx2 activity, but simultaneously confers decreased sugar sensitivity (Jang et al., 1997). This last piece of evidence shows that dual functions of hexokinase (metabolic and regulatory) are separable. These three pieces of evidence point to the existence of hexokinase-dependent pathways.

Arguments against hexokinase as a sugar sensor includes the following: 1) Hexokinase may produce signal molecule(s) but is itself not a sugar sensor/signal transducer \textit{per se}. Rather, changes in ATP, Pi, and AMP levels elicit the signal (reviewed in Halford et al., 1999). Some researchers interpret the observation that 2-deoxyGlc-6-P elicits a strong signal in the following manner: since 2-deoxyGlc-6-P is poorly metabolized, it builds up in toxic levels inside cell, thereby trapping Pi and preventing conversion of ADP to ATP. This leads to ATP depletion (reviewed in Halford et al., 1999). However, findings from other studies contradict this interpretation (Klein and Stitt, 1998). 2) Hexokinase-independent sugar-signaling/response pathways exist. Here are three examples: a) Sucrose regulates the proton-sucrose symporter, which mediates apoplastic phloem
loading (described previously in the "Sucrose transport" section). This response is sucrose-specific and hexokinase-independent, as hexoses do not elicit a symporter response, and mannoheptulose (a hexokinase inhibitor) does not inhibit the response (Chiou and Bush, 1998). b) Expression of ATB2 (described above under "Sugar-regulated genes") is repressed specifically by sucrose in a hexokinase-independent manner (Rook et al., 1998). c) The potato patatin promoter, transformed into Arabidopsis, is induced both by sucrose and by 3-O-methyl glucose (3-OMG), a glucose analog that is a poor substrate for hexokinase (Martin et al., 1997). Example "c" typifies a hexokinase-independent, but non-sucrose-specific, sugar signaling response. However, as little is known about 3-OMG metabolism in Arabidopsis, and as 3-OMG is a poor substrate for some glucose uptake systems (reviewed in Gibson, 2000), results from 3-OMG studies should be interpreted carefully.

**SNFl.** Another factor postulated to act as a major component of the sugar signal transduction pathway is SUCROSE NON-FERMENTING1 (SNF1), a serine/threonine protein kinase. SNF1 was first identified in yeast as a sugar-response factor (reviewed in Hardie et al., 1998). The yeast SNF1 complex is activated in response to glucose starvation. Upon activation, the SNF1 complex triggers a phosphorylation cascade, inducing expression of genes
required for the metabolism of non-glucose carbohydrates, such as galactose. These genes are normally repressed in the presence of glucose. SNF1 is essential for de-repression of glucose-repressed genes (reviewed in Gancedo, 1998).

SNF1 is homologous to the catalytic subunit of mammalian AMP-activated protein kinase (AMPK), an enzyme which activates key metabolic enzymes such as HMG-CoA reductase (involved in steroid synthesis), acetyl-CoA carboxylase (involved in fatty acid synthesis and oxidation), and glycogen synthase. Like SNF1, AMPK is part of a phosphorylation cascade (reviewed in Hardie et al., 1998).

Plant homologs of SNF1, called SNF1-related kinases (SnRKs), have been identified in numerous plant species, including spinach (Sugden et al., 1999), rye, wheat, barley, oat, rice, Arabidopsis, potato, and others (reviewed in Halford and Hardie, 1998). Evidence indicates that SnRKs regulate key enzymes in major biosynthetic pathways, including HMG-CoA reductase, sucrose phosphate synthase (involved in sucrose synthesis), and nitrate reductase (involved in nitrogen assimilation; reviewed in Hardie et al., 1998; Sugden et al., 1999). For example, the anti-sense expression of SnRKs affects sucrose synthase gene expression in potato (Purcell et al., 1998). Recently, researchers discovered that Arabidopsis SnRKs and yeast SNF1 interact with the Arabidopsis
protein PLEIOTROPIC REGULATORY LOCUS1 (PRL1) in the yeast two-hybrid assay and in vivo immunocomplexes (Bhalerao et al., 1999). The prl1 mutation in Arabidopsis causes de-repression of glucose-responsive genes and hypersensitivity to sugar, among other effects (Németh et al., 1998). Based on this evidence, SnRKs are thought to be components of sugar-response pathways in plants.

**cAMP.** cAMP plays a prominent role in relieving glucose repression in bacteria (reviewed in Ullmann, 1996). In 1965, scientists discovered that glucose-starved *E. coli* cells accumulate elevated levels of cAMP. In 1968, two groups independently showed that cAMP relieves glucose repression, thereby antagonizing the effect of glucose. After the identification of the cAMP receptor protein (CAP), researchers showed that the cAMP-CAP complex binds near the transcription start site of operons of glucose-repressed genes (such as *LacZ* encoding β-galactosidase, an enzyme that breaks down lactose) and activates their expression. Later, in the 1970's, studies showed that although cAMP is a major modulator of glucose repression, glucose repression can be relieved independently of cAMP (reviewed in Ullmann, 1996).

A cAMP signal is also involved in glucose repression in fission yeast. Interestingly, the signaling roles of cAMP and glucose in fission yeast are directly opposite to those in bacteria. Whereas
cAMP stimulates expression of glucose-repressed genes in bacteria, cAMP causes glucose repression in fission yeast. Glucose inhibits cAMP synthesis in bacteria, whereas glucose induces synthesis of cAMP in fission yeast. Fission yeast (as well as all eukaryotes) lack a CAP-like cAMP-binding transcription factor; the cAMP signal is instead transmitted by a cAMP-dependent protein kinase (reviewed in Ronne, 1995).

The function of cAMP signaling in budding yeast (Saccharomyces cerevisiae, simply referred to as "yeast" in the rest of this thesis) is not well understood. As in fission yeast, cAMP synthesis is transiently induced by glucose in budding yeast, and after induction it affects transcription of some glucose-repressed genes. However, glucose repression via the SNF1 complex can occur even when cAMP-dependent kinase activity is present at constitutive low levels; therefore, cAMP is not required for SNF1-mediated glucose repression in S. cerevisiae. The SNF1 and cAMP repression systems are postulated to act in parallel (reviewed in Ronne, 1995).

Sugar-sensing/response mutants in Arabidopsis

In an attempt to learn more about the mechanisms of sugar sensing and response, several labs are working to isolate, characterize, and clone genes defective in sugar-sensing/response
mutants using the plant model system *Arabidopsis thaliana*. Some labs have isolated mutants based on altered responses to high exogenous glucose or sucrose levels. Other labs isolated mutants based on altered expression patterns of sugar-regulated genes. Mutants isolated based on altered responses to high exogenous sugar levels include *sugar-insensitive* (*sis*) mutants (Gibson et al., 2001; Laby et al., 2000), *glucose-insensitive* (*gin*) mutants (Arenas-Huertero, et al., 2000; Zhou et al., 1998), *sucrose-insensitive growth* (*sig*) mutants (Pego et al., 2000), and *sucrose super-sensitive* (*sss*) mutants (Pego et al., 2000). Similarly, *mannose-insensitive germination* (*mig*) mutants were isolated based on their ability to develop on otherwise inhibitory concentrations of mannose (Pego et al., 2000). The *sugar-insensitive2* mutant, a major subject of this thesis, belongs to this category of mutants.

Other sugar-sensing/response mutants were isolated based on altered expression patterns of sugar-regulated genes. The *sucrose-uncoupled* (*sun*) mutants were isolated based on altered expression of the plastocyanin gene (*PC*). Their name is derived from the observation that their far-red light responses are uncoupled with sucrose dependence (Dijkwel et al., 1997). Three independent sets of mutants were isolated based on altered expression of β-amylase: *high level of beta-amylase activity* (*hba1*) mutants (Mita et al.,
1997a), low-level beta-amylase (iba1) mutants (Mita et al., 1997b), and reduced beta-amylase (ram) mutants (Laby et al., 2001). The reduced sugar response (rsk) mutants demonstrate altered expression of patatin in response to sucrose induction (Martin et al., 1997). The impaired sucrose induction (isi) mutants show reduced induction of the ApL3 gene (encoding a subunit of ADP-glucose pyrophosphorylase) when supplied with exogenous sucrose (Rook et al., 2001).

Phytohormone defects in sugar-sensing/response mutants in Arabidopsis

Many sugar-sensing/response mutants have been isolated in Arabidopsis that show alterations in phytohormone response or metabolism. For example, four sugar-sensing/response mutants, isolated independently in different labs, have defects in the ABSCISIC ACID-INSENSITIVE4 (ABI4) gene. The mutants allelic to abi4 are the sugar-insensitive5 (sis5) mutant (Laby et al., 2000), the sucrose-uncoupled6 (sun6) mutant (Huijser et al., 2000), the glucose-insensitive6 (gin6) mutant (Arenas-Huertero et al., 2000), and the impaired sucrose induction3 (isi3) mutant (Rook et al., 2001). The abi4 mutation confers the ability to germinate on abscisic acid (ABA; Finkelstein, 1994). The abscisic acid-deficient2 (aba2)
mutant, originally characterized by its inability to produce ABA (Léon-Kloosterziel et al., 1996), was also isolated in three different labs in independent screens for sugar-sensing/response mutants. The mutants allelic to *aba2* are the *sugar-insensitive4 (sis4)* mutant (Laby et al., 2000), the *impaired sucrose induction4 (isi4)* mutant (Rook et al., 2001), and the *glucose-insensitive1 (gin1)* mutant (Rook et al., 2001). In addition to *abi4* and *aba2*, the *abal* and *aba3* mutants exhibit a strong *sugar-insensitive (sis)* phenotype, and the *abi5* mutant demonstrates a weak *sis* phenotype (Arenas-Huertero et al., 2000; Laby et al., 2000). These results suggest the existence of connections between abscisic acid and sugar sensing/response pathways. However, the ABA and sugar response pathways retain some distinctions. For example, the *abi1, abi2, and abi3* mutants demonstrate wild-type responses on exogenous sugar (Arenas-Huertero et al., 2000; Laby et al., 2000). Also, *sis3* shows wild-type or near wild-type abscisic acid sensitivity and wilting responses (Donna Pattison, unpublished results).

Other Arabidopsis sugar-sensing/response mutants have defects in ethylene responses. Our lab identified a mutant, *sugar-insensitive1 (sis1)*, the defect of which lies in the *CONSTITUTIVE RESPONSE1 (CTRL)* gene (Gibson et al., 2001). The *ctrl* mutant displays constitutive response to ethylene (Kieber et al., 1993). The
ethylene-overproducer1 (eto1) mutant, which overproduces ethylene (Guzman and Ecker, 1990), also exhibits a sis phenotype (Gibson et al., 2001; Zhou et al., 1998).

Sugar-sensing/response mutants may demonstrate altered responses in other phytohormones as well. The prll mutant (mentioned previously in the "SNF1" section) exhibits hypersensitivity to sugar, cytokinin, ethylene, abscisic acid, and auxin (Németh et al., 1998).

These discoveries of phytohormone alterations in sugar-sensing/response mutants point to the existence of connections between phytohormone and sugar responses in plants.

Other connections between sugar and phytohormone responses

One of the first connections to be identified between sugar and phytohormone response pathways was that between sugar and gibberellin (Radley, 1969). Sugar regulates gibberellin synthesis in barley grains by a feedback loop involving α-amylase. The feedback loop operates as follows: 1) Gibberellin induces expression of α-amylase in barley embryos. 2) α-amylase breaks down starch storage reserves in the barley endosperm into soluble sugars.
3) The soluble sugars are thought to repress gibberellin synthesis/release (reviewed in Thomas and Rodriguez, 1994). Sugars also repress transcription of α-amylase genes, independently of sugars' effects on gibberellin biosynthesis (Perata et al., 1997). Recently, it was shown that sugar has different effects on gibberellin action (induction of α-amylase) in different tissue layers of germinating barley grains (Perata et al., 1997).

Very recently, it was discovered that glucose allows germination on ABA (Finkelstein and Lynch, 2000; Garciarrubio et al., 1997). This finding suggests that glucose can overcome the inhibitory effects of ABA during germination. Previously, it was postulated that gibberellin (GA) is required to overcome the inhibitory effects of ABA during germination (Koornneef and van der Veen, 1980). This hypothesis was based on experiments showing that although GA biosynthetic mutants will not germinate unless supplied with exogenous GA (Koornneef and van der Veen, 1980), ABA biosynthetic mutants do germinate (Koornneef et al., 1982; Léon-Kloosterziel et al., 1996). Furthermore, double mutants defective in both GA and ABA biosynthesis germinate in the absence of exogenous GA (Koornneef et al., 1982). In light of these observations, if the above hypothesis is true that glucose can overcome the inhibitory effects of ABA on germination, then this
suggests that glucose may reduce the requirement for GA for germination. In this way, glucose may allow germination in the absence of gibberellin. This leads to the hypothesis that glucose might allow germination on paclobutrazol, an inhibitor of gibberellin biosynthesis. This hypothesis was tested in this work by examining the combinatorial effects of glucose and paclobutrazol on seed germination. Surprisingly, the results obtained were the opposite of those expected. Namely, glucose exacerbates rather than alleviates the negative effects of paclobutrazol on seed germination.

In order to better understand sugar-sensing/response mechanisms, the work described in this thesis uses molecular genetic techniques to characterize and map the Arabidopsis sugar-sensing/response mutant *sugar-insensitive2* (*sis2*). Results of this work show that the *sis2* mutant is insensitive to the inhibitory effects of glucose on germination, which cannot be accounted for simply by precocious germination. The *sis2* mutant may have wild-type endogenous sugar levels, although this is difficult to determine. Interestingly, the *sis2* mutant demonstrates novel phytohormone responses. The *sis2* mutant is resistant to gibberellin and brassinosteroid biosynthesis inhibitors during germination, but
shows little to no resistance to these inhibitors during later
developmental stages. The *sis2* mutant is also resistant to the
combined negative effects of glucose and paclobutrazol on
germination. The finding that glucose greatly exacerbates the
negative effects of paclobutrazol on seed germination is also a result
of this work.

The work described in this thesis is largely based on previous
foundational work, described in the following section.

**PREVIOUS WORK ON THE *sis2* MUTANT**

The characterization of the *sis2* mutant described in this thesis
is based largely on foundational work done previously by Dr. Sue
Gibson, Dr. Donggiun Kim, and Dr. Ron Laby. Their foundational
work includes isolation of the *sis2* mutant; initial characterization of
the *sis2* mutant’s responses to sugar, to paclobutrazol, and to
ethylene; and mapping the *sis2* mutation.

*Isolation of the *sis2* mutant* (performed by Dr. Sue Gibson).

Over 95% of wild-type *Arabidopsis thaliana* seeds germinate on
minimal media (Kranz and Kirchheim, 1987) supplemented with
0.3 M glucose or sucrose, but the seedlings fail to develop further.
Cotyledons of these seedlings turn purple and fail to expand, and
true leaves do not develop (Gibson et al., 2001; Huijser et al., 2000; Laby et al., 2000; Zhou et al., 1998). To identify sugar-sensing/response mutants, ~28,000 ethyl methane sulfonate (EMS)-mutagenized M2 Arabidopsis thaliana seeds were sown on plates containing minimal Arabidopsis media (Kranz and Kirchheim, 1987) supplemented with 0.3 M glucose. Putative mutants ("putants") were isolated based on their ability to form expanded cotyledons and true leaves. Putant seedlings were transferred to soil and grown to maturity. Their seeds were harvested and re-assayed. The majority of seeds from one putant, named sugar-insensitive2 (sis2), retained the mutant phenotype on 0.3 M glucose (Figure 2).

The sis2 mutant is resistant to other sugars and sorbitol. (Experiment performed by Dr. Sue Gibson). The sis2 mutant demonstrates resistance not only to 0.3 M glucose (on which it was isolated), but also to 0.3 M sucrose (Figures 2 and 3). In addition, the sis2 mutant exhibits osmotolerance when grown on 0.4 M sorbitol (Figure 2) or 0.5 M sorbitol (Figure 3), a sugar analog that cannot be transported into plant cells. As 0.3 M sorbitol does not significantly affect the wild type (Figure 2; Laby et al., 2000), higher concentrations of sorbitol are used when testing for osmotolerance.
Figure 2. The *sis2* mutant demonstrates resistance to the inhibitory effects of glucose, sucrose, mannose, and sorbitol on seedling development. Seeds were sown on the indicated media and grown for 13 days under 90-120 μmol photons m⁻² sec⁻¹ continuous fluorescent light prior to scoring. Values indicate the mean ± SD (n = 3 plates each containing 50-100 seeds). Col WT, wild-type Columbia. Experiment performed by Dr. Sue Gibson.
Figure 3. The *sis2* mutant demonstrates resistance to the inhibitory effects of high levels of exogenous sucrose and sorbitol on seedling development. Seeds were sown on the indicated media and grown for 13 days under 90-120 μmol photons m⁻² sec⁻¹ continuous fluorescent light prior to photographing. Col WT, wild-type Columbia. Red scale bars = 2 mm. Experiment performed by Dr. Sue Gibson.
In order to distinguish an osmotolerant mutant from wild type, it is most useful to examine a concentration high enough to significantly affect wild type, but not too high to mask the resistant phenotype of the osmotolerant mutant. The \textit{sis2} mutant's sugar insensitivity does not solely result from its osmotolerance, as \textit{sis2} exhibits an altered response to mannose at a concentration too low (1.7 mM) to exert an osmotic effect (Figure 2). On minimal media, the \textit{sis2} mutant does not look different from wild type, having expanded cotyledons and true leaves (Figure 2).

Young \textit{sis2} seedlings exhibit wild-type anthocyanin and chlorophyll production on low and high sucrose. (Experiment performed by Dr. Donggiun Kim.) The \textit{sis2} mutant was examined for alterations in two typical sugar-regulated processes, anthocyanin and chlorophyll production (Krapp et al., 1991; Tsukaya et al., 1991). The effects of sugars on anthocyanin and chlorophyll production are described above under "Sugar-regulated genes". Anthocyanin and chlorophyll levels in the \textit{sis2} mutant parallel those of wild-type plants both on low and high sucrose (Figure 4). Since \textit{sis2} is not defective in these two sugar-regulated processes, presumably the \textit{sis2} mutation does not affect sugar transport, as otherwise all sugar responses in the \textit{sis2} mutant would be affected.
Figure 4. Young sis2 seedlings exhibit wild-type anthocyanin and chlorophyll accumulation on low and high sucrose. Anthocyanin levels were normalized relative to the amounts found in wild-type seedlings grown on 0.18 M sucrose. Values indicate the mean ± SD (n = 3 for chlorophyll, n = 8-9 for anthocyanin). Col WT, wild-type Columbia; fwt, fresh weight. Experiment performed by Dr. Donggiun Kim.
Phytohormone responses of sis2 (initial characterization).

(Experiments performed by Dr. Sue Gibson and Dr. Ron Laby.) As described previously in the "Phytohormone defects in sugar-sensing/response mutants" section, sugar-response mutants may show alterations in phytohormone response or metabolism (Arenas-Huerto et al., 2000; Gibson et al., 2001; Huijser et al., 2000; Laby et al., Németh et al., 1998; Rook et al, 2001; Zhou et al., 1998). Therefore, characterization of the sis2 mutant's responses to phytohormones was begun.

An easy method of categorizing phytohormone response mutants is to determine whether these mutants are able to germinate on paclobutrazol. Paclobutrazol, a cytochrome p450 monooxygenase inhibitor, inhibits biosynthesis of gibberellin (Table 1; Hedden and Graebe, 1985), a phytohormone which promotes germination (Figure 5). Paclobutrazol therefore inhibits germination of wild-type seeds. Paclobutrazol provides a useful tool for characterizing phytohormone responses because several phytohormones that play roles in germination (Figure 5) affect the seeds' ability to germinate on paclobutrazol: abscisic acid, ethylene, gibberellin, and brassinosteroids. For example, mutants defective in abscisic acid (ABA) biosynthesis (aba mutants), and some
**Table 1. Phytohormones and phytohormone biosynthesis inhibitors employed in this work**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Abbreviations</th>
<th>Function</th>
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<tbody>
<tr>
<td>Abscisic acid</td>
<td>ABA</td>
<td>inhibits germination; promotes dormancy; mediates stomatal closure</td>
</tr>
<tr>
<td>1-aminocyclopropane-1-carboxylic acid</td>
<td>ACC</td>
<td>an ethylene precursor</td>
</tr>
<tr>
<td>Ancymidol</td>
<td>Anc</td>
<td>inhibits cytochrome p450 monooxygenases in gibberellin biosynthetic pathway</td>
</tr>
<tr>
<td>Aminoethoxyvinylglycine</td>
<td>AVG</td>
<td>inhibits ethylene biosynthesis</td>
</tr>
<tr>
<td>Brassinazole</td>
<td>Brz</td>
<td>inhibits cytochrome p450 monooxygenases in brassinosteroid biosynthetic pathway</td>
</tr>
<tr>
<td>Cytokinin</td>
<td></td>
<td>inhibits root elongation, among other functions</td>
</tr>
<tr>
<td>Ethylene</td>
<td></td>
<td>promotes germination; inhibits hypocotyl elongation in the dark</td>
</tr>
<tr>
<td>24-epibrassinolide</td>
<td>EBR</td>
<td>a brassinosteroid; promotes germination, hypocotyl elongation, cell expansion</td>
</tr>
<tr>
<td>Gibberellin</td>
<td>GA</td>
<td>promotes germination, hypocotyl elongation, flowering, stem elongation</td>
</tr>
<tr>
<td>Indole-3-acetic acid</td>
<td>IAA</td>
<td>inhibits root elongation, among other functions</td>
</tr>
<tr>
<td>Methyl-jasmonate</td>
<td>inhibits root elongation, among other functions</td>
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<td></td>
</tr>
<tr>
<td>Paclobutrazol</td>
<td>Pac</td>
<td></td>
</tr>
<tr>
<td></td>
<td>inhibits cytochrome p450 monooxygenases in gibberellin biosynthetic pathway</td>
<td></td>
</tr>
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Figure 5. A model for the effects of phytohormones and glucose on germination. Gibberellin promotes germination (Karssen et al., 1989), whereas abscisic acid (ABA) inhibits germination (Koornneef et al., 1984). Ethylene (Ghassemian et al., 2000; Karssen et al., 1989) and brassinosteroids (Steber and McCourt, 2000) promote germination under certain conditions. Our lab found that glucose inhibits germination in a concentration-dependent manner (Figure 8).
ABA-insensitive (abi) mutants, can germinate on paclobutrazol (or on a similar gibberellin biosynthesis inhibitor, uniconazol) (Laby et al., 2000; Léon-Kloosterziel et al., 1996; Nambara et al., 1991). Mutations conferring a constitutive ethylene response (ctr1) or ethylene overproduction (eto1) also confer the ability to germinate on paclobutrazol, as does the application of ethylene to wild-type seeds (Gibson et al., 2001). Many of the ethylene and abscisic acid mutants that can germinate on paclobutrazol are also characterized by sugar insensitivity. These mutants include eto1 and ctr1 (Gibson et al., 2001; Zhou et al., 1998); abal, aba2, and aba3 (Arenas-Huertero et al., 2000; Laby et al., 2000); and abi4 (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001).

Dr. Sue Gibson discovered that the sis2 mutant germinates on paclobutrazol (Figure 6). The spindly-3 (spy-3) mutant, a constitutive gibberellin response mutant, is used as a control in many experiments in this work (such as Figure 6). The spy-3 mutant was isolated based on its ability to germinate on 120 µM paclobutrazol. It is insensitive to paclobutrazol during all developmental stages tested (Jacobsen and Olszewski, 1993).

An important observation is worthy of note here. Cytochrome p450 monooxygenase inhibitors such as paclobutrazol are not as
Figure 6. The *sis2* mutant germinates on paclobutrazol, an inhibitor of gibberellin biosynthesis. Seeds were sown on Arabidopsis minimal media plates supplemented with 120 μM paclobutrazol. Plates were incubated under continuous fluorescent light at approx. 21°C. Seed germination was scored after 10 days. Values indicate the mean ± SD (n = 3 for Col WT and *sis2*, n = 1 for *spy-3*). Col WT, wild-type Columbia; *spy-3, spindly-3* mutant. Experiment performed by Dr. Sue Gibson. Additional replicates were performed by Lydia Sommerlad.
specific as their developers claim. Paclobutrazol, developed as an inhibitor of gibberellin biosynthesis (Hedden and Graebe, 1985), also inhibits ABA biosynthesis by 35% (Norman et al., 1986). Results of this work also provide evidence against the specificity of cytochrome p450 monooxygenase inhibitors (see "Conclusions"). This lack of specificity may be due to the fact that cytochrome p450 monooxygenases are components of myriads of biochemical pathways, including gibberellin (Hedden and Kamiya, 1997), ABA (Krochko et al., 1998), and brassinosteroids (Asami et al., 2000; Clouse and Sasse, 1998) biosynthetic pathways, among others.

The response of sis2 to ethylene was examined next. (Experiment performed by Dr. Ron Laby.) Ethylene response was examined by measuring hypocotyl elongation in the dark on the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG; Table 1). Ethylene inhibits hypocotyl elongation in the dark. Wild type, sis2, constitutive response1 (ctrl), and ethylene-overproducer1 (eto1) were grown for comparison. The ctrl mutant behaves as if there is always ethylene present, and therefore has a short, fat hypocotyl with an exaggerated apical hook, regardless of whether AVG is present (Figure 7; Gibson et al, 2001; Kieber et al., 1993). The eto1 mutant etiolates in the presence of AVG (which inhibits ethylene synthesis even in eto1), but in the absence of AVG, eto1
Figure 7. The sis2 mutant demonstrates wild-type hypocotyl elongation in the dark in the presence and absence of aminoethoxyvinylglycine (AVG), an ethylene biosynthesis inhibitor. Seeds were sown on plates containing minimal media plus 0.03 M glucose, with 10 μM AVG added (AVG+) or no AVG (AVG-). Plates were cold-treated at 4°C in darkness for 3 days, then transferred to room temperature (~25°C) under fluorescent light for 1 hour, then grown in darkness at room temperature for 5 days before scoring hypocotyl lengths. Values indicate the mean ± SD (n = 5). Col WT, wild-type Columbia. Experiment performed by Dr. Ron Laby.
has a short, fat hypocotyl (Figure 7; Guzman and Ecker, 1990). The 
sis2 mutant demonstrates a wild-type hypocotyl elongation response
on AVG (Figure 7), indicating that the sis2 mutant does not likely
overproduce or respond constitutively to ethylene.

Characterization of the responses of sis2 to all other major
phytohormones is part of this work and is described in the
"Phenotypic characterization of the sis2 mutant" chapter.

*Mapping of the SIS2 gene* (performed by Dr. Donggiun Kim).
The sis2 mutation is likely to be the result of a point mutation, as it
was isolated from a pool of ethyl methane sulfonate (EMS)-
mutagenized M2 seeds. Therefore, a map-based positional cloning
approach is being used to clone SIS2. Preliminary results show that
the SIS2 gene maps to the bottom of chromosome 1, 11.4 cM away
from the marker nga111 (Dr. Donggiun Kim, unpublished results).
See Appendix I for a more detailed description of this mapping
work. For more information describing the positional cloning
technique, see (Bell and Ecker, 1994; Gibson and Somerville, 1993;
Konieczny and Ausubel, 1993).

The previous work just described — isolation of the sugar-
insensitive2 (sis2) mutant, initial characterization of its responses
to sugar, ethylene, and paclobutrazol (a gibberellin biosynthesis
inhibitor), and mapping the *sis2* mutation — laid the foundation on which this work is based. This work includes the complete characterization of the *sis2* mutant, described in the next chapter. Results demonstrate that *sis2* is insensitive to the inhibitory effects of glucose on germination, which cannot be accounted for simply by precocious germination. The *sis2* mutant may have wild-type endogenous sugar levels, although this is difficult to determine. Interestingly, *sis2* demonstrates novel phytohormone responses. The *sis2* mutant is resistant to the inhibitory effects of gibberellin and brassinosteroid biosynthesis inhibitors during germination, but shows little to no resistance to these inhibitors during later developmental stages. The *sis2* mutant is also resistant to the combined negative effects of glucose and paclobutrazol on germination. The finding that glucose greatly exacerbates the negative effects of paclobutrazol on seed germination is also a result of this work. These results suggest a novel connection between phytohormone and sugar response pathways.
MATERIALS AND METHODS

Plant Material, Growth Conditions, and Media

Wild-type seeds of *Arabidopsis thaliana* var. Columbia were obtained from Dr. Chris Somerville (Carnegie Institute, Palo Alto, CA, USA).

The *sis2*, *ctl-12/sis1-1*, and *aba2-3/sis4-1* mutants were isolated from pools of ethyl methyl sulfonate (EMS)-mutagenized M2 seeds of *Arabidopsis thaliana* var. Columbia obtained from Dr. Chris Somerville (Carnegie Institute, Palo Alto, CA, USA). The *sk390* and *abi4-103/sis5-3* mutants were isolated from pools of EMS-mutagenized M2 seeds of *Arabidopsis thaliana* var. Columbia obtained from Lehle seeds (Tucson, AZ, USA). The *det2-1* (CS6159), *etr1-3* (CS3070), and *spy-3* (CS6268) mutants of *Arabidopsis thaliana* var. Columbia, and the *abi2-1* (CS23) and *gal-5* (CS3106) mutants of *Arabidopsis thaliana* var. Landsberg erecta, were obtained from the Arabidopsis Biological Resource Center at Ohio State University (USA). The *abi3* mutant of *Arabidopsis thaliana* was obtained from Dr. Ruth Finkelstein (University of California at Santa Barbara, CA, USA).

Non-backcrossed lines (~M6 to ~M10) of the *sis2* mutant were employed in all experiments. To validate results obtained from non-
backcrossed *sis2* lines, the *sk591* mutant (a potential allele of *sis2*) was examined in many experiments in parallel with *sis2*. The *sk591* mutant demonstrates practically identical phythormone and sugar responses as *sis2*, including dual paclobutrazol-resistant and sugar-insensitive phenotypes (data not shown).

Plant growth media consisted of Arabidopsis minimal media (Kranz and Kirchheim, 1987) supplemented with 0.65% or 0.7% agar. Seeds were surface-sterilized in 50% bleach + 0.02% triton, washed three times with sterile, double-deionized water, and resuspended in sterile 0.15% agar for sowing. Seeds were then cold-treated at 4°C in darkness for 2-4 days to break dormancy, either in the agar (before sowing) or on plates (after sowing), as indicated. After cold treatment and sowing, plates were incubated under continuous fluorescent light at 21°C.

Abscisic acid (mixed isomers), 1-aminocyclopropane-1-carboxylic acid, aminoethoxyvinylglycine, ancymidol, N-6-benzyladenine (6-benzylaminopurine, or cytokinin), gibberellic acid (GA₃), glucose, indole-3-acetic acid, mannose, sorbitol, and sucrose were obtained from Sigma (St. Louis, MO, USA). Methyl jasmonate was obtained from Aldrich (Milwaukee, WI, USA). 3-0-methyl glucose was obtained from ICN (Costa Mesa, CA, USA). 24-epibrassinolide was obtained from CIDtech Research (Cambridge,
Ontario, Canada). Paclobutrazol was obtained from ChemService, Inc. (West Chester, PA, USA). Brassinazole was graciously given by Dr. Tadao Asami (Institute of Physical and Chemical Research [RIKEN], Saitama, Japan).

**Isolation of *sis2* and sugar sensitivity assays**

(Experiments performed by Dr. Sue Gibson; included for sake of completeness.) For the mutant screen, ~28,000 M2 seeds were sown on minimal media plates supplemented with 0.3 M glucose. Plates were incubated for 14 days at 22°C under 50-65 μmol photons m⁻² s⁻¹ continuous light. Plants that formed relatively normal shoot systems (seedlings that had expanded cotyledons and true leaves) were transplanted to soil and grown to maturity. Seeds were harvested from these putative mutants and re-screened to weed out wild-type plants that escaped the initial selection. During the re-screens, seeds/seedlings were scored for germination, cotyledon expansion, and true leaf formation. Germination is defined as the emergence of any part of the seedling from the seed coat. Cotyledons are the two small leaf-like structures that emerge with a germinating seedling and expand to look similar to true leaves. Cotyledon expansion is defined as the enlargement and growth of cotyledons to a size much greater than cotyledons found on a
newly-emerged seedling. True leaf formation is defined as the formation of true leaves that are clearly visible to the naked eye.

For glucose, sucrose, mannose, and sorbitol sensitivity assays, seeds were sown on plates containing one of the following media: minimal, 0.3 M sucrose, 0.3 M glucose, 1.7 mM mannose, 0.27 M sorbitol + 0.03 M glucose, or 0.4 M sorbitol + 0.03 M glucose. Plates were incubated under 90-120 μmol photons m\(^{-2}\) sec\(^{-1}\) continuous fluorescent light for 13 days prior to scoring. Seeds/seedlings were scored for germination, cotyledon expansion, and true leaf formation.

For sucrose sensitivity assays of wild type (± GA, ± EBR), sis2, and spy-3, seeds were sown on 0.33 M sucrose with or without 10 μM gibberellin (GA) or 0.1 μM 24-epibrassinolide (EBR). Seeds were grown for 14 days under 40-75 μmol photons m\(^{-2}\) sec\(^{-1}\) continuous fluorescent light prior to scoring germination, cotyledon expansion, and true leaf formation.

**Chlorophyll and anthocyanin determination**

(Experiment performed by Dr. Donggiun Kim; included for sake of completeness.) To measure chlorophyll levels, seeds/seedlings were grown for 2 weeks under 60-80 μmol photons
m\(^{-2}\) s\(^{-1}\) continuous light on minimal media plates supplemented with 0.03 M or 0.15 M sucrose. Chlorophyll levels were determined as described (Wintemans and de Mots, 1965). To measure anthocyanin levels, seeds/seedlings were grown on minimal media plates for 2 weeks, transferred to media plates supplemented with 0.03 M or 0.18 M sucrose, and grown for an additional week under 60-80 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) continuous light. Anthocyanin levels were determined as described (Rabino and Mancinelli, 1986). Values for anthocyanin measurements were scaled relative to levels in wild-type seedlings on 0.18 M sucrose.

**Germination experiments**

*Paclobutrazol.* For germination on paclobutrazol (Figure 6, experiment performed by Dr. Sue Gibson, included for sake of completeness), seeds were sown on Arabidopsis minimal media plates supplemented with 120 \(\mu\)M paclobutrazol. Plates were incubated under continuous fluorescent light at approximately 21\(^\circ\)C. Germination (defined as the emergence of any part of the seedling from the seed coat) was scored after 10 days.

*Glucose or sorbitol.* For germination of wild type and sis2 on glucose vs. sorbitol (Figures 8 and 10), seeds were surface-sterilized
and cold-treated in 0.15% agar for 3 days at 4°C in darkness. Then seeds were sown on minimal media plates supplemented with varying concentrations of glucose or sorbitol, as indicated. Plates were incubated at 21°C under 40-60 μmol photons m⁻² sec⁻¹ continuous fluorescent light. Germination was scored every 12 hours, beginning with 34 hours post-imbibition, for four days.

ABA. For germination on ABA, seeds were surface-sterilized and cold-treated in 0.15% agar for 3 days at 4°C in darkness. Then seeds were sown on media containing 0 to 5 μM ABA (as indicated) and incubated at 21°C under 40-60 μmol photons m⁻² sec⁻¹ continuous fluorescent light. Germination was scored almost daily; germination after 5 days is shown in the figure.

Paclobutrazol, ancytidol, and brassinazole. For germination on paclobutrazol, ancytidol, and brassinazole, seeds were surface-sterilized and sown on media supplemented with 240 μM paclobutrazol, 600 μM ancytidol, or 100 μM brassinazole. Seeds were cold-treated on plates for 3 days in darkness at 4°C. Then plates were incubated at 21°C under 40-60 μmol photons m⁻² sec⁻¹ continuous fluorescent light. Germination was scored daily.

Glucose and paclobutrazol. For germination of wild type, sis2, ctrl1, spy-3, sk390, aba2, abi3, and abi4 on glucose and
paclobutrazol (Figures 18 and 26), seeds were surface-sterilized and sown on media supplemented with 3.4 μM paclobutrazol and 7 to 30 mM glucose, as indicated. Seeds were cold-treated on plates at 4°C for 2.5 days in complete darkness (Figure 18) or for 4 days in darkness* (uncovered in the refrigerator, Figure 26). (*Refrigerator is dark except when someone opens the refrigerator door, and the light comes on. Plates were not covered with foil or placed in a cardboard box for cold treatment because this makes the plates too wet — condensation forms on the lid and drops down onto the media. This disturbs the media concentration.) Then plates were incubated at 21°C under 40-75 μmol photons m⁻² sec⁻¹ of continuous fluorescent light. Germination was scored daily for 5 days, then at regular intervals until 18 days post-imbibition.

*Paclobutrazol and sorbitol, mannose, 3-0-methyl glucose, or sucrose.* For wild-type germination on paclobutrazol and sorbitol, mannose, 3-0-methyl glucose, or sucrose, seeds were surface-sterilized and sown on media supplemented with 3.4 μM paclobutrazol and one of the following: 60 mM sorbitol, 2 or 3 mM mannose, 15 to 60 mM 3-0-methyl glucose, or 15 to 30 mM sucrose. Seeds were cold-treated on plates at 4°C in darkness* (uncovered in the refrigerator). Cold treatment lasted for 2 days (mannose, 3-OMG), 3 days (sorbitol), or 4 days (sucrose). Then plates were
incubated at 21°C under continuous fluorescent light of intensity in the range 70-170 μmol photons m⁻² sec⁻¹. Specific light intensities are indicated in each figure legend. Germination was scored daily for 5 days, then at regular intervals afterwards.

GA/EBR/ACC with paclobutrazol and glucose. For wild-type germination on paclobutrazol and glucose with gibberellin (GA), 24-epibrassinolide (EBR), or 1-aminocyclopropane-1-carboxylic acid (ACC), seeds were surface-sterilized and sown on media containing 120 μM paclobutrazol + 30 mM glucose + 10μM GA or 1μM EBR or 50 μM ACC. Seeds were cold-treated on plates at 4°C in darkness* (uncovered in the refrigerator) for 4 days. Then plates were incubated at 21°C under 100-150 μmol photons m⁻² sec⁻¹ of continuous fluorescent light. Germination was scored daily for 5 days, then at 8 days post-imbibition.

etrl, gal-5, and det2 on glucose. For germination of the mutants etrl, gal-5, and det2 on glucose, seeds were surface-sterilized and sown on media containing 0.1 M glucose. Wild-type seeds were surface-sterilized and sown on media containing 0.1 M glucose ± 120 μM paclobutrazol. Seeds were cold-treated on plates at 4°C in darkness* (uncovered in the refrigerator) for 4 days. Then plates were incubated at 21°C under 90-100 μmol photons m⁻² sec⁻¹
of continuous fluorescent light. Germination was scored daily for 5 days, then at regular intervals until 20 days post-imbibition.

**Hypocotyl elongation experiments**

*Aminoethoxyvinylglycine (AVG).* (Experiment performed by Dr. Ron Laby; included for sake of completeness.) For hypocotyl elongation on aminoethoxyvinylglycine (AVG), an ethylene biosynthesis inhibitor, seeds were sown on plates containing minimal media plus 0.03 M glucose, with 10 µM AVG added (AVG+) or no AVG (AVG-). Plates were cold-treated at 4°C in darkness for 3 days, then transferred to room temperature (~25°C) under fluorescent light for 1 hour, then grown in darkness at room temperature for 5 days before scoring hypocotyl lengths.

*Glucose and sorbitol.* For hypocotyl elongation on glucose and sorbitol, wild-type seeds were surface-sterilized and cold-treated in 0.15% agar for 6 days at 4°C in darkness. After cold treatment, seeds were sown atop mesh filters on minimal media plates. Plates were given 30 minutes of light under the sterile hood at room temperature, then were wrapped in foil and incubated horizontally at 21°C in darkness for 52 hours to allow complete germination. Next, seedlings on filters were transferred to plates supplemented 0.03 M to 0.4 M glucose and/or 0.1 M to 0.3 M sorbitol. Seedlings
were exposed to light under the sterile hood during the media shift, which lasted ~30-60 min. Seedlings were grown vertically in darkness for 5 additional days at 21°C before measuring hypocotyl lengths.

*Paclobutrazol, ancyridol, and brassinazole.* For hypocotyl elongation on paclobutrazol, ancyridol, and brassinazole, seeds were surface-sterilized and cold-treated in 0.15% agar for 2 days at 4°C in darkness. After cold treatment, seeds were sown atop mesh filters on minimal media plates. Plates were given 1 hour of light under the sterile hood at room temperature, then were wrapped in foil and incubated horizontally at 21°C in darkness for 2 days to allow complete germination. Next, seedlings on filters were transferred to plates supplemented with 0.25 μM paclobutrazol, 0.5 μM ancyridol, or 1.0 μM brassinazole, with or without 1 μM gibberellin or 0.1 μM 24-epibrassinolide. Seedlings were exposed to light under the sterile hood during the media shift, which lasted ~30-60 min. Seedlings were grown vertically in darkness at 21°C for 5 additional days before measuring hypocotyl lengths. P values were calculated according to the Student's t-test.
Root elongation experiments

Seeds were surface-sterilized and cold-treated in 0.15% agar at 4°C in darkness for 2-4 days (2 days, IAA; 3 days, cytokinin; 4 days, methyl-jasmonate). After cold-treatment in 0.15% agar, seeds were sown on plates containing 0-1000 nM IAA, 0-4 μM cytokinin, or 0-150 μM methyl-jasmonate. Plates were incubated at 30-45 μmol photons m⁻² sec⁻¹ continuous fluorescent light under a yellow filter (as IAA is light-sensitive) at 20°C for 8 days prior to scoring root lengths. P values for the methyl-jasmonate experiment were calculated according to the Student's t-test.

Time to flowering and stem elongation experiments

(The stem elongation experiment was performed by Dr. Sue Gibson; included for sake of completeness.) Seeds were surface-sterilized and sown directly on plates supplemented with 0.03 M sucrose. Plates were incubated at 21°C under 40-60 μmol photons m⁻² sec⁻¹ of continuous fluorescent light for 8 days (time to flowering) or 11 days (stem elongation). Seedlings were transplanted to large test tubes containing media supplemented with 0-0.5 μM paclobutrazol and grown at 26°C under 55-75 μmol photons m⁻² sec⁻¹ continuous light. Plants were checked daily for
bolting using 2.75X magnifying goggles (optivisors). After bolting, the length of primary inflorescence was measured at 24 hour intervals from ~1 cm to ~10 cm. Rosette leaves were counted after stems had grown up to 10 cm tall. Bolting is defined as the appearance of a bud at the center of the rosette. After bolting, the plant ceases to produce rosette leaves; therefore, counting the number of rosette leaves is a way to measure developmental time to flowering. P values were calculated according to the Student's t-test.

Sugar level measurements

Seeds were surface-sterilized and cold-treated in 0.15% agar at 4°C in darkness for 3 days, then sown on 3MW Gel Blot Paper (Midwest Scientific, Valley Park, MO, USA) on minimal media plates. Plated seeds were given 30 minutes of light under the sterile hood at room temperature, then grown at 21°C in darkness to prevent photosynthesis. Seeds were collected after 24, 48, or 72 hours, frozen, and ground. Sugars (fructose, sucrose, and glucose) were extracted and measured with a Glucose HK assay kit (Sigma, St. Louis, MO, USA). The Glucose HK assay reagent was supplemented with 1 U/sample phosphoglucose isomerase and 400 U/sample invertase. P values were calculated according to the Student's t-test.
Wilting test

Wild-type, *sis2*, and *aba2* seeds were sown according to genotype in separate pots containing premium potting soil (Metro mix). Seeds/seedlings/plants were grown under continuous light at room temperature and watered frequently until they developed to maturity. Five weeks after sowing, adult (flowering, mostly full-grown, green, non-senescing) plants were cut off at the base of the rosette and placed in front of a gentle fan. All of the plants from a single pot (typically 6-9 plants/pot) were weighed together at one-minute intervals for a period of 30 minutes. The rate of weight loss due to water loss was calculated for the first ten-minute period. P values were calculated using the Student's *t*-test. Data collection and analysis were aided by Catherine McCollum.
PHENOTYPIC CHARACTERIZATION OF THE sis2 MUTANT

As described in the introduction, the sis2 mutant was isolated in our lab from a pool of ethyl methane sulfonate (EMS)-mutagenized Arabidopsis thaliana M2 seeds on the basis of its resistance to high levels of exogenous glucose. Previous work shows that unlike wild type, the sis2 mutant is able to form a substantial shoot system on high concentrations of exogenous glucose, sucrose, and sorbitol, and also displays resistance to mannose. Previous work also shows that the sis2 mutant is resistant to the inhibitory effects of paclobutrazol, a gibberellin biosynthesis inhibitor, on germination. This previous work laid the foundation for the work described in this chapter.

This chapter describes the phenotypic characterization of the sis2 mutant, with particular attention to sugar and phytohormone responses and metabolism. Before examining the sis2 mutant's responses to sugars, wild-type responses to sugar and sorbitol were examined during two developmental stages: germination and hypocotyl elongation. These experiments were done first because published reports do not clarify whether the effects of sugars on these processes are sugar-specific or due to osmotic effects. After the effects on germination were found to be sugar-specific, the sis2
mutant's responses to sugar during germination were examined. In addition, sugar levels were measured in *sis2* seeds. Phytohormone responses of *sis2* examined include ethylene (hypocotyl elongation); abscisic acid (germination and wilting); indole-3-acetic acid, cytokinin, and methyl-jasmonate (root elongation); gibberellin (germination, hypocotyl elongation, flowering time, stem elongation); and brassinosteroids (germination and hypocotyl elongation).

**Sugar responses of *sis2***

*Wild-type germination, hypocotyl elongation, and flowering on glucose and sorbitol.* Evidence from several labs suggests that glucose and sucrose inhibit several plant developmental processes, including germination (Lydia Sommerlad, Kelly Biddle, and Dr. Sue Gibson, unpublished results) and hypocotyl elongation (Dijkwel et al., 1997; Jang et al., 1997; Zhou et al., 1998). However, the published experiments lack appropriate osmotic controls. Therefore, our lab determined whether the inhibitory effects of glucose and sucrose on these developmental processes are sugar-specific or osmotic. To do this, the responses of wild type seeds/seedlings to glucose and sorbitol (an osmotic control) were compared during germination and hypocotyl elongation.
Germination results indicate that sorbitol exerts only very subtle effects on seed germination rates, but glucose inhibits germination in a concentration-dependent manner (Figure 8).

An important side note is worthy of mention here. The germination assay on glucose/sorbitol just described is different from sugar-insensitivity (sis) assays described in the introduction. In sis assays, seeds are sown on very high levels of glucose or sucrose (0.3 M) or sorbitol (0.4 M). Seeds/seedlings are allowed to grow for two weeks, and then are examined for percent germination, as well as percent cotyledon expansion and percent true leaf formation. In these experiments, seeds are scored only to determine whether they are able to germinate within two weeks. No attempt is made to determine the rate at which seeds germinate. The germination assay on glucose described above utilizes low, near-physiological levels of glucose (0.03 M to 0.1 M). In this assay, germination is scored daily or twice daily from the time the seeds are sown. The goal of this germination assay is to compare germination rates between wild-type and mutant lines.

Hypocotyl elongation results indicate that osmotica, particularly sorbitol, inhibit hypocotyl elongation (Figure 9). A noteworthy result of the hypocotyl elongation experiment is that
Figure 8. Germination of wild-type seeds is delayed by glucose in a concentration-dependent manner. In contrast, sorbitol has only a very minor effect on germination rates. Seeds were cold-treated for 3 days at 4°C in darkness, then sown on the indicated media and incubated at 21°C in 40-60 μmol photons m⁻² sec⁻¹ of continuous fluorescent light. Germination was scored every 12 hours, as shown. Values indicate the mean ± SD (n = 3 plates, each containing ~50 seeds). This experiment was repeated multiple times, with similar results. Additional replicates were performed by Kelly Biddle. Col WT, wild-type Columbia; Glc, glucose; Sorb, sorbitol.
Figure 9. Hypocotyl elongation of wild-type seedlings is inhibited by osmoticia (particularly sorbitol). Seeds were cold-treated for 6 days at 4°C in darkness and sown atop mesh filters on minimal media plates. Plates were given 30 minutes of light under the sterile hood at room temperature, then were incubated at 21°C in darkness for 52 hours to allow complete germination. Next, seedlings (filters) were transferred to the indicated media and grown in darkness for 5 additional days at 21°C before measuring hypocotyl lengths. Values indicate the mean ± SD (n = 24). The experiment was repeated twice, with similar results. Col WT, wild-type Columbia.
glucose alone has less of an inhibitory effect on hypocotyl elongation than does an equimolar concentration of glucose and sorbitol (Figure 9). This suggests that glucose has beneficial effects on plant growth that reduce the negative effect of the osmotic stress caused by high exogenous glucose levels.

*Germination of sis2 on glucose and sorbitol.* The response of *sis2* to glucose was examined during seed germination, a developmental stage affected in a sugar-specific manner. Results show that *sis2* mutant is resistant to the inhibitory effects of glucose on germination (Figure 10). In contrast, *spindy-3 (spy-3)*, a constitutive gibberellin response mutant (Jacobsen and Olszewski, 1993), demonstrates wild-type sensitivity to the inhibitory effects of glucose on germination (Figure 10). The faster rate at which *sis2* germinates on glucose cannot simply be due to precocious germination, as *spy-3* has been reported to germinate precociously on minimal media (Tong-Seung Tseng, pers. comm.) but does not germinate more quickly than wild type on glucose (Figure 10). In addition, the *spy-3* mutant does not demonstrate a *sis* phenotype (Figure 11). Note that *SIS2* is not allelic to *SPY-3*, as the two genes map to different chromosomes (*SIS2*, chromosome 1; *SPY-3*, chromosome 3 [Dr. Donggiun Kim, unpublished results; Jacobsen and Olszewski, 1993]).
Germination of Col WT, sis2, and spy-3 on Glc vs. Sorb, 46 hours post-imbibition

Figure 10. The sis2 mutant is resistant to the inhibitory effects of glucose on germination. Seeds were cold-treated for 3 days at 4°C in darkness, then sown on the indicated media and incubated at 21°C in 40-60 μmol photons m⁻² sec⁻¹ of continuous fluorescent light. Germination was scored every 12 hours, but only the 46 hours post-imbibition time point is shown here. Essentially no seeds had germinated by the 34-hour post-imbibition time point (not shown). Values indicate the mean ± SD (n = 3 plates, each containing ~50 seeds). This experiment was repeated multiple times, with similar results. Col WT, wild-type Columbia; Glc, glucose; Sorb, sorbitol.
Figure 11. The spy-3 mutation, exogenous gibberellin, and exogenous 24-epibrassinolide do not confer a sugar-insensitive (sis) phenotype. The sis2 mutant demonstrates a sis phenotype. Seeds were sown on media containing 0.33 M sucrose, plus 10 μM gibberellin (GA) or 0.1 μM 24-epibrassinolide (EBR) where indicated. Seeds were grown for 14 days under 40-75 μmol photons m\(^{-2}\) sec\(^{-1}\) continuous fluorescent light prior to scoring. Values indicate the mean ± SD (n = 3 plates, each of a different seed line, with ~50 seeds/plate). The experiment was repeated once, with similar results. Additional replicates of wild type on high sucrose + GA were performed by Dr. Sue Gibson. Col WT, wild-type Columbia; Suc, sucrose.
**Internal sugar levels.** It is of interest to determine why the *sis2* mutant exhibits osmotolerance, or resistance to high levels of exogenous sorbitol, in addition to sugar insensitivity. One trait that can confer osmotolerance is high endogenous sugar levels. Therefore, sugar levels were measured in germinating *sis2*, wild-type, and *spy-3* seeds. In general, sugar levels decrease over the period of 24 hours to 72 hours post-imbibition (Figure 12). Unfortunately, sugar levels vary widely depending on the seed line (Figure 12). This makes it difficult to determine whether there is a significant difference between sugar levels in *sis2* and wild type. In addition, these measurements account for the seed as a whole but do not distinguish between possible differences in sugar levels according to tissue type.
Figure 12. Sugar levels in seeds decrease over the period of 24 hours to 72 hours post-imbibition. Seeds were cold-treated at 4°C in darkness for 3 days, then sown on 3MW Gel Blot Paper on minimal media plates. Plated seeds were given 30 minutes of light under the sterile hood at room temperature, then grown at 21°C in darkness to prevent photosynthesis. Seeds were collected after 24, 48, or 72 hours, frozen, and ground. Sugars (fructose, sucrose, and glucose) were extracted and measured with a Glucose HK assay kit (Sigma). Values indicate the mean ± SD (n = 3 samples/genotype, of seeds harvested at the same time and grown in parallel). According to the Student's t-test, sugar levels in sis2 are not significantly different from wild type (P values = 0.655, 0.137, and 0.886, for 24, 48, and 72 hours post-imbibition, respectively). In addition, spy-3 values are not significantly different from wild type (P values = 0.227, 0.473, and 0.886, for 24, 48, and 72 hours post-imbibition, respectively). This experiment was repeated once, using three different seed lines representing each genotype. Results of this repetition vary widely, depending on the seed line. P values indicate significant differences even between seed lines of the same genotype. In addition, P values indicate inconsistency as to whether significant differences exist among the Col WT and sis2 comparisons. Col WT, wild-type Columbia; fwt, fresh weight.
Phytohormone responses of *sis2*

As described in the introduction, several sugar-response mutants show alterations in phytohormone response or metabolism. Therefore, the responses of *sis2* to the following phytohormones were examined: ethylene, abscisic acid (ABA), indole-3-acetic acid (IAA, the major form of auxin), cytokinin, methyl-jasmonate, gibberellin, and brassinosteroids. Refer to Table 1 for a summary of the functions of these phytohormones.

*Ethylene.* Ethylene responses of *sis2* were analyzed previously and are described under "Previous work on the *sis2* mutant."

*Abscisic acid (ABA).* ABA responses of *sis2* were examined in two ways: 1) rate of wilting, to look for defects in ABA biosynthesis and sensitivity, and 2) germination on ABA, to determine ABA sensitivity. 1) Wilting is slowed down when plants close their stomata, a process mediated by ABA (Léon-Kloosterziel et al., 1996). Mutants deficient in ABA biosynthesis (*aba* mutants) fail to close their stomata, resulting in faster water loss (Koornneef et al., 1982; Léon-Kloosterziel et al., 1996). In addition, the *abscisic acid-insensitive* (*abi*) mutants *abi1* and *abi2* demonstrate a wilty phenotype (Koornneef et al., 1984). Wilting results for the *sis2* mutant indicate that *sis2* is only slightly, if at all, more wilty than wild type (Table 2). In contrast, *sis2* is much less wilty than the
<table>
<thead>
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<th></th>
<th>First 10 min.</th>
<th>Last 20 min.</th>
<th>Entire 30 min.</th>
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<tr>
<td>Col wt</td>
<td>0.50 ± 0.10</td>
<td>0.33 ± 0.05</td>
<td>0.38 ± 0.07</td>
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<tr>
<td>sis2</td>
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<td>0.42 ± 0.07</td>
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<tr>
<td>aba2</td>
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<td>0.79 ± 0.07</td>
<td>1.02 ± 0.11</td>
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**Table 2.** The *sis2* mutant demonstrates a nearly wild-type wilting response, unlike the wilty *aba2* mutant. Adult five-week old plants were cut off at the base of the rosette and placed in front of a gentle fan. All of the plants from a single pot (typically 6-9 plants/pot) were weighed together at one-minute intervals for a period of 30 minutes. The rate of weight loss due to water loss was calculated during three periods: the first 10 minutes, the last 20 minutes, and the entire 30 minutes. Values indicate the mean ± SD (n=24). According to the Student's t-test, wilting values for wild type and *sis2* are hardly different (P values 0.0509, 0.130, 0.0717, for the first 10 minutes, last 20 minutes, and entire 30 minutes, respectively). The data collection and analysis for this experiment were aided by Catherine McCollum.
*aba2* mutant (Table 2). Wilting test work was aided by Catherine McCollum.

Germination of wild-type Arabidopsis seeds is inhibited by ABA (Koornneef et al., 1984). In contrast, the ABA-insensitive mutants, *abil* through *abi5*, germinate on ABA (Finkelstein, 1994; Koornneef et al., 1984). In order to determine whether *sis2* may have a defect in ABA sensitivity, germination of *sis2* was examined on a range of ABA concentrations. Results show that *sis2* demonstrates little, if any, ABA insensitivity during germination (Figure 13). Interestingly *ctr1*, a constitutive ethylene response mutant, the extensive characterization of which shows its major role in ethylene response but no direct role in ABA signaling, is resistant to ABA (Figure 13).

**IAA, cytokinin, and methyl-jasmonate.** Root-elongation responses of *sis2* to indole-3-acetic acid (IAA), cytokinin, and methyl-jasmonate were examined, as these three phytohormones exert strong effects on root growth. The *sis2* mutant demonstrates wild-type root length inhibition by IAA and cytokinin, and slight resistance to root length inhibition by methyl-jasmonate (Figure 14). On minimal media, *sis2* has similar root elongation to wild type (Figure 14).
Figure 13. The *sis2* mutant demonstrates slight ABA insensitivity. Seeds were cold-treated for 3 days at 4°C in darkness, then sown on the indicated media and incubated at 21°C under 40-60 μmol photons m⁻² sec⁻¹ continuous fluorescent light. Germination was scored after 5 days. Values indicate the average of two plates of each genotype (each containing ~50 seeds, except for *abi2-1*, where n = 1 plate). This experiment was repeated once, with similar results. Col WT, wild-type Columbia.
IAA

Root length (mm)

IAA conc. (nM)

- Col WT
- sis2

Cytokinin

Root length (mm)

Cytokinin conc. (μM)

- Col WT
- sis2

Methyl-jasmonate

Root length (mm)

Methyl-jasmonate conc. (μM)

- Col WT
- sis2
Figure 14. The *sis2* mutant demonstrates wild-type root length inhibition by indole-3-acetic acid (IAA) and cytokinin, and slight resistance to methyl-jasmonate. Seeds were sown on the indicated media, supplemented with 0.015 M sucrose. Plates were incubated at 30-45 μmol photons m⁻² sec⁻¹ continuous fluorescent light under a yellow filter for 8 days prior to scoring root lengths. Values indicate the mean ± SD (n = 20, IAA; n = 25, cytokinin; n = 30, methyl-jasmonate). Student's t-test indicates that root lengths of *sis2* and wild type are significantly different on methyl-jasmonate (all P values under 0.002 except for 0.5 μM methyl-jasmonate, where P value = 0.0545). Each of these experiments was repeated once, with similar results. Col WT, wild-type Columbia.
Gibberellin and brassinosteroids. To look for alterations in gibberellin and brassinosteroi'd responses, the responses of sis2 were examined to the gibberellin biosynthesis inhibitors paclobutrazol and ancydrol (Hedden and Graebe, 1985), and to the brassinosteroid biosynthesis inhibitor brassinazole (Asami et al., 2000). All of these three inhibitors are cytochrome p450 monooxygenase inhibitors (Table 1). The response of sis2 to paclobutrazol was examined during some of the developmental stages promoted by gibberellin: germination (Karssen et al., 1989), hypocotyl elongation (reviewed in Arteca, 1996), flowering (Wilson et al., 1992), and stem elongation (Peng et al., 1997). The response of sis2 to ancydrol was examined only during germination and hypocotyl elongation. The response of sis2 to brassinazole was tested during two stages promoted by brassinosteroids: hypocotyl elongation (reviewed in Li and Chory, 1999) and germination (Steber and McCourt, 2000). The spy-3 mutant demonstrates resistance to paclobutrazol at all developmental stages tested (Jacobsen and Olszewski, 1993) and therefore is included as a control in these experiments.

Results demonstrate that the sis2 mutant is resistant to the inhibitory effects of paclobutrazol, ancydrol, and brassinazole on seed germination (Figure 15). However, the sis2 mutant
Figure 15. The *sis2* mutant is resistant to the inhibitory effects of paclobutrazol, ancymidol, and brassinazole on seed germination. Seeds were sown on media supplemented with the following inhibitors: none (MIN, or minimal), 600 μM ancymidol (Anc), 240 μM paclobutrazol (Pac), or 100 μM brassinazole (Brz). Next, seeds were cold-treated on plates for 3 days in darkness at 4°C. Then, plates were incubated at 21°C under 40-60 μmol photons m⁻² sec⁻¹ continuous fluorescent light. Germination was scored daily. Values represent one plate of each genotype (~50 seeds per plate). Multiple seed lines of each genotype were examined in two repetitions of this experiment, with similar results. Col WT, wild-type Columbia.
demonstrates slight resistance to paclobutrazol, ancymidol, and brassinazole during hypocotyl elongation (Figure 16). In addition, the sis2 mutant demonstrates little to no resistance to paclobutrazol during later developmental stages, including time to flowering (Figure 17) and stem elongation (Figure 18, experiment performed by Dr. Sue Gibson). In contrast, spy-3 exhibits marked resistance to the inhibitory effects of paclobutrazol, ancymidol, and brassinazole on germination as well as hypocotyl elongation (Figures 15, 16), and to paclobutrazol on flowering and stem elongation (Figures 17, 18; Jacobsen and Olszewski, 1993).

Under normal growth conditions (on minimal media or in soil), the sis2 mutant demonstrates wild-type hypocotyl elongation, flowering time, and stem elongation (Figures 16, 17, 18). In contrast, the spy-3 mutant reportedly demonstrates faster hypocotyl elongation, early flowering, and faster stem elongation than wild type under normal growth conditions (Jacobsen and Olszewski, 1993).

In summary, sis2 demonstrates resistance to paclobutrazol, ancymidol, and brassinazole (cytochrome p450 monooxygenase inhibitors) primarily during germination. The sis2 mutant also demonstrates insensitivity to sugars during early developmental
Figure 16. The *sis2* mutant demonstrates slight resistance to paclobutrazol, ancydimol, and brassinazole during hypocotyl elongation. Seeds were cold-treated for 2 days in darkness at 4°C, then sown atop mesh filters on minimal media plates. Plates were given 1 hour of light under the sterile hood at room temperature, then incubated at 21°C in darkness for 2 days to allow complete germination. Seedlings (filters) were then transferred to the indicated media and returned to darkness at 21°C for an additional 5 days before scoring hypocotyl lengths. Brz, = 1.0 µM brassinazole, EBR = 0.1 µM 24-epibrassinolide, GA = 1.0 µM gibberellin, Pac = 0.25 µM paclobutrazol, Anc = 0.5 µM ancydimol. Values indicate the mean ± SD (n = 20). According to the Student’s t-test, hypocotyl lengths on Brz, Pac, and Anc are significantly different between wild type and *sis2* (all P values less than 0.0099). Col WT, wild-type Columbia; MIN, minimal media. This experiment was repeated once, with similar results.
Figure 17. The sis2 mutant demonstrates little to no resistance to the inhibitory effects of paclobutrazol on time to flowering. Time to flowering was measured both by the number of days until bolting, as well as by the number of rosette leaves. (As described in the introduction, Arabidopsis stops producing rosette leaves after flowering. In this way, the total number of rosette leaves gives a measure of the number of developmental units, or phytomers, completed prior to flowering.) Seeds were sown on plates supplemented with 0.03 M sucrose and incubated at 21°C under 40-60 μmol photons m⁻² sec⁻¹ of continuous fluorescent light for 8 days. Seedlings were transplanted to large test tubes containing media supplemented with the indicated concentrations of paclobutrazol and grown at 26°C under 55-75 μmol photons m⁻² sec⁻¹ continuous fluorescent light. Plants were checked daily for bolting (the appearance of a bud at the center of the rosette). Rosette leaves were counted after stems had grown several centimeters tall. Values indicate the mean ± SD (n = 15). According to the Student's t-test, flowering time results for sis2 are not consistently different from wild type (P values for rosette leaf count are 0.177, 0.849, and 1.3E-08 on 0, 0.1, and 0.5 μM Pac, respectively. However, the striking difference in the number of rosette leaves of sis2 and wild type on 0.5 μM paclobutrazol is not reproducible. P values for days to bolting of sis2 vs. wild type are 0.073, 0.542, and 0.030 on 0, 0.1, and 0.5 μM Pac, respectively). In contrast, the Student's t-test shows significant difference between flowering time results for spy-3 and wild type (all P values are below 0.003, except for rosette leaf count on 0 μM Pac, where P value = 0.602). Both graphs represent the same experiment. Col WT, wild-type Columbia; Pac, paclobutrazol. This experiment was repeated once, with mostly similar results. One additional replicate was performed by Dr. Sue Gibson.
Figure 18. The sis2 mutant is sensitive to the inhibitory effects of paclobutrazol on stem elongation. Seeds were sown on plates supplemented with 0.03 M sucrose and incubated at 21°C under 40-60 μmol photons m\(^{-2}\) sec\(^{-1}\) of continuous fluorescent light for 11 days; then seedlings were transferred to large test tubes containing media supplemented with the indicated concentrations of paclobutrazol. After bolting, the length of primary inflorescence was measured at 24 hour intervals from ~1 cm to ~10 cm. Values indicate the mean ± SD (n = 14-15). Col WT, wild-type Columbia; Pac, paclobutrazol. Experiment performed by Dr. Sue Gibson.
stages. The dual sugar-insensitive and paclobutrazol-resistant phenotypes of the *sis2* mutant suggest the existence of connections between sugar and phytohormone signaling/response pathways. To explore these connections, several experiments were performed, as described in the following chapter.
COMBINATORIAL INHIBITORY EFFECTS OF PACLOBUTRAZOL
AND GLUCOSE ON SEED GERMINATION

As the dual paclobutrazol-resistant and sugar-insensitive phenotypes of the \textit{sis2} mutant suggest the existence of novel connections between the sugar and phytohormone response pathways, it was of interest to pursue studies to further explore these connections. Germination is a useful developmental stage for exploring these connections because it is the earliest developmental stage at which glucose exerts sugar-specific effects (as shown in the previous chapter) and the stage in which \textit{sis2} demonstrates insensitivity to paclobutrazol, a gibberellin biosynthesis inhibitor.

Another motivating factor to explore these connections was the recent discovery that glucose rescues germination on otherwise inhibitory concentrations of abscisic acid (ABA; Finkelstein and Lynch, 2000). As described in the introduction, this observation led, through a series of hypotheses and observations, to the following hypothesis tested in this chapter, namely, that glucose might allow germination on paclobutrazol. This hypothesis was tested by examining the combinatorial effects of glucose and paclobutrazol on seed germination. Surprisingly, the results obtained were the opposite of those expected. Results
demonstrated that glucose exacerbates rather than alleviates the negative effects of paclobutrazol on seed germination.

To explore the connections between paclobutrazol and glucose responses, first the sugar-specific nature of the inhibition of germination by paclobutrazol and glucose was analyzed, and secondly the phytohormone(s) involved were determined. The sugar-specific nature was analyzed by employing glucose, sorbitol, mannose, sucrose, and 3-O-methyl glucose (a glucose analog) in combination with paclobutrazol. The phytohormone analysis involved determining whether the gibberellin, ethylene, and brassinosteroids rescue germination on paclobutrazol and glucose; and comparing rates of germination of numerous phytohormone response/synthesis mutants. The results are presented in the following paragraphs.

Sugar-specificity of glucose and paclobutrazol

Glucose and paclobutrazol. First, the effect of paclobutrazol combined with glucose was examined on wild-type seed germination. These results show that increasing the paclobutrazol concentration (from 3.4 μM to 120 μM) in the absence of glucose does not greatly increase the inhibition of germination (Figure 19), contrary to a published report (Debeaujon and Koornneef, 2000).
Figure 19. Increasing the paclobutrazol concentration (from 3.4 μM to 120 μM) in the absence of glucose does not greatly increase the inhibition of germination. Wild-type seeds were sown on plates containing the indicated media and cold-treated at 4°C in darkness for 2.5 days. Then plates were incubated at 21°C under 40-70 μmol photons m⁻² sec⁻¹ of continuous fluorescent light. Germination was scored daily for 5 days, then at regular intervals until 18 days post-imbibition. Values for one wild-type seed line are shown (n = 1 plate/media, ~50 seeds/plate). Multiple wild-type seed lines var. Columbia were examined in three repetitions of the experiment, with similar results. Additional replicates were performed by Dr. Sue Gibson. p = μM paclobutrazol.
However, combining as little as 3.4 µM paclobutrazol with 15 mM glucose results in a significant inhibitory effect on wild-type germination (Figure 20). Note that 15 mM glucose has hardly any noticeable effect on germination by itself (Figure 20). These results show that the combination of glucose and paclobutrazol has a much greater inhibitory effect on wild-type seed germination than either paclobutrazol alone or glucose alone.

*Sorbitol and paclobutrazol.* To ascertain whether the heightened inhibitory effect of glucose combined with paclobutrazol is a sugar-specific or an osmotic effect, wild-type germination was examined on media containing paclobutrazol combined with either glucose or sorbitol. As shown in Figure 21, the inhibitory effect of sorbitol and paclobutrazol on wild-type germination is much less severe than the inhibitory effect of glucose and paclobutrazol. Thus, the osmotic stress caused by glucose is not strong enough to account for the multiplicative inhibitory effect seen in the presence of glucose and paclobutrazol. Therefore, the effect of glucose and paclobutrazol is sugar-specific.
**Figure 20.** Germination of wild-type seeds on paclobutrazol and glucose is inhibited at 15 mM glucose when combined with as little as 3.4 μM paclobutrazol. Seeds were sown on plates containing the indicated media and cold-treated on plates at 4°C in darkness for 2.5 days. Then plates were incubated at 21°C under 40-70 μmol photons m⁻² sec⁻¹ of continuous fluorescent light. Germination was scored daily for 5 days, then at regular intervals until 18 days post-imbibition. Values for one wild-type seed line are shown (n = 1 plate/media, ~50 seeds/plate). Multiple wild-type seed lines var. Columbia were examined in three repetitions of the experiment, with similar results. Additional replicates were performed by Dr. Sue Gibson. p = μM paclobutrazol, Glc = mM glucose.
Figure 21. The inhibitory effect of sorbitol and paclobutrazol on wild-type germination is much less severe than the inhibitory effect of glucose and paclobutrazol. Seeds were sown on plates containing the indicated media and cold-treated at 4°C in darkness for 3 days. Then plates were incubated at 21°C under 70-160 µmol photons m⁻² sec⁻¹ of continuous fluorescent light. Germination was scored daily for 5 days, then at regular intervals until 11 days post-imbibition. Values for one wild-type seed line (var. Columbia) are shown (n = 1 plate/media, ~50 seeds/plate). Three wild-type seed lines var. Columbia were examined in each of three repetitions of the experiment, with similar results. p = µM paclobutrazol, Glc = mM glucose, Sorb = mM sorbitol.
Hexokinase dependence. As described in the "Hexokinase" section of the introduction, sugar-specific effects can be either hexokinase-mediated or hexokinase-independent. It was interesting to determine whether the sugar-specific inhibition of germination by paclobutrazol and glucose is hexokinase-mediated because that may contribute to our understanding of the mechanism and connection(s) involved. To determine whether the effect of paclobutrazol and glucose is hexokinase-mediated, wild-type seed germination was examined on media containing paclobutrazol combined with one of the following sugars/sugar analogs: glucose, mannose (another hexose that can be phosphorylated by hexokinase), 3-O-methyl glucose (3-OMG, a sugar analog that cannot be phosphorylated by hexokinase), sucrose (a disaccharide), or sorbitol (an osmotic control). Results for sorbitol were discussed above and are shown in Figure 21. As shown in Figure 22, mannose increases the inhibitory effect of paclobutrazol on wild-type germination. This effect is almost as great as the effect of glucose combined with paclobutrazol. As the metabolism of mannose has not yet been characterized in Arabidopsis, it remains unclear why mannose exerts the effect that it does.
Figure 22. Mannose increases the inhibitory effect of paclobutrazol on wild-type germination. Seeds were sown on plates containing the indicated media and cold-treated at 4°C in darkness for 2 days. Then plates were incubated at 21°C under 115-170 μmol photons m⁻² sec⁻¹ of continuous fluorescent light. Germination was scored daily for 5 days, then at regular intervals until 19 days post-imbibition. Low concentrations of mannose were used because mannose is a highly potent inhibitor of germination. Values for one wild-type seed line (var. Columbia) are shown (n = 1 plate/media, ~50 seeds/plate). Three wild-type seed lines var. Columbia were examined in each of two repetitions of the experiment, with similar results. Pac = μM paclobutrazol, Man = mM mannose.
As shown in Figure 23, 3-OMG and paclobutrazol inhibit wild-type germination at least as strongly (if not more strongly) than glucose and paclobutrazol. This suggests that the effect of glucose is not hexokinase-mediated. However, since little is known about 3-OMG metabolism in Arabidopsis, and since 3-OMG is a poor substrate for some glucose uptake systems (reviewed in Gibson, 2000), the conclusion drawn from this 3-OMG study should be analyzed carefully. As shown in Figure 24, sucrose and paclobutrazol have a similar inhibitory effect on wild-type germination as glucose and paclobutrazol. This suggests that the effect of glucose is not mediated by a hexose transporter. However, it is possible that sucrose exerts its effect by being cleaved by an extracellular invertase, releasing glucose, which is imported by a hexose transporter.

Phytohormones affected by paclobutrazol and glucose

After the sugar-specific nature of germination inhibition by glucose and paclobutrazol was determined, the phytohormone(s) involved during the inhibition of germination by paclobutrazol and glucose were examined next. This is important due to the possibility that paclobutrazol, known to affect gibberellin biosynthesis, may affect biosynthesis of other phytohormones as well.
A. Experiment 1

Wild-type germination on Pac + Glc/3-OMG

B. Experiment 2

Wild-type germination on Pac + Glc/3-OMG
Figure 23. 3-0-methylglucose (3-OMG) and paclobutrazol inhibit wild-type germination at least as strongly (if not more strongly) than glucose and paclobutrazol. Results of two independent experiments are depicted. Seeds were sown on plates containing the indicated media and cold-treated at 3°C in darkness for 2 days.

Then plates were incubated at 21°C under 115-170 μmol photons m⁻² sec⁻¹ (Top) or 75-150 μmol photons m⁻² sec⁻¹ (Bottom) of continuous fluorescent light. Germination was scored daily for 5 days, then at regular intervals until 18 to 19 days post-imbibition. Values for one wild-type seed line (var. Columbia) are shown (n = 1 plate/media, ~50 seeds/plate). The seed line in the top figure is different from the seed line in the bottom figure, and both figures are from different repetitions of the experiment. Three wild-type seed lines var. Columbia were examined in each of two repetitions of the experiment. Pac = μM paclobutrazol, Glc = mM glucose, 3-OMG = mM 3-0-methylglucose.
Figure 24. Sucrose and paclobutrazol have a similar inhibitory effect on wild-type germination as glucose and paclobutrazol. Seeds were sown on plates containing the indicated media and cold-treated at 4°C in darkness for 2 days. Then plates were incubated at 21°C under 110-160 μmol photons m⁻² sec⁻¹ of continuous fluorescent light. Germination was scored daily for 5 days, then at regular intervals until 18 days post-imbibition. Values for one wild-type seed line (var. Columbia) are shown (n = 1 plate/media, ~50 seeds/plate). Three wild-type seed lines var. Columbia were examined in each of two repetitions of the experiment, with similar results. Pac = μM paclobutrazol, Glc = mM glucose, Suc = mM sucrose.
Exogenous gibberellin, brassinosteroids, and ethylene. Three phytohormones, namely gibberellin, brassinosteroids, and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), endogenously promote germination under normal conditions (Figure 5). These three phytohormones also rescue germination of wild-type seeds on paclobutrazol (Hedden and Graebe, 1985; Gibson et al., 2001; Lydia Sommerlad, unpublished results). To determine which phytohormone(s) rescue germination on paclobutrazol and glucose, germination of wild-type seeds was examined on media containing paclobutrazol and glucose supplemented with gibberellin, 24-epibrassinolide (a brassinosteroid), or ACC. As shown in Figure 25, gibberellin, 24-epibrassinolide, and ACC fully rescue germination of wild-type seeds on paclobutrazol and glucose.

Gibberellin-, brassinosteroid-, and ethylene-deficient or insensitive mutants. Another way to determine which phytohormone(s) may be involved in the inhibition of germination by paclobutrazol and glucose is to examine germination responses of mutants lacking or insensitive to ethylene, brassinosteroids, or gibberellin. This provides an alternative approach to the examination of wild-type germination on paclobutrazol and glucose.
Figure 25. Gibberellin, 24-epibrassinolide, and 1-aminocyclopropane-1-carboxylic acid (ACC, an ethylene precursor) rescue germination of wild-type seeds on paclobutrazol and glucose. Seeds were sown on plates containing the indicated media and cold-treated at 4°C in darkness for 4 days. Then plates were incubated at 21°C under 100-150 μmol photons m⁻² sec⁻¹ of continuous fluorescent light. Germination was scored daily for 5 days, then at 8 days post-imbibition. Values for one wild-type seed line (var. Columbia) are shown (n = 1 plate/media, ~50 seeds/plate). Three wild-type seed lines var. Columbia were examined in each of two repetitions of the experiment, with similar results. (ACC was only examined in one experiment.) +Glc, 30 mM glucose; +Pac, 120μM paclobutrazol; +GA, 10 μM gibberellin, +EBR, 1 μM 24-epibrassinolide; +ACC, 50 μM 1-aminocyclopropane-1-carboxylic acid.
Germination responses of the following three mutants were examined on glucose: *ethylene resistance1 (etr1)*, insensitive to ethylene (Bleecker et al., 1988; Chang et al., 1993); *de- etiolated2 (det2)*, defective in brassinosteroid biosynthesis (Li et al., 1996); and *gal-5*, defective in gibberellin biosynthesis (Chien and Sussex, 1996). The *gal-5* allele is a leaky allele of the gibberellin biosynthetic mutant *gal*; it germinates without the addition of exogenous gibberellin, unlike tight *gal* mutants (Chien and Sussex, 1996). The *etr1* mutant is super-sensitive to sugars during early shoot development (Gibson et al., 2001; Zhou et al., 1998). As shown in Figure 26, *etr1* and *gal-5* germinate more slowly than wild type on glucose, and *det2* germinates at a similar rate to wild type on glucose. In addition, *gal-5* germinates slower than wild type without glucose (Figure 26). However, *gal-5* germinates faster on glucose than wild type germinates on glucose and paclobutrazol (Figure 26). These results are discussed in the concluding chapter.

*Gibberellin and ethylene constitutive response mutants; abscisic acid-, sugar-, and paclobutrazol-insensitive mutants.*

Another experiment to determine which phytohormone(s) rescue germination on paclobutrazol and glucose is to examine germination responses of mutants having constitutive responses to ethylene or gibberellin, and/or insensitivity to sugar, paclobutrazol, and/or
Figure 26. The etr1 and gal-5 mutants germinate more slowly than wild type on glucose, and det2 germinates at a similar rate to wild type on glucose. However, gal-5 germinates faster on glucose than wild type germinates on glucose and paclobutrazol. Sorbitol (0.1 M and 0.2 M) exerted little if any delay on germination compared to that on minimal media (not shown); hence, the effect of glucose on etr1, det2, and gal-5 germination is sugar-specific. Seeds were sown on plates containing the indicated media and cold-treated at 4°C in darkness for 4 days. Then plates were incubated at 21°C under 90-100 μmol photons m⁻² sec⁻¹ of continuous fluorescent light. Germination was scored daily for 5 days, then at regular intervals until 20 days post-imbibition. Values indicate averages ± SD (n = 3 plates/seed line, 1 seed line/genotype, ~50 seeds/plate). The experiment was repeated once, with similar results. Glc = glucose; Pac = 120 μM paclobutrazol; Col WT, wild-type Columbia.
abscisic acid. All of these mutants have different defects that increase their ability to germinate. Therefore, comparing the rates of germination of these mutants on paclobutrazol and glucose may indicate which phytohormone(s) are affected by paclobutrazol and glucose. In turn, this information may help identify which phytohormone(s), if any, are responsible for the sis2 mutant's ability to germinate on paclobutrazol and other gibberellin and brassinosteroid biosynthesis inhibitors. The mutants examined in this experiment include sis2, sk390, abi3, abi4, aba2, ctrl, and spy-3. (Refer to Table 3 for a description of the sugar and hormone phenotypes of these mutants.) Germination of these mutants was examined on paclobutrazol and glucose. Results show that wild type has the greatest sensitivity to paclobutrazol and glucose, and the sis mutant sk390 is almost as sensitive as wild type (Figure 27). The paclobutrazol-resistant mutants spy-3 and abi3, as well as mutants which demonstrate both paclobutrazol resistance and sugar insensitivity (sis2, abi4, aba2, and ctrl), demonstrate resistance to the combined inhibitory effects of paclobutrazol and glucose on seed germination (Figure 27). Thus, constitutive ethylene and gibberellin responses, and defects in abscisic acid biosynthesis or sensitivity, confer the ability to germinate on paclobutrazol and glucose. This does not identify exactly which phytohormone(s)
Table 3. Summary of phenotypes displayed by mutants defective in ABA, ethylene, gibberellin, or sugar biosynthesis or response. Phenotypes of sis2 and sk390 were determined by Dr. Sue Gibson and Lydia Sommerlad (unpublished results). The aba2 mutant is sugar-insensitive, but the abi3 mutant is not (Arenas-Huerta et al., 2000; Laby et al., 2000). In addition, the abi4 mutant is sugar-insensitive (Arenas-Huerta et al., 2000; Huijser et al., 2000; Laby et al., 2000). The mutants aba2 (Léon-Kloosterziel et al., 1996), abi3 (Nambara et al., 1991; Steber et al., 1998), and abi4 (Laby et al., 2000) germinate on paclobutrazol. The aba2 mutant does not germinate on ABA (Laby et al., 2000), whereas abi3 (Koornneef et al., 1984) and abi4 (Finkelstein, 1994) germinate on ABA. The ctrl mutant is both sugar-insensitive and paclobutrazol resistant (Gibson et al., 2001). The ctrl mutant germinates on ABA (Figure 13; Beaudoin et al., 2000). The spy-3 allele germinates on ABA (Figure 13; Steber et al., 1998; Swain et al., 2001) and paclobutrazol (Figure 6; Jacobsen and Olszewski, 1993) but does not demonstrate a sis phenotype (Figure 11).
Germination after 4 days

Germination after 8 days
Figure 27. Mutants which demonstrate both paclobutrazol resistance and sugar insensitivity (ctrl, sis2, aba2, and abi4), as well as the paclobutrazol-resistant mutants spy-3 and abi3, demonstrate resistance to the inhibitory effects of paclobutrazol and glucose on seed germination. In contrast, the sis mutant sk390 is almost as sensitive as wild type. Seeds were sown on plates containing the indicated media and cold-treated at 4°C in darkness for 2 days. Then plates were incubated at 21°C under 50-75 μmol photons m⁻² sec⁻¹ of continuous fluorescent light. Germination was scored daily for 5 days, then at regular intervals until 18 days post-imbibition. Values for one seed line of each genotype are shown (n = 1 plate/media, ~50 seeds/plate). The experiment was repeated once, with similar results. Pac = μM paclobutrazol, Glc = mM glucose; Col WT, wild-type Columbia.
are responsible for the sis2 mutant's ability to germinate on paclobutrazol and other gibberellin and brassinosteroid biosynthesis inhibitors. However, sugar insensitivity alone does not fully account for the sis2 mutant's ability to germinate on these inhibitors, as sis2 is markedly more resistant than sk390 to the combined inhibitory effects of paclobutrazol and glucose on seed germination.
CONCLUSIONS

As described in the introduction, the sis2 mutant was isolated by Dr. Sue Gibson from a pool of ethyl methane sulfonate (EMS)-mutagenized Arabidopsis thaliana M2 seeds on the basis of its resistance to high levels of exogenous glucose. Previous work shows that unlike wild type, the sis2 mutant is able to form a substantial shoot system on high concentrations of exogenous glucose, sucrose, and sorbitol, and also displays resistance to mannose. Previous work also shows that the sis2 mutant is resistant to the inhibitory effects of paclobutrazol, a gibberellin biosynthesis inhibitor, on germination. This work examines other sugar responses of sis2 and looks at the response of sis2 to all classical phytohormones. Results of this work show that the sis2 mutant is insensitive to the inhibitory effects of glucose on germination, which cannot be accounted for simply by precocious germination. The sis2 mutant may have wild-type endogenous sugar levels, although this is difficult to determine. Interestingly, the sis2 mutant demonstrates novel phytohormone responses. The sis2 mutant is resistant to the inhibitory effects of gibberellin and brassinosteroid biosynthesis inhibitors during germination, but shows little to no resistance to these inhibitors during later developmental stages. The sis2 mutant
is also resistant to the combined negative effects of glucose and paclobutrazol on germination. These results suggest a novel connection between gibberellin and sugar response pathways.

**Why the sis2 mutant demonstrates resistance to gibberellin and brassinosteroid biosynthesis inhibitors primarily during germination**

The next few paragraphs will attempt to answer why the sis2 mutant demonstrates resistance to gibberellin and brassinosteroid biosynthesis inhibitors primarily during germination, with little to no resistance to these inhibitors during later developmental stages.

As described in the introduction, abscisic acid-deficient (aba), ethylene-overproducing (eto1), ethylene constitutive response (ctr1), and some abscisic acid-insensitive (abi) mutants germinate on the gibberellin biosynthesis inhibitors paclobutrazol or uniconazol (Gibson et al., 2001; Laby et al., 2000; Léon-Kloosterziel et al., 1996; Nambara et al., 1991). In addition, aba mutants, eto1, ctr1, and some abi mutants display sugar insensitivity (Arenas-Huertero et al., 2000; Gibson et al., 2001; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001; Zhou et al., 1998). As the sis2 mutant displays sugar insensitivity and germinates on paclobutrazol,
the responses of *sis2* to ethylene and abscisic acid (ABA) were examined to determine whether *sis2* might be allelic to one of the above mutants. Results indicate that *sis2* is probably not an *eto* or *ctr* mutant because it demonstrates wild-type hypocotyl elongation responses on the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (Figure 7). The *sis2* mutant is probably not an *abi* mutant, as it demonstrates only slight resistance to ABA during germination (Figure 13). The *sis2* mutant is not *aba* during adult developmental stages, as it does not demonstrate a wilty phenotype. It remains possible that *sis2* has a unique *aba* mutation that causes ABA biosynthesis defects apparent only during germination. Measurement of ABA levels in germinating *sis2* seeds will be necessary in the future to test this possibility. There is precedent for the idea that a mutant may display an altered phenotype only during germination: the ABA-insensitive mutant *abi4* demonstrates resistance to ABA during germination, but wild-type responses to ABA during later developmental stages (Finkelstein, 1994).

Alternatively, *sis2* might be affected in gibberellin response, as paclobutrazol reportedly inhibits gibberellin biosynthesis. However, *sis2* shows resistance to paclobutrazol primarily during germination,
unlike the constitutive gibberellin response mutant *spindly-3 (spy-3)* which shows resistance to paclobutrazol during all developmental stages examined (Figures 6, 15, 16, 17, 18; Jacobsen and Olszewski, 1993). Thus, *sis2* is unlikely to be a constitutive gibberellin response mutant, unless the *sis2* mutation causes an altered phenotype only during germination.

The lack of specificity of cytochrome p450 monooxygenase inhibitors makes it difficult to determine the phytohormone(s) responsible for the *sis2* mutant's ability to germinate on the three inhibitors tested (paclobutrazol, ancymidol, and brassinazole). Scientists claim that ancymidol and uniconazol specifically inhibit gibberellin biosynthesis (Asami et al., 2000; Norman et al., 1986), and that brassinazole, a recently developed brassinosteroid biosynthesis inhibitor, has effects distinct from uniconazol (Asami et al., 2000). However, this thesis provides evidence that these inhibitors are not highly specific. For example, exogenous brassinosteroids partially alleviate the inhibitory effects of paclobutrazol and ancymidol, and exogenous gibberellin partially alleviates the inhibitory effects of brassinazole during hypocotyl elongation (Figure 16). Furthermore, the gibberellin constitutive response mutant *spindly-3 (spy-3)* is resistant to brassinazole
(Figures 15, 16). This may be partly explained by the observation that both brassinosteroids and gibberellin promote hypocotyl elongation (reviewed in Arteca, 1996; Li and Chory, 1999), so they may compensate for each other, even if they act independently. Their ability to compensate for one another makes it difficult to determine whether gibberellin or brassinosteroideal responses are affected in the sis2 mutant.

In any case, no matter which response pathway(s) confer the sis2 mutant's paclobutrazol resistance during germination, the dual sugar-insensitive and paclobutrazol-resistant phenotypes of the sis2 mutant suggest the existence of connections between the signaling/response pathways involved (sugar response and gibberellin? response). The two pathways are unlikely to be completely linear, some mutants exhibit sugar insensitivity but wild-type response to paclobutrazol, such as sis3 (Donna Pattison, unpublished results), and conversely, the spy-3 mutant exhibits paclobutrazol resistance (Jacobsen and Olszewski, 1993) but wild-type sugar sensitivity (Lydia Sommerlad, unpublished results). A model of one possible connection between sugar and gibberellin responses, based on these results, is shown in Figure 28.
Figure 28. A model for the connections between sugar response and gibberellin response. The sis2 mutant exhibits both sugar-insensitive and paclobutrazol-resistant phenotypes. The spy-3 mutation confers paclobutrazol resistance (Figure 6) but wild-type sugar sensitivity (Figure 11). The sis3 mutation confers sugar insensitivity but wild-type paclobutrazol sensitivity (Donna Pattison, unpublished results). Courtesy of Dr. Sue Gibson.
Why ctri germinates on ABA

As ethylene, gibberellin, and brassinosteroids promote germination, one possible model is that they compensate for one another, even if acting independently in parallel. If one phytohormone is absent or reduced, constitutive signaling or overproduction of another may allow normal or above-normal germination. For example, exogenous brassinosteroids promote germination in the gibberellin-insensitive mutant sleepy which otherwise cannot germinate unless its seed coat is cut (Steber and McCourt, 2000). A related scenario is that when abscisic acid, which inhibits germination, is absent, gibberellin is not required for germination (Koornneef et al., 1982). An alternative explanation of these observations is that the signaling pathways of these hormones interact, rather than act independently on the same process. Epistatic analysis between ABA-insensitive and ethylene-insensitive mutations suggests that ethylene promotes germination by decreasing sensitivity to endogenous ABA (Beaudoin et al., 2000) and may prevent ABA-induced seed dormancy (Ghassemian et al., 2000). On the signaling level, it suggests that ethylene signaling inhibits ABA signaling in seeds (Beaudoin et al., 2000). Either interpretation — independent compensation for one another, or direct interaction between signaling pathways — could account for
the observation that the *ctr1* mutant germinates on ABA (Figure 13). These interpretations may also help explain why the *spy-3* mutant is slightly resistant to ABA during germination (Figure 13).

**Why *etrl* and *gal-5* germinate more slowly than wild type on glucose**

Figure 26 shows that the mutants *etrl* and *gal-5* germinate more slowly than wild type on glucose, whereas *det2* retains wild-type sensitivity to sugars during germination. However, *gal-5* also germinates more slowly than wild type even without glucose. In contrast, *det2* and *etrl* demonstrate wild-type germination without glucose. An explanation of these observations is provided as follows.

As the *etrl* mutant demonstrates super-sensitivity to glucose and sucrose during early seedling development (Gibson et al., 2001; Zhou et al., 1998), it is not surprising that *etrl* demonstrates super-sensitivity to glucose during germination. Conversely, evidence shows that exogenous ethylene, constitutive ethylene response mutations (*ctrl*), and ethylene overproduction mutations (*etol*) confer resistance to the inhibitory effects of high exogenous sugar levels on early seedling development (Gibson et al., 2001; Zhou et al., 1998). Perhaps exogenous ethylene and the *ctrl* and *etol*
mutations confer resistance to glucose during germination.

Preliminary results indicate that this is true for the ctrl mutant
(Kelly Biddle and Dr. Sue Gibson, unpublished results).

The brassinosteroid mutants isolated to date, including det2,
demonstrate unaltered germination phenotypes (Steber and
McCourt, 2000). None of the brassinosteroid biosynthetic mutants
characterized to date exhibit altered responses to sugars.
Furthermore, exogenous brassinosteroids do not confer a sis
phenotype (Figure 11). Therefore, it is not surprising that det2
demonstrates wild-type sensitivity to glucose during germination
(Figure 26).

The gal-5 mutant germinates more slowly than wild type with
or without glucose (Figure 26), due to its defect in gibberellin
biosynthesis. (Tight gal alleles completely prevent germination in
the absence of exogenous gibberellin.) This may explain why gal-5
is less able to withstand the inhibitory effect of glucose on
germination. Another aspect of this experiment is the addition of
paclobutrazol to wild type, which was intended to mimic the gal-5
mutation. However, results demonstrate that the gal-5 mutant
germinates slower on minimal media than wild type on
paclobutrazol, but faster on glucose than wild type on glucose and
paclobutrazol (Figure 26). Perhaps the concentration of
paclobutrazol added did not reduce gibberellin levels in wild type as low as in the \textit{gal-5} mutant. If true, this presents difficulty in explaining why paclobutrazol and glucose have a stronger effect on wild type than glucose has on the \textit{gal-5} mutant.

\textbf{Why glucose and paclobutrazol have opposite effects from glucose and ABA}

At first glance, the observation that glucose rescues germination on abscisic acid (ABA) but worsens the inhibitory effect of paclobutrazol on germination seems inconsistent with what we know about the effects of ABA, paclobutrazol, and glucose on seed germination. However, understanding the complexity behind these interactions may help to resolve the discrepancy. Germination is not just a single event; it is a highly complex process, comprised of many different steps, some of which occur sequentially and depend on the outcome of previous step(s). Many environmental and chemical factors, including light, chilling treatment, sugar, gibberellin (GA), abscisic acid (ABA), and ethylene play roles in germination. Some factors involved during germination may interact with each other during one stage of germination, but in another stage, they may not interact at all. Other factors may have different roles depending on the tissue type. For example, cereal
grains (seeds) have multiple α-amylase genes, and each gene is regulated differently by sugars, gibberellin, and/or ABA, in a tissue-specific manner (Perata et al., 1997; reviewed in Thomas and Rodriguez, 1994). Gibberellin promotes α-amylase expression in both the aleurone (surrounding the endosperm) and scutellar (surrounding the embryo) epithelial layers of germinating cereal grains (Perata et al., 1997). ABA represses α-amylase expression both in the aleurone and the scutellar epithelium, and this repression is reversible upon the addition of GA (Perata et al., 1997). Glucose inhibits GA-induced α-amylase expression downstream of the gibberellin signal, but in the scutellar epithelium only. Glucose does not affect GA-induced α-amylase expression in the aleurone (Perata et al., 1997). Furthermore, this glucose repression of α-amylase cannot be counteracted by additional GA (Perata et al., 1997). This implies that glucose and ABA repress α-amylase expression independently (Perata et al., 1997). Thus, glucose, GA, and ABA may interact (or not interact) in different tissues at different times and in different ways. Therefore, it is not unreasonable to expect that glucose may act differently in combination with paclobutrazol (a GA biosynthesis inhibitor) than
with ABA during germination, depending on the different steps and tissues involved.

In closing, sugars play an important role in many plant developmental processes, including germination and early shoot development (this work; Gibson et al., 2001; Laby et al., 2000). The *sis2* mutant is resistant to the effects of sugars on these two processes, and to the effects of the paclobutrazol and ancymidol (gibberellin biosynthesis inhibitors) and brassinazole (a brassinosteroids biosynthesis inhibitor) primarily during germination. Furthermore, the *sis2* mutant is resistant to the combined negative effects of glucose and paclobutrazol on seed germination. These results suggest a novel connection between phytohormone and sugar response pathways. The *sis2* mutant provides a useful genetic tool for elucidating the ways in which sugar and phytohormone response pathways interact to control germination.

Immediate future work may include cloning the *SIS2* gene and analyzing its expression patterns. Expression patterns may vary depending on the treatment (different sugars, sorbitol, sugar analogs, and/or phytohormone biosynthesis inhibitors), the concentration of chemical(s) added, and the developmental stage at
which treatment is applied. As more genes responsible for sugar-
sensing/response mutations are cloned, long-term future work may
involve determining at the protein level which sugar-
sensing/response factors interact with each other. Since
connections have been postulated between sugar and phytohormone
sensing/response pathways, some components of the sugar signal
transduction pathway may interact with certain components of
phytohormone signaling pathways. Eventually, more of the
interactions belonging to the highly complex "web" of sugar and
phytohormone signaling may be understood.
APPENDIX I.

Recombination mapping of $sk591$

As described under "Previous work on the sis2 mutant", a map-based positional cloning approach is being used to identify $SIS2$, as the sis2 mutation is most likely a point mutation created by ethyl methane sulfonate (EMS) mutagenesis. The $SIS2$ gene maps to the bottom of chromosome 1, 11.4 cM away from the marker nga111 (Dr. Donggiun Kim, unpublished results). A related mapping project performed for this thesis, mapping $sk591$, is described in the following paragraphs.

This appendix describes the mapping of a potential $SIS2$ allele, $SK591$. The $sk591$ mutant is thought to be allelic to sis2 because both mutants have dual sugar-insensitive and paclobutrazol-resistant phenotypes (Dr. Sue Gibson and Lydia Sommerlad, unpublished results). In addition, the responses of $sk591$ and sis2 to all other major phytohormones and sugars are virtually identical (Lydia Sommerlad, unpublished results). Like sis2, $sk591$ was isolated from a pool of EMS-mutagenized M2 seeds on the basis of its sugar-insensitive (sis) phenotype (by Sean Kincaid, a former undergraduate in the Gibson lab). As $sk591$ and sis2 were isolated
from independent M2 pools, their mutations are most likely independent.

The *sk591* mutation was mapped during this work because *sk591* has a stronger mutant phenotype than *sis2*. A stronger mutant phenotype allows more stringent selection of homozygous mutants from the F2 seed pool in a *sis* screen, resulting in less contamination of the mapping population by escapees. "Escapees" refer to wild-type seeds that show a *sis* phenotype on high sugar (usually 1-5% of wild-type var. Columbia seeds escape selection), and to heterozygous seedlings, which may show a partial *sis* phenotype.

To create the *sk591* mapping population, crosses were performed between the homozygous *sk591* mutant (var. Columbia, abbreviated Col) and wild type of two different ecotypes. Two *sk591* (Col) x wild-type (Wassilewskija) crosses and two *sk591* (Col) x wild-type (Hi-O) crosses were performed. F1 seeds resulting from each cross were collected separately. Twenty-three F1 seeds were planted and grown to maturity. F2 seeds from each F1 plant were collected separately, yielding twenty-three F2 seed lines. Approximately one hundred seeds from each F2 seed line were screened on 0.3 M sucrose for a *sis* phenotype. 372 F2 seedlings which exhibited expanded cotyledons and true leaves after two
weeks of growth on 0.3 M sucrose were chosen for the mapping population. These seedlings were transplanted to soil and grown to maturity. F3 seeds were collected from individual F2 plants, and one hundred seeds from each F3 seed line were re-screened on 0.3 M sucrose for a *sis* phenotype. If a majority of F3 seeds from an individual F2 plant showed a *sis* phenotype, the F2 plant was considered homozygous for the *sk591* mutation. If the majority of the F3 seeds from an individual F2 plant did not show a *sis* phenotype, that F2 plant was deemed an escapee. The crosses and the selection of the F2 mapping population were performed by Dr. Sue Gibson. Re-screening of F3 seeds was performed by Daniel Verduzco.

Mapping the *sk591* mutation was performed using SSLP (Simple Sequence Length Polymorphism) and CAPS (Cleaved Amplified Polymorphic Sequence) markers. Loci near the top, middle, and bottom of each of the five chromosomes in the Arabidopsis genome (a total of sixteen loci) were examined for linkage to *sk591* (by Daniel Verduzco). Only the loci near the bottom of the chromosome 1 showed linkage to the *sk591* mutation. This finding rules out the possibility that an ecotype effect unlinked to the *sk591* mutation was selected for during the *sis* screen. Ecotype effects (phenotypic differences between
ecotypes) between the Columbia and Landsberg *erecta* ecotypes have been identified (Laby et al., 2001), so it is possible that there are ecotype effects between Columbia and other non-similar ecotypes such as Wassilewskija. Creating a mapping population from crosses of *sk591* (Col) with two different ecotypes helps to avert the problem of mapping an ecotype effect rather than the *sk591* mutation. This technique works if Col/Wassilewskija and Col/Hi-O do not have ecotype effects at the exact same locus.

Current mapping data indicates that the *sk591* mutation lies between the PAB5 and nga111 markers — these markers are 6 cM apart (Daniel Verduzco and Lydia Sommerlad, unpublished results). As both *sk591* and *sis2* map near the nga111 marker, this further supports the hypothesis that *sk591* and *sis2* might be allelic.

The best approach to determine whether *sk591* and *sis2* are allelic is to clone the *SK591* gene, then to amplify and sequence this same gene from the *sis2* mutant to test whether a mutation occurred in this gene in *sis2* as well. If *sk591* and *sis2* have mutations in the same gene, two conclusions can be drawn. First, the fact that two different, independently-isolated mutants with similar phenotypes have mutations in the same gene indicates, with a high degree of certainty, that the mutation in that gene is responsible for the mutants' sugar-insensitive and paclobutrazol-
resistant phenotypes. Secondly, it confirms that \textit{sis2} and \textit{sk591} are allelic.

As the gene(s) where the \textit{sk591} and \textit{sis2} mutations occur have not yet been determined, it has not yet been determined conclusively whether \textit{sis2} and \textit{sk591} are allelic.
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