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Mannose Inhibits Arabidopsis Germination via a Hexokinase-Mediated Step[1]

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Low concentrations of the glucose (Glc) analog mannose (Man) inhibit germination of Arabidopsis seeds. Man is phosphorylated by hexokinase (HXK), but the absence of germination was not due to ATP or phosphate depletion. The addition of metabolizable sugars reversed the Man-mediated inhibition of germination. Carbohydrate-mediated regulation of gene expression involving a HXK-mediated pathway is known to be activated by Glc, Man, and other monosaccharides. Therefore, we investigated whether Man blocks germination through this system. By testing other Glc analogs, we found that 2-deoxyglucose, which, like Man, is phosphorylated by HXK, also blocked germination; no inhibition was observed with 6-deoxyglucose or 3-*O*-methylglucose, which are not substrates for HXK. Since these latter two sugars are taken up at a rate similar to that of Man, uptake is unlikely to be involved in the inhibition of germination. Furthermore, we show that mannoheptulose, a specific HXK inhibitor, restores germination of seeds grown in the presence of Man. We conclude that HXK is involved in the Man-mediated repression of germination of Arabidopsis seeds, possibly via energy depletion.

Among the many regulatory systems and signals in plants, carbon-metabolite-mediated gene regulation has been receiving growing attention in the past few years (for reviews, see Farrar, 1991; Sheen, 1994; Koch, 1996; Jang and Sheen, 1997; Smeekens and Rook, 1997). Many plant genes are controlled by sugars and are involved in such diverse processes as photosynthesis, storage protein accumulation, and starch, lipid, and nitrogen metabolism (Hattori et al., 1990, 1991; Nakamura et al., 1991; Karrer and Rodriguez, 1992; Krapp et al., 1993; McLaughlin and Smith, 1994; Chevalier et al., 1996). In general, when sugar concentrations in the plant increase, there is repression of the genes involved in photosynthesis and in the mobilization of starch, lipid, and protein storage reserves. At the same time, genes required for storing carbon metabolites for future use are induced. There are numerous examples of genes whose expression is regulated by sugars, and carbon-metabolite-mediated regulation of gene expression appears to be a central and fundamental mechanism common to all higher plants (Koch, 1996).

Whereas the understanding of sugar regulation of gene expression in plants is still in its early stages, a considerable amount of information is available from research in bacteria (Magasanik, 1961; Ullmann, 1985; Saier, 1989) and yeast (Gancedo, 1992; Johnston and Carlson, 1992; Trumbly, 1992; Thevelein, 1994; Ronne, 1995). In yeast the repression of Glc-repressible genes is triggered by HXK, the enzyme that acts first in glycolysis by phosphorylating Glc to Glc-6-P, originating a signal that is perceived by the GLC7 complex (Tu and Carlson, 1995). This activates the downstream SSN6/TUP1 complex, which, by binding to the transcription factor MIG1, modulates the chromatin structure, thus repressing gene expression. However, the connection between these components is unknown. In the absence of Glc a pathway involving several protein complexes, including the SNF2-containing complex, reverses the SSN6/TUP1-mediated block of gene expression so that genes can be transcribed. An important question that remains is how HXK triggers the signaling cascade. Neither Glc-6-P nor other downstream glycolytic intermediates are capable of triggering this repression. The HXK-dependent pathway is clearly of great importance and seems to be at least partially conserved in eukaryotes. HXK has been implicated in sugar-mediated gene regulation in yeast (Thevelein, 1994), animals (Vaulont and Kahn, 1994), and plants (Jang and Sheen, 1997).

In plants HXK activity has long been known as an important regulator of glycolysis, but recently several lines of evidence have suggested that it also has a sensor function in the sugar signal transduction pathway (Graham et al., 1994; Jang and Sheen, 1994; Jang et al., 1997). This points to possible similarities with the yeast and animal systems, in which HXK and glucokinase have an identical function (Entian and Fröhlich, 1984; Pilkins et al., 1994; Heimberg et al., 1996). By analogy with the yeast system, and taking into account the large number and different functions of genes regulated by sugars, there are probably several interacting, highly complex sugar-signaling pathways in plants. To determine the molecular components of these pathways it became evident that a genetic approach would be of great interest.

We developed a screening strategy for the isolation of sugar-sensing mutants, and isolated a number of *sun* (Suc uncoupled) mutants (Dijkwel et al., 1996, 1997; Smeekens and Rook, 1997; Van Oosten et al., 1997). During the char-

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Abbreviation: HXK, hexokinase.

acterization of these mutants, germination on several different sugars was tested. Unexpectedly, low concentrations of Man, a Glc analog that can also be phosphorylated by HXK, blocked germination of Arabidopsis seeds. Man has recently been shown to be capable of specifically repressing several plant genes via the HXK pathway (Graham et al., 1994; Jang and Sheen, 1994) with greater efficiency than Glc. Here we provide evidence that Man represses germination through this HXK pathway.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Columbia (glabrous) ecotype of Arabidopsis (Lehle Seeds, Round Rock, TX) was used in all experiments except those involving *sun* mutants, which were isolated in a C24 ecotype background. The corresponding ecotypes were used as controls in the experiments described here. Seeds were surface-sterilized for 12 min in 20% commercial bleach, and rinsed four times with sterile, ultrapure water (Milli-Q, Millipore). Seeds were then sown onto sterile Murashige and Skoog (1962) medium containing vitamins (Duchefa, Haarlem, The Netherlands), and solidified with 0.7% plant agar (Duchefa). The different sugars and metabolites were added to this medium as indicated below. Sowing was carried out in a small volume of 0.1% agarose that was allowed to dry. Plates were placed at 4°C in the dark for 2 d to promote germination, and were then transferred to 22°C and a 16-h/8-h light/dark cycle at d 0.

Germination Assays

All measurements of germination frequencies were obtained at d 8 unless stated otherwise. In the absence of a universal definition, in this paper we define germination as the emergence of 1 mm or more of the radicle from the seed coat.

ATP Measurements

Approximately 50 seeds or seedlings were harvested from the agar plates and immediately frozen and ground in liquid nitrogen. The samples were then centrifuged for 5 min at 14,000 rpm in microtubes. One-hundred microliters of the supernatant was added to 100 μL of 25-times-diluted ATP assay mix solution from a bioluminescent assay kit (Sigma). Light emission was immediately measured 3 times for 10 s each in a luminometer (model 1253, Bio-Orbit, Turku, Finland), and the average value was taken. Protein quantification was performed according to the method of Bradford (1976), using 100 μL of sample and 1 mL of Bradford reagent, and allowing the reaction to proceed for 15 min.

RESULTS

Man Represses Germination of Arabidopsis Seeds

Growth of Arabidopsis seeds on several different sugars was tested. It was found that Man, a Glc epimer at the second carbon atom, repressed germination in a concentration-dependent manner (Fig. 1). In this and subsequent experiments, the addition of increasing concentrations of Man to the agar medium led to a decrease in the percentage of seeds that germinated. In the absence of sugars in the medium the germination frequency was nearly 100%. However, even with a concentration as low as 7.5 mM, germination was virtually abolished by Man. At lower concentrations the seeds germinated but growth was halted at an early stage. This effect was shown not to be osmotic, since germination and growth were normal when 15 mM mannitol or sorbitol was substituted for Man. The addition of similar concentrations of Glc to the medium also did not affect germination frequencies. Like other metabolizable sugars, Glc induces increased growth of Arabidopsis seedlings (Rook et al., 1998).

Phosphate and ATP Levels and Repression of Germination

Upon entry into the plant cell, Man is phosphorylated by HXK, yielding Man-6-P, a process that requires ATP. Man-6-P is then only slowly processed by the plant, since the enzymes required for this are either absent or exist in very low concentrations (Sheu-Hwa et al., 1975; Walker and Sivak, 1986). Due to this property, Man has been used in the past to provoke ATP and phosphate depletion in adult leaves of some plant species (Siegl and Stitt, 1990; Van Quy and Champigny, 1992). The Man concentrations used in these studies (50–200 mM) were much higher than the ones we used (7.5 mM). We carried out a series of experiments to determine whether the repression of germination by Man could be due to phosphate or ATP depletion.

Since phosphate is readily taken up from the medium into plant tissues, even at concentrations as low as 0.01 μM (Russel and Martin, 1953; Barber and Loughman, 1967; Bieleski, 1973; Lin and Hanson, 1974; Lin, 1979), we added up to 75 mM phosphate to medium containing 7.5 mM Man, and found that the repression of germination remained unaltered. This was attempted with combinations of various phosphate concentrations, different phosphate salts, and at different pH values, with consistent results (data not

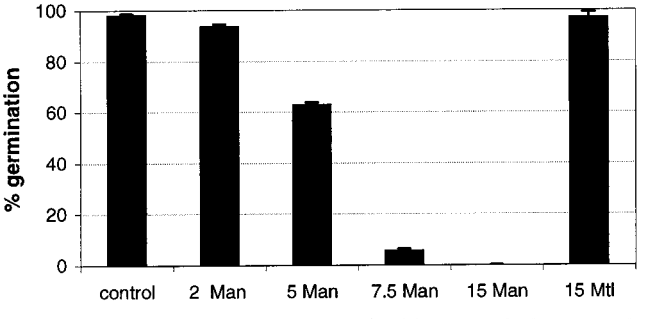


Figure 1. Man represses germination of wild-type Arabidopsis seeds in a concentration-dependent manner. Seeds were plated in the absence of sugar (control) and on 2, 5, 7.5, and 15 mM Man. Fifteen millimolar mannitol (15 Mtl) was taken as an osmotic control. Approximately 200 seeds were used for each data point in each experiment. Values presented are the average of three independent experiments. Germination was scored at d 8.

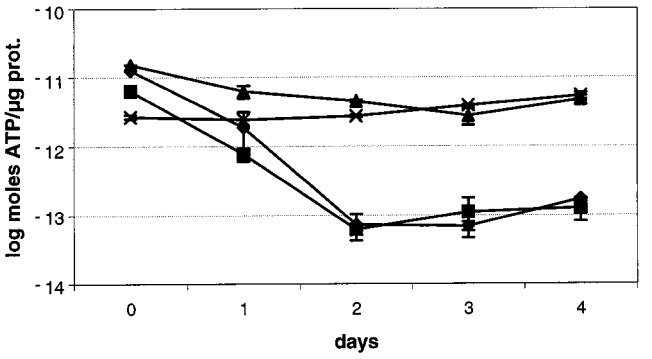


Figure 2. Man does not deplete ATP in wild-type Arabidopsis seeds. Seeds were plated out at d −2 in the absence (control, ♦) or in the presence of 0.5 (■), 7.5 (▲), or 50 (×) mM Man. After being submitted to a 48-h cold treatment at 4°C to promote germination, they were placed at 22°C at d 0. Measurements were taken at 24-h intervals to determine the ATP and protein levels in the seedlings. Approximately 50 seeds or seedlings were used for each measurement per experiment. prot., Protein.

shown). Possible ATP depletion was tested by directly measuring ATP levels. Seeds were plated out both in the absence and in the presence of 0.5, 7.5, and 50 mM Man. The lowest concentration of Man (0.5 mM) still allowed germination, but 7.5 and 50 mM repressed it entirely. A concentration of 50 mM Man has previously been used to provoke ATP depletion in detached adult wheat leaves (Van Quy and Champigny, 1992). The ATP levels were then measured at 24-h intervals for several days (Fig. 2).

Upon germination in the absence of sugars, ATP levels expressed on a protein basis decreased 100- to 1000-fold, but if germination was blocked by Man, ATP levels did not change. When germination was repressed, the overall protein content measured in the soluble fraction was lower than when germination occurred. This explains why the [ATP]/μg protein ratio shown in Figure 2 decreased in germinated seedlings (control and 0.5 mM Man) and should not be directly compared with that of the samples that did not germinate (7.5 and 50 mM Man). We conclude that at the seed stage the presence of Man in the external medium did not greatly affect the internal ATP concentration, since the ATP levels in seeds treated with 7.5 or 50 mM Man were not greatly affected. This suggests that it was not due to a lack of ATP that germination was blocked. At d 0 and 1, before germination had occurred, the ATP levels were in the same range in all seeds, showing that there was sufficient ATP to allow germination, even in the seeds on 50 mM Man. This was observed in several independent experiments.

To further investigate a possible effect of Man on ATP or phosphate levels, seeds were plated out on Murashige and Skoog medium containing 7.5 mM Man and 75 mM phosphate. In the absence of Man, germination took place on d 2. In the presence of Man, germination still had not occurred by d 4. The nongerminated seeds were briefly rinsed with sterile water and transferred to Man-free, 75 mM phosphate Murashige and Skoog medium. If phosphate or ATP depletion were preventing germination we would not expect seeds to germinate upon transfer. If, on the contrary, phosphate and ATP levels remained sufficiently high, and if Man were repressing germination via another mechanism, germination upon transfer would be expected. Upon transfer the seeds did germinate, although with signs of stress, as indicated by elevated anthocyanin levels (not shown). These results suggest that in the presence of Man there was sufficient ATP and phosphate in the seeds to allow germination.

Further evidence for this came from the observation that the addition of Glc to Man-containing medium (Fig. 3) was capable of restoring germination. This effect was already visible when 2.5 mM Glc was added together with 7.5 mM Man, and became stronger with increasing Glc/Man concentration ratios. Identical results were obtained when Glc was substituted for other metabolizable sugars such as Suc and Fru (data not shown). Seeds did not germinate on medium containing 7.5 mM Man with 60 mM mannitol, showing that this effect is specific for Glc.

Glc Analogs and Germination

After testing several Glc analogs, we found that 5 mM 2-deoxyglucose also repressed Arabidopsis germination. The addition of Glc (30 mM) was capable of fully reversing this effect, as it had been with Man (not shown). In addition to Man and 2-deoxyglucose, two other Glc analogs were tested for their effect on the germination of Arabidopsis seeds. Whereas 10 mM Man or 2-deoxyglucose repressed germination, the same concentration of 6-deoxyglucose, 3-*O*-methylglucose, Fru, or Glc did not (Fig. 4). All of these Glc analogs are taken up by the plant (Lin et al., 1984; Komor et al., 1985), but 6-deoxyglucose and 3-*O*-methylglucose cannot be phosphorylated by HXK. Man and 2-deoxyglucose are phosphorylated by this enzyme into Man-6-P and 2-deoxyglucose-6-P, respectively (Sols et al., 1958; Dixon and Webb, 1979), in the same manner in which HXK normally converts Glc to Glc-6-P. However, whereas Glc-6-P is further processed by the cell, yielding

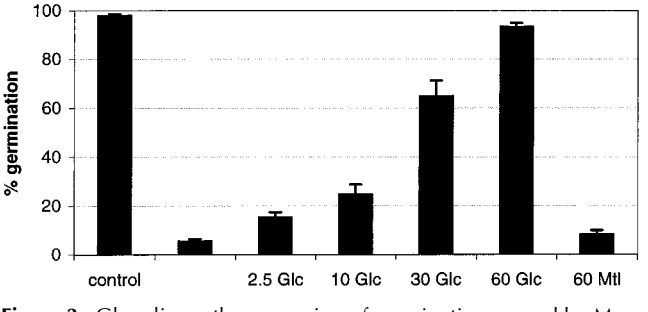


Figure 3. Glc relieves the repression of germination caused by Man. All values are expressed as millimolar concentrations. In lane 1, seeds were germinated in the absence of any sugar (control), whereas in the remaining lanes 7.5 mM Man was present. Lanes 2 to 6, Germination was gradually restored with the addition of increasing Glc concentrations. In lane 7, 60 mM mannitol (60 Mtl) was added together with Man as an osmotic control. Germination was scored at d 8, and approximately 200 wild-type seeds were taken for each data point. The results represent the average of three independent experiments.

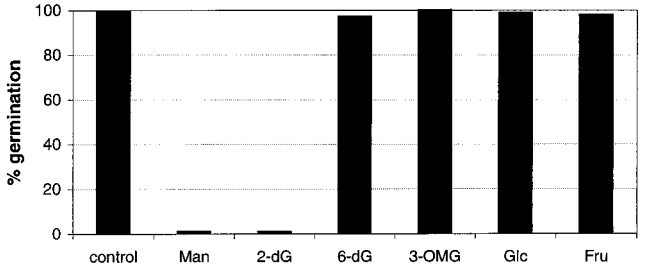


Figure 4. Effect of Glc analogs on germination. Approximately 200 wild-type seeds were plated on media containing Man, 2-deoxyglucose (2-dG), 6-deoxyglucose (6-dG), 3-*O*-methylglucose (3-OMG), Fru, or Glc in 10 mM concentrations. In lane 1, seeds were sown in the absence of external sugars (control).

energy and serving as a carbon source, Man-6-P and 2-deoxyglucose-6-P do not enter glycolysis at a significant rate (Egyud and Whelan, 1963; Bessel and Thomas, 1973; Herold and Lewis, 1977; Harris et al., 1986). This difference in the effects on germination between these two classes of Glc analogs suggested the possible involvement of HXK, but not Glc transporters, in the repression of germination by Man.

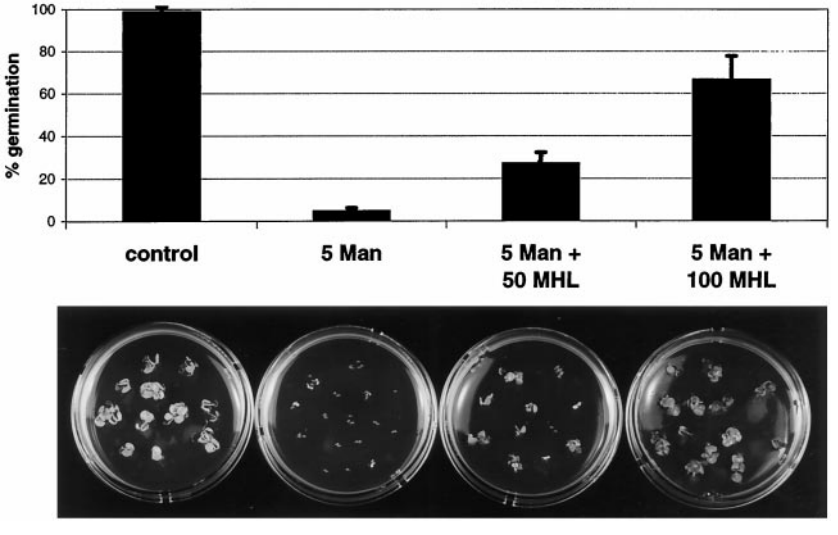
Mannoheptulose Inhibits the Effect of Man on Germination

Mannoheptulose is a specific, competitive HXK inhibitor (Sols et al., 1958; Coore and Randle, 1963; Salas et al., 1965) and is not metabolized by the plant cell. We confirmed this by plating seeds on a medium containing 100 mM mannoheptulose and allowing them to grow. The external addition of metabolizable sugars is known to lead to increased growth rate, greater root elongation, and increased starch and anthocyanin accumulation (Rook et al., 1998). However, in the present study, seedlings grown in the presence of 100 mM mannoheptulose remained similar to those grown in the absence of external sugars. Furthermore, we observed no effect of 100 mM mannoheptulose on germination frequencies of Arabidopsis seeds. If HXK was involved in carbohydrate-mediated repression of germination then this repression should be relieved by inhibiting this enzyme. For this purpose we plated seeds on 5 mM Man-containing medium in the absence and in the presence of 50 and 100 mM mannoheptulose (Fig. 5), and found that this HXK inhibitor did overcome the effect of Man on germination in a concentration-dependent manner. A 10- to 20-fold molar excess of mannoheptulose was required for this effect, suggesting that mannoheptulose may have lower affinity for HXK than Man, as was observed for yeast HXK (Sols et al., 1958). These results suggest the involvement of HXK in signaling Man-induced repression of germination.

Germination of *sun6* on Man

In the *sun6* mutant 3% Suc is not capable of repressing the gene for plastocyanin and several other genes, as it is in the wild-type plant. Although Suc is a disaccharide, it can readily be converted to Glc and Fru, both of which are HXK substrates. Van Oosten et al. (1997) showed that in *sun6* photosynthesis is no longer repressed by the Glc analog 2-deoxyglucose, and that *sun6* seedling development is insensitive to high Glc concentrations that arrest development in wild-type seedlings. They proposed that the *sun6* mutation affects a process involved in the HXK-mediated signal transduction pathway. If this were the case, and if Man inhibits germination via HXK, one would expect *sun6* to germinate on Man-containing medium. Therefore, we plated seeds in the presence of 7.5, 10, and 15 mM Man and measured the germination frequencies at d 8. A greater insensitivity to Man was observed for *sun6* in the entire range of concentrations tested but was most clear with 7.5 mM Man (Fig. 6). *sun6* displayed a percentage of germination several times higher than that of the wild type, suggesting that Man blocks germination via a HXK-mediated pathway, as our previous work indicated.

Figure 5. Mannoheptulose (MHL), a specific HXK inhibitor, is capable of restoring germination to Man-repressed wild-type seeds. Seeds were plated in the absence of sugars (control) and on 5 mM Man to which 50 and 100 mM mannoheptulose was added. Approximately 150 seeds were used for each data point and germination was scored at d 8.



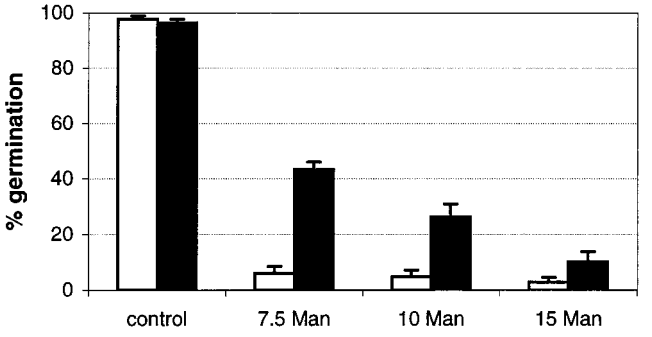


Figure 6. Germination frequency of *sun6* (black bars) and wild type (white bars) in the absence of external sugars (control) and on 7.5, 10, and 15 mM Man-containing medium. Approximately 80 seeds per line were used in each experiment and results represent the average of three independent experiments.

DISCUSSION

Carbon metabolite levels directly and indirectly influence virtually every metabolic process in the life of a plant. Much has been published about how metabolites regulate a given process at the enzyme level, but far less is known about how this regulation occurs at the gene-expression level. Carbon-metabolite-mediated regulation of gene expression seems to be of fundamental importance for plant functioning. In yeast, plant, and animal systems an important role for HXK has been proposed in sensing and signaling of the sugar status. Exactly how HXK transmits this signal to downstream elements in the pathway is unknown.

We have shown that the HXK substrate Man represses germination of Arabidopsis seeds at concentrations as low as 5 mM in the growth medium. All metabolizable sugars tested were able to reverse this effect. This was also true for seeds whose germination had been inhibited by the Glc analog 2-deoxyglucose (data not shown). Although monosaccharides such as Glc and Fru can also be phosphorylated by HXK and should have an effect similar to that of Man or 2-deoxyglucose on gene expression, they can be further metabolized and used as energy and carbon sources. With Man and 2-deoxyglucose this occurs to a much lesser extent, so we propose that this is why germination is restored by metabolizable sugars. 6-Deoxyglucose and 3-*O*-methylglucose, two other Glc analogs, were taken up by the plant with the same efficiency as Glc, but unlike Glc, Man, or 2-deoxyglucose, they could not be phosphorylated by HXK. The finding that these HXK-nonphosphorylatable Glc analogs did not repress germination shows that it is not via sugar uptake that Man represses germination. Furthermore, it suggests that repression occurs via a HXK-mediated pathway.

In the presence of 7.5 mM Man, both ATP and phosphate were present in sufficient amounts to allow germination. Although Man has been reported to provoke ATP and phosphate depletion in detached leaves of adult plants, in our system we did not observe any significant reduction in seed ATP levels at the time of germination. This may be explained by the significantly higher Man concentrations that are used for phosphate and ATP depletion. In addition, to our knowledge, phosphate and ATP depletion have never been measured previously in Arabidopsis (or in any other plant at the seed stage). By plating seeds on 7.5 mM Man and transferring them to Man-free medium at d 4, we were able to show that they remained fully viable and capable of germinating.

Phosphate has previously been implicated in metabolite-mediated gene regulation (Sadka et al., 1994; Takeda et al., 1994; Berger et al., 1995), but this was not supported by our results. Several different phosphate salts at various concentrations and pH values were tested, but even when 75 mM phosphate was added to the medium, the effect of 7.5 mM Man was not overcome. Jang and Sheen (1994) and Graham et al. (1994) previously reached this same conclusion. By coelectroporation of phosphate with Man into a maize protoplast culture, Jang and Sheen (1994) showed that phosphate did not relieve the repressive effect of Man on expression of photosynthesis genes. It is possible that the genes studied by these different groups belong to two different branches of the sugar-signaling pathway, one branch involving phosphate and the other not. The genes involved in our system probably belong to the latter.

To confirm that HXK is involved in the repression of germination by Man, we carried out experiments using mannoheptulose, a specific competitive inhibitor of this enzyme. The observation that seeds germinate on Man when mannoheptulose is present is strong evidence that a metabolic signal is capable of halting germination in Arabidopsis, and that this signal is transmitted via a HXK-mediated pathway. Since it is by this enzyme that Man is phosphorylated and since Man-6-P is only slowly metabolized, HXK is likely to be the first component of the pathway. This supports previous independent studies by Graham et al. (1994) in cucumber and by Jang and Sheen (1994, 1997) and Jang et al. (1997) in maize and Arabidopsis, which proposed an identical function for HXK in carbohydrate-induced gene regulation.

Our results suggest that in Arabidopsis seeds the phosphorylation of Man by HXK triggers a signaling cascade leading to the repression of genes needed for germination. The possibility that this signal could also activate genes whose products block germination must not be excluded. Further research is needed to determine the nature of the genes involved. Free Man is not found in green plants except in trace amounts in some species, during the breakdown of reserve mannans of seeds, and during storage in certain vegetative organs (Herold and Lewis, 1977). In Arabidopsis Man leads to the accumulation of Man-6-P, which is thought to be only slowly metabolized. Seed lipid and protein reserves are mobilized to generate Suc and amino acids for germination, which are then transported and used for growth. The expression of several genes involved in this process is repressed by sugars, including Man (Graham et al., 1994). One could speculate that Man blocks germination by interfering with this process. Man present in the medium is phosphorylated by HXK, potentially halting the mobilization of seed reserves. The addition of an external energy and carbon source, such as Glc, Fru, or Suc, would restore germination. Although compet-

ing with Man for HXK, mannoheptulose contributes to germination by reducing the HXK-mediated signal.

Carbon-metabolite-mediated gene regulation in plants is expected to be complex in view of the dozens of components known to be involved in yeast (Thevelein, 1994; Smeekens and Rook, 1997). A higher degree of complexity is expected due to the greater metabolic complexity and multicellular nature of plants, and also because plants are capable of synthesizing their own sugars via photosynthesis. By investigating the effect of Man on germination, we are probably only looking at a HXK-specific sugar-signaling pathway or a HXK-mediated metabolic effect.

The availability of the sugar-signaling *sun6* mutant, which was previously isolated in our laboratory (Dijkwel et al., 1997), allowed us to further test whether Man blocks germination via a HXK-mediated process. *sun6* was isolated for its insensitivity to Suc, a disaccharide that can readily be converted to Glc and Fru, both of which are HXK substrates. Photosynthesis and expression of photosynthesis genes show a reduced sensitivity to repression by the Glc analog 2-deoxyglucose in *sun6* compared with the wild type. Seedling development in this mutant has also been found to be insensitive to high concentrations of Glc (Van Oosten et al., 1997), and Van Oosten et al. (1997) proposed that the *sun6* mutation affects a process involved in the HXK-mediated signal transduction pathway. When tested for its capacity to germinate on Man, we found that *sun6* displays a significantly greater insensitivity to this Glc analog than the wild type. This further supports the conclusion that Man blocks germination via a HXK-mediated pathway. *sun6* may be mutated in HXK itself or in any of the pathway's downstream components. We are currently attempting to clone the gene.

Based on the findings in the present study, we have carried out screens on Arabidopsis ethyl methanesulfonate and T-DNA- and transposon-tagged mutant collections, and have isolated putative mutants in the HXK-mediated sugar-sensing and -signaling pathway. The basis of this screen is that such mutants are expected to germinate on Man-containing medium and a considerable number of *mig* (Man insensitive germination) mutants have been obtained.

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