Multiple Signaling Pathways in Gene Expression during Sugar Starvation. Pharmacological Analysis of *din* Gene Expression in Suspension-Cultured Cells of Arabidopsis[1]

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We have identified many dark-inducible (*din*) genes that are expressed in Arabidopsis leaves kept in the dark. In the present study we addressed the question of how plant cells sense the depletion of sugars, and how sugar starvation triggers *din* gene expression in suspension-cultured cells of Arabidopsis. Depletion of sucrose in the medium triggered marked accumulation of *din* transcripts. Suppression of *din* gene expression by 2-deoxy-Glc, and a non-suppressive effect exerted by 3-*O*-methyl-Glc, suggested that sugar-repressible expression of *din* genes is mediated through the phosphorylation of hexose by hexokinase, as exemplified in the repression of photosynthetic genes by sugars. We have further shown that the signaling triggered by sugar starvation involves protein phosphorylation and dephosphorylation events, and have provided the first evidence that multiple pathways of protein dephosphorylation exist in sugar starvation-induced gene expression. An inhibitor of serine/threonine protein kinase, K-252a, inhibited *din* gene expression in sugar-depleted cells. Okadaic acid, which may preferentially inhibit type 2A protein phosphatases over type 1, enhanced the transcript levels of all *din* genes, except *din6* and *din10*, under sugar starvation. Conversely, a more potent inhibitor of type 1 and 2A protein phosphatases, calyculin A, increased transcripts from *din2* and *din9*, but decreased those from other *din* genes, in sugar-depleted cells. On the other hand, calyculin A, but not okadaic acid, completely inhibited the gene expression of chlorophyll *a/b*-binding protein under sugar starvation. These results indicate that multiple signaling pathways, mediated by different types of protein phosphatases, regulate gene expression during sugar starvation.

Sugars are major respiratory substrates in plant cells. However, plants easily fall into sugar starvation under conditions such as leaf senescence (Hensel et al., 1993), darkness (Brouquisse et al., 1998), and in post-harvest stages (Davies et al., 1996), all of which inevitably result in a significant decrease in photosynthesis.

Sugar starvation induces enzymatic activities related to the degradation of proteins (James et al., 1993; Moriyasu and Ohsumi, 1996), and the catabolism of fatty acids (Dieuaide et al., 1992) and amino acids (Brouquisse et al., 1992). These studies imply that plants survive sugar starvation by substituting protein and lipid catabolism for sugar catabolism (Journet et al., 1986; Yu, 1999). Besides these biochemical changes, sugar starvation has been shown to induce the expression of various genes (Yu et al., 1991; Graham et al., 1994; Chevalier et al., 1995; Koch, 1996; Prata et al., 1997). However, little is known about the mechanisms controlling gene expression associated with sugar starvation.

The mechanism of sugar-modulated gene expression has been studied extensively in yeast (Gancedo, 1998). Hexokinase and SNF1 protein kinase are known to play critical roles in sugar signaling in yeast (Gancedo, 1998). Several reports have suggested that plants have evolved a similar sugar sensing mechanism (Smeekens and Rook, 1997). One well-characterized example in plants involves a hexokinase-mediated sugar sensing system in the repression of photosynthetic genes by hexose (Jang and Sheen, 1994; Jang et al., 1997; Moore and Sheen, 1999). In a similar manner, the importance of phosphorylation of hexose by hexokinase was proposed for sugar suppression of non-photosynthetic genes (Graham et al., 1994; Prata et al., 1997; Umemura et al., 1998). Another key component in sugar signaling, a homolog of SNF1, has been isolated from a variety of plants (Halford and Hardie, 1998). Several plant homologs have been shown to complement *snf1* mutations in yeast, suggesting that there might be an SNF1-dependent sugar-signaling pathway in plants (Halford and Hardie, 1998; Halford et al., 1999). However, no study has presented evidence for the involvement of plant SNF1 homologs in the regulation of gene expression under sugar starvation (Halford and Hardie, 1998).

Besides the sugar-sensing system mediated by hexokinase, the existence of a Suc-specific sensor and a

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hexose transporter-associated sensor has been suggested (Smeekens and Rook, 1997; Lalonde et al., 1999). Despite considerable progress in recent years, many crucial elements in these pathways are still unknown (Koch et al., 2000; Pego et al., 2000).

In our attempt to understand the response of plants to photosynthetically unfavorable light conditions, we have isolated and characterized dozens of dark-inducible (*din*) genes from Arabidopsis and radish, the transcripts of which accumulate in leaves kept in the dark (Azumi and Watanabe, 1991; Fujiki et al., 1997, 2000; Shimada et al., 1998; Nakabayashi et al., 1999; Nozawa et al., 1999). We found that application of 3% (w/v) Suc to detached leaves prevented dark-induced expression of *din* genes, suggesting that sugar deprivation plays a key role in *din* gene expression in leaves exposed to unfavorable light conditions (Fujiki et al., 2000).

In this study we took a pharmacological approach to identify signaling processes in sugar starvation-inducible gene expression in suspension-cultured cells of Arabidopsis, using a set of *din* genes (Table I) and the chlorophyll *a/b*-binding protein (*Cab*) gene as model genes. We found that the sugar sensing system for the suppression of *din* genes by sugars involved phosphorylation of hexose by hexokinase, as previously shown for the *Cab* gene. In addition, we have shown that protein phosphorylation and dephosphorylation events are involved in sugar starvation-induced gene expression. Furthermore, we have found that multiple pathways, coordinated by different protein phosphatases, control gene expression during sugar starvation. Application of okadaic acid enhanced transcript levels for all *din* genes, except *din6* and *din10*, whereas calyculin A increased transcript levels for *din2* and *din9*, but decreased those for other *din* genes during sugar starvation. In contrast, okadaic acid had no inhibitory effect on *Cab* gene expression, whereas calyculin A had a strong inhibitory effect, independent of sugar. These results reveal that multiple regulatory pathways lead to sugar starvation-induced gene expression, and that *din* genes constitute useful molecular markers for analysis of such regulation.

Table I. *Dark-inducible genes in Arabidopsis examined in this study*

Gene Name	Gene Product	Reference
din1	Sulfide dehydrogenase (*SEN1*)	Shimada et al. (1998)
din2	β-Glucosidase	Fujiki et al. (1997)
din3	BCKDH E2-subunit[a]	Fujiki et al. (2000)
din4	BCKDH E1β-subunit	Fujiki et al. (2000)
din6	Asparagine synthetase (*ASN1*)	Nozawa et al. (1999)
din9	Mannose-6-phosphate isomerase	Fujiki et al. (1997)
din10	Seed imbibition protein	Fujiki et al. (1997)

[a] BCKDH, Branched-chain α-keto acid dehydrogenase.

RESULTS

Expression of *din* Genes in Suc-Starved Cells

Northern-blot analysis was performed to examine the accumulation of transcripts from *din* genes in Arabidopsis suspension-cultured cells subjected to Suc starvation. Cells were transferred to a Suc-free medium, and then collected from the medium after varying periods of time up to 72 h. Transcripts from all *din* genes examined began to accumulate immediately after the depletion of Suc, and transcript levels reached a maximum at 12 h of Suc starvation. (Fig. 1).

In contrast, when cells were incubated in a Suc-free medium for 12 h and then returned to a Suc-containing medium, the transcripts accumulated in sugar-starved cells disappeared within 4 h of Suc feeding (data not shown). These results suggest that the expression of *din* genes is repressed by Suc. Because it is well known that the expression of photosynthetic genes is repressed by sugars (Jang and Sheen, 1994), we examined the expression of the *Cab* gene as a typical example of a sugar-repressible gene. The transcript level of the *Cab* gene in Suc-fed cells was maintained at a basal level (Fig. 1, time 0). Once Suc was removed from the medium, the repression by sugar was eliminated, and the transcript levels of the *Cab* gene began to increase with kinetics similar to those observed for *din* genes. This implied that *din* genes and the *Cab* gene share, in part, a common mechanism for sugar-repressible gene expression.

Effect of Glc Analogs on the Expression of *din* Genes

Several studies using Glc analogs and inhibitors of hexokinase have described a putative role for hexokinase and/or the phosphorylation of hexose in sugar repression of gene expression (Jang and Sheen, 1994; Prata et al., 1997; Umemura et al., 1998). We examined whether the phosphorylation of hexose by hexokinase is involved in the regulation of *din* gene expression by using Glc analogs. Seven-day-old cells were washed with a Suc-free medium and incubated for 12 h with a fresh medium containing 10 mM Glc, 10 mM 3-*O*-methyl-Glc (3-OMG), or 0.5 mM 2-deoxy-Glc (2-d-Glc). 3-OMG cannot be phosphorylated by hexokinase, and thus generates no sugar repression signal through hexokinase (Jang and Sheen, 1994, and refs. therein). In contrast, 2-d-Glc can be phosphorylated by hexokinase in plant cells, and in turn can initiate hexokinase-mediated sugar signaling, but cannot be easily metabolized further (Jang and Sheen, 1994; and refs. therein). The application of 2-d-Glc abolished the accumulation of transcripts from the *din* genes, whereas 3-OMG did not suppress *din* gene expression (Fig. 2). These expression patterns apparently resembled that of the *Cab* gene, which is repressed by sugars in a hexokinase-dependent manner (Jang and Sheen, 1994; see also

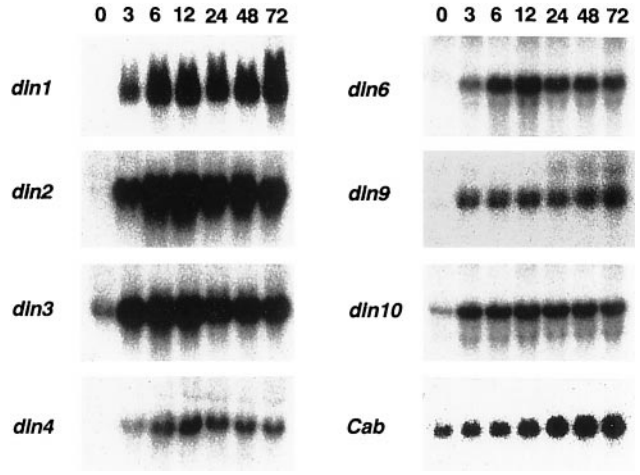

Figure 1. Time course analysis of the expression of *din* genes during Suc starvation. Total RNA was isolated from cells incubated in the absence of Suc for varying lengths of time up to 72 h. An equal amount of RNA (20 μg) was loaded in each lane and analyzed by northern-blot hybridization. In this study we examined the expression of the *Cab* gene as a control for sugar-repressible genes.

Fig. 2), suggesting that *din* genes and the *Cab* gene are subject to a common sugar-sensing mechanism through the phosphorylation of hexose. However, it should be noted that the expression of the *Cab* gene was not completely suppressed even in the presence of 2% (w/v) Suc, 10 mM Glc, or 0.5 mM 2-d-Glc in our experimental system (Fig. 2). The *Cab* gene is known to be positively regulated by light (Terzaghi and Cashmore, 1995; Argüello-Astorga and Herrera-Estrella, 1998), and so the expression of this gene might have been, in part, induced by light since the cells were incubated under illumination in our system. In contrast, *din* genes are not positively regulated by light (Fujiki et al., 2000).

The application of 0.5 mM 2-d-Glc incompletely suppressed the expression of *din2* and *din10* (Fig. 2, lane 4). Because expression of *din2* and *din10* was strongly suppressed in the presence of 2% (w/v) Suc (Fig. 2, lane 1), the suppression of *din2* and *din10* may require a higher concentration of sugar than did other *din* genes.

Requirement of Protein Synthesis for the Expression of *din* Genes during Sugar Starvation

We examined whether protein synthesis is required in the process of *din* gene expression during sugar starvation. Addition to the culture medium of 20 μM cycloheximide, an inhibitor of cytosolic translation, completely blocked the sugar starvation-induced accumulation of transcripts from all *din* genes except *din10* and partly abolished *din10* expression (Fig. 3, lane 3). This result suggested that the sugar starvation-induced expression of *din* genes requires the synthesis of new proteins.

With respect to *din10* expression, a higher concentration of cycloheximide was required for its complete inhibition in sugar-starved cells (data not shown). In addition, it should be noted that the suppression of *din10* expression by Suc seemed to be partially relieved by 20 μM cycloheximide (Fig. 3, lane 4). These results suggest that regulation of the expression of *din10* differs, to a certain degree, from that of other *din* genes. Sheu et al. (1996) reported that cycloheximide, at concentrations ranging from 20 to 300 μM, blocked the suppression of a rice α-amylase gene (*αAmy3*) by Suc, resulting in marked accumulation of its transcripts. In contrast, we found that cycloheximide at 200 μM, but not at 20 μM, inhibited the accumulation of *din10* transcripts (data not shown). These results imply that the regulation of *din10* gene expression by sugars is distinct not only from that of other *din* genes, but also from that of an αAmy3.

Effects of Inhibitors of Protein Kinases and Phosphatases on the Expression of *din* Genes

Protein phosphorylation and dephosphorylation events are known to regulate numerous biological processes (Luan, 1998). By using various inhibitors of protein kinases and phosphatases, we examined whether protein phosphorylation and dephosphorylation events are involved in the expression of *din* genes during Suc starvation. These inhibitors were dissolved in dimethyl sulfoxide, and used at concentrations known to be effective in Arabidopsis cell culture (Christie and Jenkins, 1996). In our preliminary experiments, dimethyl sulfoxide alone did not

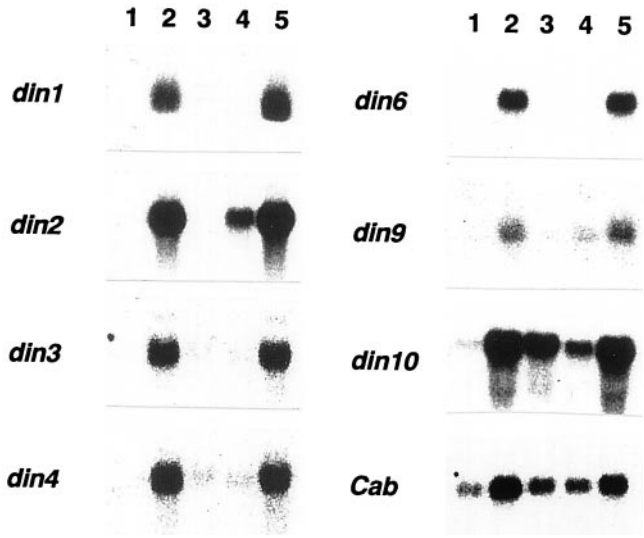

Figure 2. Effects of Glc analogs on the expression of *din* genes. Seven-day-old cells were rinsed and incubated for 12 h with fresh medium containing 10 mM Glc (lane 3), 0.5 mM 2-d-Glc (lane 4), or 10 mM 3-OMG (lane 5). RNA isolated from non-treated (2% [w/v] Suc, lane 1) and Suc-starved (lane 2) cells was used as a control. Each lane was loaded with 10 μg of RNA.

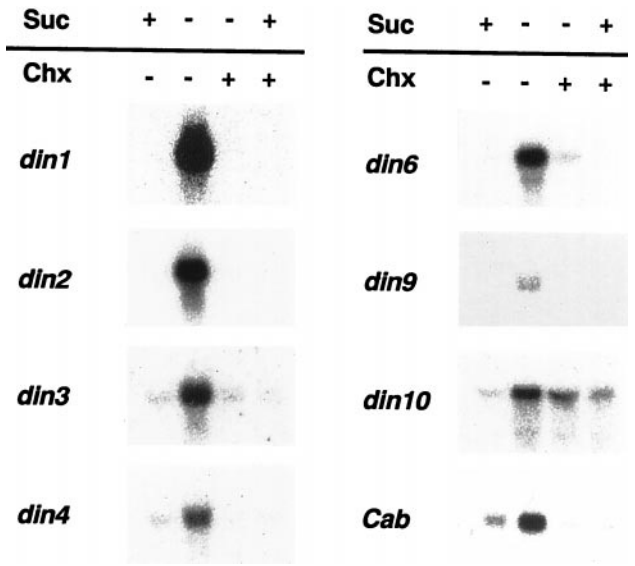

Figure 3. Effect of cycloheximide on the expression of *din* genes during Suc starvation. For the treatment of cycloheximide, cells were pre-incubated for 1 h with 20 μM of cycloheximide (chx). Cells were incubated for 12 h either in a Suc-free (−) or a Suc-containing medium (+), with or without cycloheximide. Each lane was loaded with 10 μg of RNA.

alter the expression pattern of *din* genes in cells with or without Suc (data not shown).

Incubation of cells with 4 μM K-252a, a general Ser/Thr protein kinase inhibitor, prevented the accumulation of *din* transcripts in sugar-starved cells. In contrast, 75 μM genistein, a Thy/His kinase inhibitor, had no effect on gene expression (Fig. 4A). These results suggest that Ser/Thr protein kinases, but not Thy/His kinases, are involved in the processes of *din* gene expression during sugar starvation. Accumulation of the transcripts from the *Cab* gene was completely inhibited by K-252a, but not by genistein (Fig. 4A).

Figure 5 summarizes the relative mRNA levels of *din* genes and the *Cab* gene in cells treated with protein phosphatase inhibitors. The application of 1 μM okadaic acid, known to preferentially inhibit protein phosphatase type 2A (PP2A) over type 1 (PP1; Cohen et al., 1990), enhanced transcript levels of all *din* genes, except *din6* and *din10*, in sugar-depleted cells (Figs. 4A and 5A). On the other hand, okadaic acid had little inhibitory effect on *din6* and *din10* expression under sugar starvation. The *Cab* gene expression similarly was not influenced by okadaic acid (Figs. 4A and 5A).

We also examined the effect of 1 μM calyculin A, a more potent inhibitor of PP1 and PP2A (Cohen et al., 1990; Figs. 4B and 5B). Calyculin A enhanced transcript levels of *din2* and *din9*, but reduced those of other *din* genes in Suc-starved cells. In contrast, transcript levels of all *din* genes were enhanced by the addition of calyculin A in the Suc-fed cells. In particular, sugar-mediated suppression of *din2* and *din9* seemed to be profoundly influenced by calyculin A (Fig. 5B). Furthermore, the expression patterns of *din* genes were completely different from that of the *Cab* gene, since the basal transcript level of *Cab* in the sugar-fed cells was significantly decreased by calyculin A, and the induction of *Cab* expression by sugar starvation was completely inhibited by calyculin A (Figs. 4B and 5B). These results suggest that there are multiple pathways for the regulatory processes in sugar-modulated gene expression, i.e. with respect to protein dephosphorylation events.

DISCUSSION

We have previously shown that the expression of a variety of genes is induced in leaves kept in the dark, and in senescing leaves (Azumi and Watanabe, 1991;

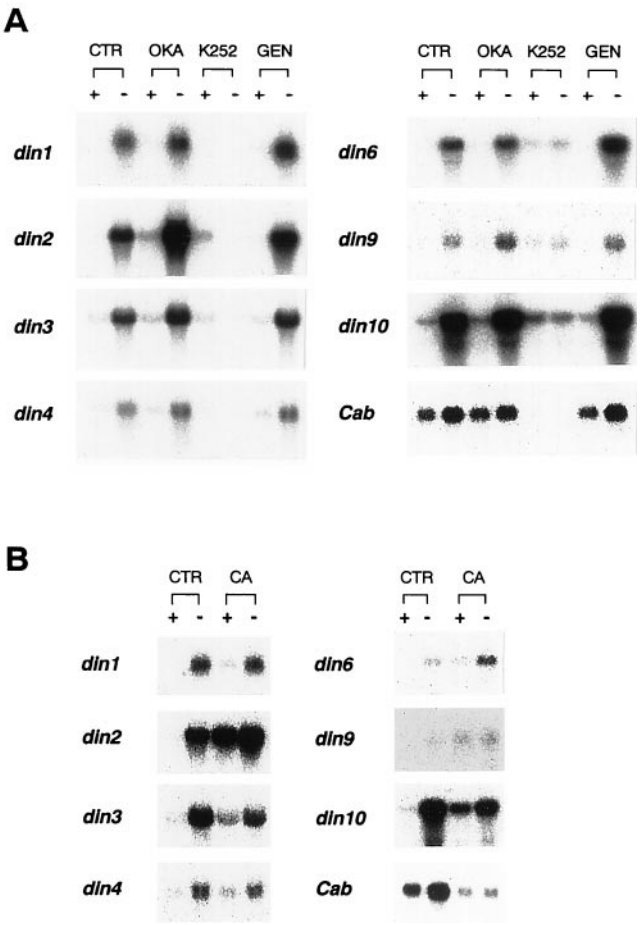

Figure 4. Effects of inhibitors of protein phosphatases and protein kinases on the expression of *din* genes. A, Cells were pre-incubated for 1 h with 1 μM okadaic acid (OKA), 4 μM K-252a (K252), or 75 μM genistein (GEN). Cells were rinsed and incubated with each inhibitor for 12 h in a Suc-free (−) or a Suc containing (+) medium. Cells were also incubated for 12 h in the absence of inhibitors (CTR). B, Cells were incubated with 1 μM of calyculin A (CA) for 12 h, either in a Suc-free (−) or a Suc-containing (+) medium. Each lane was loaded with 10 μg of RNA.

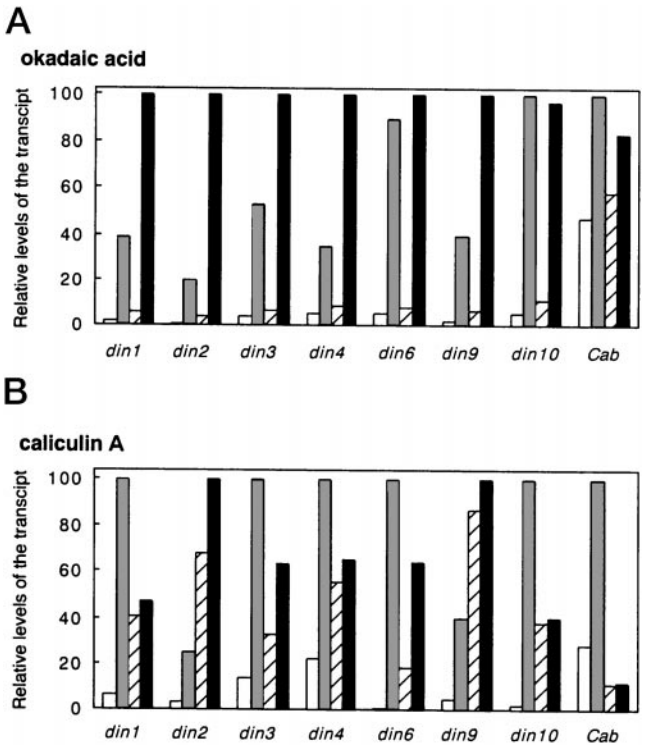

Figure 5. Effects of protein phosphatase inhibitors on the relative mRNA levels of *din* genes. Relative levels of the transcripts were estimated by quantitation of signals on the blotted membranes (Fig. 4), with a Bio Imaging Analyzer BAS2000 (Fuji Photo Film, Tokyo). The results were expressed as a percentage of the respective maximum level for each gene. Cells were incubated in a Suc-containing (white bars) or a Suc-free (shaded bars) medium for 12 h in the absence of inhibitors. For the treatment of inhibitors, cells were incubated with okadaic acid (A) or calyculin A (B) for 12 h in a Suc-containing (hatched bars) or a Suc-free (black bars) medium.

Fujiki et al., 1997, 2000; Nakabayashi et al., 1999; Nozawa et al., 1999). Under these conditions plants suffer sugar starvation as a direct consequence of the cessation of photosynthesis. The gene products encoded by *din* genes (Table I) include proteins related to the catabolism of β-glucoside (*din2*), of amino acids (*din3*, *din4*, and *din6*), of Man (*din9*), and of the raffinose family oligosaccharides (*din10*). All of these enzymes would significantly contribute to plant survival under conditions of sugar starvation by providing alternate energy sources in place of photosynthate (Fujiki et al., 1997, 2000). Thus *din* genes will be useful molecular markers for research into sugar starvation. It is well known that sugar starvation triggers dramatic biochemical changes in the metabolism of lipids and amino acids in many plant species (Journet et al., 1986; Yu, 1999). Few experiments, however, have been undertaken to investigate the regulation of gene expression induced by sugar starvation. To investigate the molecular events occurring in sugar-starved cells, we adopted a suspension-cultured cell system, and succeeded in illustrating a part of the signaling process of gene expression including *din* genes and the *Cab* gene during sugar starvation. Transcripts from all *din* genes accumulated rapidly in Arabidopsis suspension-cultured cells transferred to a Suc-free medium. Conversely, transcripts disappeared immediately after sugar-starved cells were transferred back to a Suc-containing medium. These results confirmed our previous results that the dark-induced expression of *din* genes in leaves is repressed by Suc, but not by mannitol (Fujiki et al., 1997, 2000).

It has been suggested that hexokinase is involved in an early sensory event in the suppression of photosynthetic genes by hexose (Jang and Sheen, 1994; Jang et al., 1997; Moore and Sheen, 1999). In an alternate manner, hexokinase, which is primarily responsible for catalyzing the first step of glycolysis, may play a role in sugar-repressible gene expression through changing AMP and/or ATP levels (Halford et al., 1999). We found that 2-D-Glc, but not 3-OMG, could mimic the repression effect of Glc on the expression of *din* genes, as already exemplified for photosynthetic genes such as the *Cab* gene. These results are consistent with the notion that the phosphorylation of hexose generates the signal for the sugar-repressible expression of not only photosynthetic genes, but also of the numerous dark-inducible genes. Thus far, many studies have investigated the responses of genes to increasing levels of sugars, i.e. induction or repression of gene expression by sugars. Hexokinase is involved in one such mechanism of sensing increasing levels of sugars. In the present study, we addressed the question of how plant cells sense the depletion of sugars, and how sugar starvation leads to gene expression. We have demonstrated that the induction of *din* gene expression in Suc-depleted cells requires cytoplasmic protein synthesis. Furthermore, we found that signaling in sugar starvation includes protein phosphorylation and dephosphorylation events. These results indicate that the processes in sugar starvation-induced gene expression are complex.

The induction of *din* gene expression by Suc starvation is inhibited by K-252a. This indicates that Ser/Thr protein kinases play a role in the induction of *din* gene expression during sugar starvation. SNF1 Ser/Thr protein kinase is thought to be a metabolic sensor in yeast (Gancedo, 1998) and possibly in plants because SNF1 homologs from several plant species have been shown to complement the *snf1* mutation in yeast (Halford and Hardie, 1998). Ikeda et al. (1999) showed that transcripts from the *wpk4* gene, a SNF1 homolog in wheat, accumulate in wheat seedlings after the removal of Suc from the culture medium. Hence it will be of interest to examine whether plant SNF1 homologs regulate gene expression triggered by sugar starvation.

To date, the effects of okadaic acid and calyculin A on sugar-regulated genes have been studied only in a

limited number of cases, such as the sugar-repressible expression of an *αAmy3* (Lue and Lee, 1994), and the sugar-inducible expression of the β-amylase and sporamin genes (Takeda et al., 1994). Although these studies only considered a small number of genes, the conclusions drawn may indicate that protein dephosphorylation mediates the regulation of gene expression in the presence of sugars. However, whether protein dephosphorylation events are involved in the regulation of gene expression under sugar starvation was not examined. Furthermore, no previous study has revealed the complex nature of sugar-modulated gene expression involving multiple signaling pathways regulated by different types of protein phosphatases. In the present study we examined the expression of a variety of *din* genes and the *Cab* gene, using okadaic acid and calyculin A, and found that sugar starvation-induced gene expression was mediated by protein dephosphorylation events. Furthermore, we found that the effects of okadaic acid and calyculin A on sugar-modulated gene expression vary among the *din* genes and the *Cab* gene, indicating that the mechanism of sugar-regulated gene expression is more complicated than previously envisaged.

In sugar-depleted cells, okadaic acid enhanced the accumulation of transcripts from all *din* genes, except *din6* and *din10*. Okadaic acid, however, exerted little or no enhancement on the accumulation of transcripts from *din6*, *din10*, or *Cab* in sugar-depleted cells, indicating that these genes are regulated in a slightly different manner from other *din* genes under sugar starvation. Therefore, we propose the hypothesis that an okadaic acid-sensitive protein phosphatase negatively regulates the expression of all *din* genes, except *din6* and *din10*, in response to a sugar starvation signal, e.g. by inhibiting transcription and/or destabilizing transcripts. In addition, okadaic acid produced a weak enhancement of transcript levels of all *din* genes, even in Suc-fed cells where gene expression was suppressed by sugar (Fig. 5A). Thus another okadaic acid-sensitive protein dephosphorylation event is proposed to negatively regulate the basal machinery, rather than the sugar-specific machinery, for the accumulation of transcripts. Because this enhancement effect of okadaic acid was not strong enough to cancel the suppression effect of sugar, okadaic acid-sensitive protein dephosphorylation did not seem to play a major role in the sugar-mediated suppression of *din* genes or the *Cab* gene.

In contrast to okadaic acid, calyculin A decreased transcript levels of all *din* genes, except *din2* and *din9*, in Suc-depleted cells. This suggested that, with the exception of *din2* and *din9*, the induction of *din* genes by sugar starvation requires a calyculin A-sensitive protein phosphatase that positively regulates gene expression in response to a sugar starvation signal, e.g. by stimulating transcription and/or stabilizing transcripts. In contrast, this calyculin A-sensitive protein phosphorylation system did not seem to apply to *din2* and *din9* expression, since the expression of these genes in sugar-depleted cells was enhanced by calyculin A. However, another calyculin A-sensitive protein phosphatase seemed to negatively regulate the expression of *din2* and *din9* in the presence of sugar because transcript levels of these genes in sugar-fed cells were strongly enhanced by calyculin A (Fig. 5B). In accord with the above results, *din* genes can be divided into three groups. The first group includes *din1, din3,* and *din4*, which are negatively controlled by an okadaic acid-sensitive phosphatase, and positively controlled by a calyculin A-sensitive phosphatase in sugar-starved cells. The second group includes *din6* and *din10* genes that are little affected by an okadaic acid-sensitive phosphatase, but are positively regulated by a calyculin A-sensitive phosphatase during sugar starvation. The third group includes *din2* and *din9*, which appear to be negatively controlled in sugar-depleted cells by protein phosphatases sensitive to okadaic acid and calyculin A. In addition, the expression of *din2* and *din9* appear to be negatively controlled by another calyculin A-sensitive protein phosphatase in sugar-fed cells. The expression pattern of the *Cab* gene was, in part, similar to that of *din6* and *din10*, since okadaic acid had little effect on *Cab* gene expression. However, calyculin A had a strong inhibitory effect on *Cab* gene expression independently of the presence of Suc. Hence it is likely that a calyculin A-sensitive protein phosphatase may be responsible for the basal machinery of *Cab* gene expression under illumination.

Okadaic acid was shown, in cell-free extracts, to inhibit PP2A at a low concentration (1 nM), and PP1 at a higher concentration (1 μM; Cohen et al., 1990). Calyculin A inhibits PP2A with a potency equal to that of okadaic acid, and inhibits PP1 with 10- to 100-fold greater potency than okadaic acid. Although it is difficult to manipulate the effective concentrations of these inhibitors in vivo, the differential effects of okadaic acid and calyculin A observed in this study may be explained by the presence of hypothetical protein phosphatases (PP1 and PP2A) in plant cells that exhibit differential sensitivity to these inhibitors. Thus we suggest that PP1 and PP2A play different roles in sugar-modulated gene expression. PP2A, which may be inhibited by okadaic acid and calyculin A, appears to negatively regulate the sugar starvation-inducible expression of *din1*, *din2*, *din3*, *din4*, and *din9*, but not that of *din6*, *din10*, and *Cab* genes. Another PP2A may be involved in the basal machinery of transcription and/or the stabilization of transcripts in a negative manner, which may be responsible for the accumulation of transcripts from all *din* genes in sugar-fed cells in the presence of okadaic acid (and possibly calyculin A as well). PP1, which is inhibited by calyculin A, but not by okadaic acid, may positively regulate sugar starvation-

inducible expression of all *din* genes, except *din2* and *din9*. With respect to *din2* and *din9*, another PP1 may play a major role in the negative control of gene expression in the presence of Suc, because calyculin A, but not okadaic acid, strongly enhances transcript levels of *din2* and *din9* in sugar-fed cells (Fig. 5). However, it was not possible to clarify whether PP1, as well as PP2A, negatively controls *din2* and *din9* expression in the absence of Suc, because calyculin A potentially inhibits PP2A as okadaic acid does.

We observed that calyculin A, but not okadaic acid, has a strong inhibitory effect on *Cab* expression under illumination, which suggests that PP1 may be involved in the activation of *Cab* gene expression. In a similar manner, PP1 inhibited by calyculin A, but not by okadaic acid, appears to be essential for light-dependent activation of the expression of other photosynthetic genes (Sheen, 1993). However, the regulatory role of PP1 in light-induced expression of photosynthetic genes, including the *Cab* gene, may be different from that in the sugar-regulated expression of *din* genes since calyculin A completely inhibited the induction of the *Cab* gene, but not that of the *din* genes by sugar starvation. Kurotani et al. (1999) recently showed that protein phosphatases are responsible for the light-induced accumulation of *Cab* transcripts through stabilization of the transcripts, rather than by changing the transcription rate. It will be interesting to determine whether the inhibitors examined in this study are responsible for changing the transcription rate and/or the stability of transcripts from *din* genes.

In conclusion, the sugar-sensing mechanism mediated by the phosphorylation of hexose was found to be common to all *din* genes and to the *Cab* gene. However, we have shown for the first time that the signaling pathways leading to sugar starvation-induced gene expression differ among various genes, including the *din* genes and the *Cab* gene. We have demonstrated that protein phosphorylation and dephosphorylation play critical roles in the sugar-regulated expression of *din* genes. We propose the hypothesis that a calyculin A-sensitive protein phosphatase, probably PP1, is partly responsible for the expression of *din* genes, except *din2* and *din9* during Suc starvation. By contrast, another PP1 may be responsible for the suppression of *din2* and *din9* gene expression by sugars. On the other hand, protein phosphatases that were inhibited by okadaic acid and calyculin A, probably PP2A, may be involved in the destabilization of the transcripts, and/or in the suppression of transcription of *din* genes, except *din6* and *din10* in sugar-depleted cells. Identification and characterization of the protein kinases and phosphatases responsible for such regulation will be important in revealing the signaling pathways leading to gene expression during sugar starvation. We believe that the Arabidopsis suspension-cultured cell system and the *din* genes are useful tools for further investigation of the molecular mechanisms of sugar starvation-induced gene expression.

MATERIALS AND METHODS

Plant Materials

The Arabidopsis suspension-cultured cell line T87 (Axelos et al., 1992) was obtained from the RIKEN Plant Cell Bank (Tsukuba, Japan). Cells were grown in 80 mL of Gamborg B5 medium (Wako Pure Chemical Industries, Osaka), containing 2% (w/v) Suc and 2.5 μM 2,4-dichlorophenoxyacetic acid at 23°C under continuous illumination at a photon flux density of 40 μmol m^{-2} s^{-1}. The cell suspension was maintained by transplanting 2 mL of 12-d-old cells to fresh medium.

For Suc-starvation treatment, 7-d-old T87 cells were rinsed and incubated with fresh medium devoid of Suc. As a control, cells were washed and incubated with fresh medium containing 2% (w/v) Suc. Inhibitors were added to the culture medium at concentrations that were reported to be effective in an Arabidopsis suspension-cultured cell system (Christie and Jenkins, 1996). Cells were collected on filter paper by vacuum filtration and were immediately frozen in liquid nitrogen.

Northern-Blot Hybridization

Isolation of total RNA from T87 cells and northern-blot analysis were performed as described previously (Fujiki et al., 2000). The distribution of radioactivity on the blotted membranes was analyzed with a Bio Imaging Analyzer BAS2000 (Fuji Photo Film). Full-length cDNA inserts for *din1* (*sen1*, Oh et al., 1996) *din2, din3,* and *din4* genes, and partial cDNA fragments for *din6, din9,* and *din10* genes were used as hybridization probes (Table I). A cDNA clone for the *Cab* gene in Arabidopsis (GenBank accession no. P27521) was obtained from the Arabidopsis Biological Resource Center at Ohio State University. Experiments with inhibitors were repeated two to four times, and similar results were observed in each case.

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