Sugar-Induced Increase of Calcium-Dependent Protein Kinases Associated with the Plasma Membrane in Leaf Tissues of Tobacco[1]

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The sugar-inducible expression of genes for sporamin and β-amylase in leaf explants of sweet potato (*Ipomoea batatas*) and that of a β-glucuronidase-fusion gene, with the promoter of the gene for β-amylase in leaves of tobacco (*Nicotiana tabacum*), requires Ca^{2+} signaling (M. Ohto, K. Hayashi, M. Isobe, K. Nakamura [1995] Plant J 7: 297–307), and it was inhibited by staurosporin and K252a, inhibitors of protein kinases. Autophosphorylation activities of several potential protein kinases in leaves of tobacco were significantly higher in younger leaves than in mature leaves. However, the autophosphorylation activities of these proteins in mature leaves, especially those of the major autophosphorylatable proteins with apparent molecular masses of 56 and 54 kD, increased upon treatment of leaf discs with a 0.3 M solution of sucrose, glucose, or fructose, did not increase with sorbitol or mannitol treatments, and the increase by sucrose was inhibited by cycloheximide. Autophosphorylation of the 56- and 54-kD protein in vitro was dependent on Ca^{2+} and inhibited by staurosporine, K-252a, and by W-7. These results suggest that they belong to the family of calcium-dependent protein kinases. They were concentrated in the plasma membrane fraction and were released from membrane vesicles by high salt or with sodium carbonate. The possible functions of these sugar-inducible calcium-dependent protein kinases associated with the plasma membrane are discussed.

Many plant genes, such as genes for storage proteins and for enzymes involved in the metabolism of starch (Rocha-Sosa et al., 1989; Johnson and Ryan, 1990; Müller-Röber et al., 1990; Visser et al., 1991) and in other processes (Tsukaya et al., 1991; Cheng et al., 1992; Koch et al., 1992; Mason et al., 1992), have been shown to be inducible by high levels of sugars. In addition, genes that are repressed by high levels of sugars have also been identified (Sheen, 1990; Yu et al., 1991; Krapp et al., 1993). The interorgan transport and the availability of Suc could be important factors in the control of the expression of many genes during the growth and development of plants. However, the mechanisms underlying such regulation, in particular, the mechanisms of transduction to the nucleus of signals related to carbohydrate metabolism, are unknown at present.

The expression of genes for sporamin and β-amylase, two major proteins of tuberous roots of sweet potato (*Ipomoea batatas*), can be induced, concomitantly with the accumulation of large amounts of starch, in stems when sweet potato plantlets are cultured with a medium that contains Suc (Hattori et al., 1990) or in leaves and petioles when excised leaf-petiole cuttings are supplied with high concentrations of Suc or other metabolizable sugars (Hattori et al., 1991; Nakamura et al., 1991). In addition, the expression of GUS gene fusions, composed of the promoter fragment of a gene for sporamin (gSPO-A1:GUS; Ohta et al., 1991) or β-amylase (β-Amy:GUS; Takeda et al., 1994) and the coding sequence for β-glucuronidase (Jefferson et al., 1987), in leaves of transgenic tobacco is inducible by Suc and other metabolizable sugars, suggesting that the expression of genes for sporamin and β-amylase is regulated by the carbohydrate metabolic signal primarily at the transcriptional level. We reported previously that the sugar-inducible expression of genes for sporamin and β-amylase in sweet potato and that of a β-Amy:GUS fusion gene in tobacco were inhibited by inhibitors of protein phosphatases 1 and 2A (Takeda et al., 1994), as well as by inhibitors of calmodulin and Ca^{2+} channels (Ohto et al., 1995). Furthermore, results of experiments with tobacco plants that expressed the Ca^{2+}-specific photoprotein aequorin indicated indirectly that an increase in $[Ca^{2+}]_{cyt}$ occurs in leaves of tobacco during incubation with sugars (Ohto et al., 1995).

In this paper, we show that inhibitors of protein kinases severely inhibit the sugar-inducible expression of genes for sporamin and β-amylase. Using an autophosphorylation assay of proteins that had been separated by SDS-PAGE and blotted onto a membrane (Ferrell and Martin, 1991), we found that levels of several potential CDPKs that are

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Abbreviations: β-Amy:GUS, a fusion gene consisting of the promoter of a gene for β-amylase from sweet potato and the coding sequence for GUS; $[Ca^{2+}]_{cyt}$, the cytosolic concentration of free Ca^{2+}; CDPK, calcium-dependent protein kinase; PVDF, polyvinylidene difluoride.

associated with the plasma membrane in leaves of tobacco are subject to regulation by a carbohydrate metabolic signal. The potential roles of these sugar-inducible CDPKs are discussed.

MATERIALS AND METHODS

Plant Materials

Mature leaves of sweet potato (*Ipomoea batatas* Lam var Kokei No. 14) from shoots that had sprouted from tuberous roots, cultured in water under continuous light, were cut off with a sharp razor blade, and each leaf was separated into several parts. Leaf discs were placed on the surface of a 0.4% agar plate that contained 0.3 M Suc in water, in the absence or presence of inhibitor, for 12 h in darkness. Details of the treatment of leaf-petiole cuttings of sweet potato with a solution of Suc were described previously (Nakamura et al., 1991; Ohto et al., 1992).

Transgenic tobacco plants carrying a β-Amy:GUS fusion gene were prepared as reported previously (Takeda et al., 1994). Leaf discs prepared from several leaves of such plants were placed on a 0.4% agar plate that contained a 0.3 M solution of specified sugars, in the presence or absence of inhibitor, for 24 h in darkness.

Chemicals

W-5, W-7, H-7, H-8, and HA1004, purchased from Seikagaku Kogyo Co. (Tokyo, Japan), were dissolved in distilled water at 5 mM (W series) or 10 mM (H series) as stock solutions. KT5720, calphostin C, staurosporine, K-252a, and UCN01 were generous gifts from Dr. H. Nakano (Kyowa-Hakko Kogyo Co., Tokyo, Japan). They were dissolved in DMSO at 1 mg/mL as stock solutions. Genistein from RBI (Natick, MA) and lavendutin A and methyl 2,5-dihydroxy-cinnamate from GIBCO-BRL were dissolved in DMSO at 0.1 M, 20 mM, and 30 mM, respectively, as stock solutions. Cycloheximide was purchased from Sigma. Stock solutions of these inhibitors were diluted with water or with a solution of Suc in water. When inhibitors dissolved in DMSO were used, all treatments of tissues or reactions in vitro were carried out in the presence of 0.1% DMSO. $MgCl_2$ $6H_2O$ and $MnCl_2$ $4H_2O$ were purchased from Wako Pure Chemical Co. (Osaka, Japan) and they contained, at most, 0.01 and 0.005% (w/w) calcium, respectively.

Northern Blot Hybridization

Total RNA was prepared from tissues, and northern blot hybridization of mRNAs for sporamin and β-amylase was carried out as described previously (Ohto et al., 1992). As a control, hybridization with the ^{32}P-labeled cDNA insert of $pF_1\gamma GE-1$, which encodes the γ subunit of sweet potato mitochondrial $F_1ATPase$ (Morikami et al., 1993), was also performed. After the membranes were washed with the appropriate solutions, they were exposed to x-ray film (X-Omat; Eastman Kodak) for autoradiography or the bands corresponding to sporamin mRNA and β-amylase mRNA were subjected to determination of radioactivity

with a Bio-Imaging analyzer (Fujix BAS2000II; Fuji Photo Film Co., Tokyo, Japan).

Extraction of Proteins and Fluorimetric Assay of GUS Activity

Extraction of proteins from leaf discs and fluorimetric assays of GUS activity were carried out as described by Jefferson et al. (1987).

Protein Blots and Autophosphorylation of Proteins

Proteins in the extracts were separated by SDS-PAGE on 7.5% polyacrylamide gels and blotted onto PVDF membranes (Millipore). Autophosphorylation of proteins on PVDF membranes with $[\gamma-^{32}P]ATP$ was carried out as described by Ferrell and Martin (1991). The phosphorylation buffer contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 2 mM $MnCl_2$, unless otherwise indicated. The labeling reaction was started by the addition of 10 μCi/mL $[\gamma-^{32}P]ATP$ (specific activity, approximately 6000 Ci/mmol) and continued for 30 min at room temperature, unless otherwise indicated. After incubation and a final wash of the membranes with 1 M KOH for 10 min, bands of ^{32}P-labeled proteins were visualized by autoradiography.

Fractionation of Cells and Preparation of Membrane Vesicles

All steps were performed at 4°C. Leaves were excised from pod-grown tobacco plants at the petioles. They were dipped in a solution of 0.175 M Suc at their cut edges and incubated in this way for 24 h in darkness. Leaves treated with Suc were cut into pieces and homogenized with 4 volumes of homogenization buffer, which was composed of 60 mM Mops-KOH (pH 7.6), 4 mM EGTA, 4 mM EDTA, 8 mM KF, 8 mM $NaVO_3$, 0.25 M Suc, 5 mM DTT, 1 mM PMSF, 1.5 mM p-aminobenzamidine, and 1.5% (w/v) Polyclar AT, in a food processor for 15 s and then in a Polytron homogenizer (Kinematica, Littau, Switzerland) for 50 s. The homogenate was filtered through two layers of cheesecloth and then through a 60-μm nylon mesh. After the sample was centrifuged at 10,000g for 15 min, microsomes in the supernatant were pelleted by centrifugation at 150,000g for 40 min. Microsomal pellets were resuspended in buffer A, which was composed of 10 mM KPi (pH 7.2) and 0.25 M Suc, flash frozen in liquid N_2, and stored at −80°C until use.

A plasma membrane-enriched fraction was prepared from the microsomal fraction by aqueous two-phase (dextran/PEG) separation as described by Mito et al. (1988). Plasma membrane vesicles were resuspended in buffer A. They were treated with 2 M NaCl plus 5 mM EDTA or with 0.2 M Na_2CO_3 and incubated at 4°C for 30 min. After the samples were centrifuged at 150,000g for 30 min, the supernatants were recovered. The pellets were resuspended in buffer A at final volumes equal to those of the corresponding supernatants. Aliquots were then analyzed by SDS-PAGE, which was followed by an in situ autophosphorylation assay on PVDF membranes. Protein concentrations were determined, after precipitation with 10% TCA,

by the method of Lowry et al. (1951) with BSA as the standard. The specific activities of vanadate-sensitive ATPase, azide-sensitive ATPase, nitrate-sensitive ATPase, inosine 5'-diphosphatase, and NADPH-Cyt c reductase, which are marker enzymes of the plasma membrane, mitochondrial inner membranes, the tonoplast, the Golgi apparatus, and the ER, respectively, were determined as described by Mito et al. (1988).

RESULTS

Effects of Inhibitors of Protein Kinase on the Suc-Inducible Gene Expression

When leaf discs from tobacco plants that had been transformed with the β-Amy:GUS fusion gene were placed on agar plates that contained 0.3 M Suc in water and incubated for 24 h in darkness, a marked increase in GUS activity was induced, as described previously (Takeda et al., 1994). To examine whether the sugar-inducible expression of the β-Amy:GUS fusion gene is mediated by phosphorylation of proteins, leaf discs prepared from the mature leaves of transgenic tobacco plants that carried the β-Amy:GUS fusion gene were incubated on agar plates that contained 0.3 M Suc in the presence of the following inhibitors of protein kinases at the indicated concentration: 100 μM H-7; 100 μM HA1004; 2 μM staurosporine; 2 μM K-252a; 2 μM UCN01; 2 μM KT5720; 1.3 μM calphostin C; 100 μM genistein; and 30 μM methyl 2,5-dihydroxycinnamate. H-7, HA1004, staurosporine, K-252a, UCN01, and KT5720 are known to inhibit Ser/Thr protein kinases. Calphostin C is an inhibitor of animal protein kinase C (Tamaoki, 1991). Genistein and methyl 2,5-dihydroxycinnamate are inhibitors of various Tyr protein kinases. Among the inhibitors tested, staurosporine and K-252a strongly inhibited the Suc-induced increase in GUS activity (Fig. 1A). Methyl 2,5-dihydroxycinnamate weakly repressed the induction of GUS activity to about 80% of that of the control treatment (data not shown). Inhibitors other than these three compounds did not have any inhibitory effects (data not shown). The absence of inhibition by UCN01 and KT5720, whose structures are similar to those of staurosporine and K-252a (Kase et al., 1987; Tamaoki, 1991), suggests that the inhibition by staurosporine and K-252a was specific. The lack of inhibitory effects by other compounds could be due to their permeability problems.

We also examined the effects of inhibitors of protein kinases on the Suc-induced accumulation of mRNAs for sporamin and β-amylase in leaf-petiole cuttings of sweet potato. When mature leaves, with petioles still attached, that had been excised from field-grown sweet potato plants were dipped in a solution of 0.175 M Suc at their cut edges and incubated under these conditions in darkness, significant amounts of the mRNAs for sporamin and β-amylase were induced after 12 h, as described previously (Nakamura et al., 1991; Ohto et al., 1992). The levels of mRNAs for sporamin and β-amylase in leaf-petiole cuttings after treatment with Suc in the presence of 2 μM staurosporine or 2 μM K-252a were about 60 to 70% of the control levels in tissues that had been treated with Suc in the absence of inhibitors (data not shown). The other inhibitors tested did not inhibit the induction to any significant extent. The Suc-induced accumulation of mRNAs for sporamin and β-amylase was strongly inhibited by 2 μM staurosporine or 2 μM K-252a when leaf discs of sweet potato were used instead of the leaf-petiole cuttings (Fig. 1B). The concentration of Suc used in the treatment of leaf discs of sweet potato was 0.3 M, since Suc at this concentration is associated with the highest level of induction, which is also true in the case of the Suc-inducible expression of the β-Amy: GUS fusion gene in leaf discs from transgenic tobacco (data not shown). The weaker inhibitory effects of staurosporine and K-252a in the leaf-petiole cuttings, compared to those in the leaf discs, suggest that these inhibitors might not be efficiently transported to cells via the vascular system of petioles. The level of mRNA for the γ subunit of the F_1ATPase (Morikami et al., 1993) was unaffected by the presence of either 2 μM staurosporine or 2 μM K-252a (data not shown). These results suggest that phosphorylation of proteins by a protein kinase(s) that is sensitive to staurosporine or K-252a is involved in the Suc-inducible expression of genes for sporamin and β-amylase.
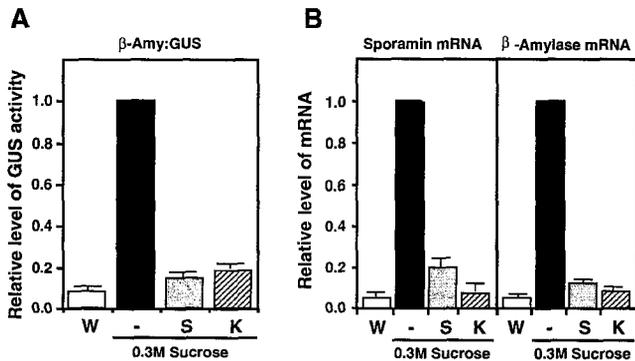
Figure 1. Effects of inhibitors of protein kinases on Suc-inducible gene expression. A, Leaf discs of transgenic tobacco with the β-Amy: GUS fusion gene were treated with 0.3 M Suc for 24 h in the absence (−) or in the presence of 2 μM staurosporine (S) or 2 μM K-252a (K). Leaf discs treated with water (W) were also analyzed. The specific activity of GUS, relative to that measured after the control treatment with Suc in the absence of inhibitors (−), was determined. Results are the means with SD of two independent experiments. B, Leaf discs of sweet potato were treated with 0.3 M Suc for 12 h in the absence (−) or in the presence of 2 μM staurosporine (S) or 2 μM K-252a (K). Twenty milligrams of total RNA from leaf discs were analyzed for the levels of mRNAs for sporamin and β-amylase by northern blot hybridization. Results are the means with SD from two independent experiments.

Autophosphorylation of Proteins from Leaves of Tobacco

Ferrell and Martin (1991) described a method for detecting the autophosphorylating activities of protein kinases that have been separated from other proteins by SDS-PAGE and blotted onto PVDF membranes. To examine whether treatments of tissues with sugars caused any changes in levels of protein kinases, we used this method to examine protein kinases in extracts from leaf discs of

A
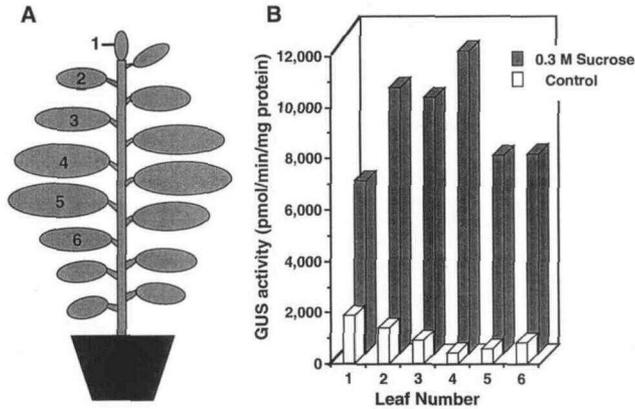
Figure 2. Suc-induced expression of the β-Amy:GUS fusion gene in leaves from various parts of tobacco plants. A, Leaves of pod-grown tobacco plants that had been transformed with the β-Amy:GUS fusion gene were numbered as indicated and used to prepare leaf discs. The length (cm) and width (cm), respectively, of each leaf were: leaf 1, 8 and 4; leaf 2, 16 and 8.5; leaf 3, 23 and 11; leaf 4, 26 and 11; leaf 5, 18 and 8; leaf 6, 12 and 7. B, Leaf discs cut from each leaf were either immediately used for extraction of proteins (open bars) or treated with 0.3 M Suc for 24 h in darkness (filled bars). The specific activity of GUS in extracts was determined. Similar results were obtained in three separate experiments.

tobacco plants that had been transformed with the β-Amy: GUS fusion gene. Leaf discs were prepared from leaves from different parts of plants, which were numbered as shown in Figure 2A. Leaf discs from each leaf were divided into two groups; discs in one group were immediately used for extraction of proteins, and discs in another group were treated with 0.3 M Suc for 24 h. The specific activity of GUS from nontreated, control tissues was highest in the youngest leaves (Fig. 2B, leaf No. 1) and it decreased with the increasing age of leaves from the top to the seventh leaf (Fig. 2B, leaf No. 4). The specific activity of GUS in leaves treated with water for 24 h was similar to that in nontreated tissues (data not shown). When leaf discs were treated with 0.3 M Suc, a marked increase in GUS activity in leaf discs was observed for every leaf (Fig. 2B). These results suggest that the highest activity of GUS in control discs cut from the youngest leaf may reflect the cellular response to Suc that is transported from the source leaves.
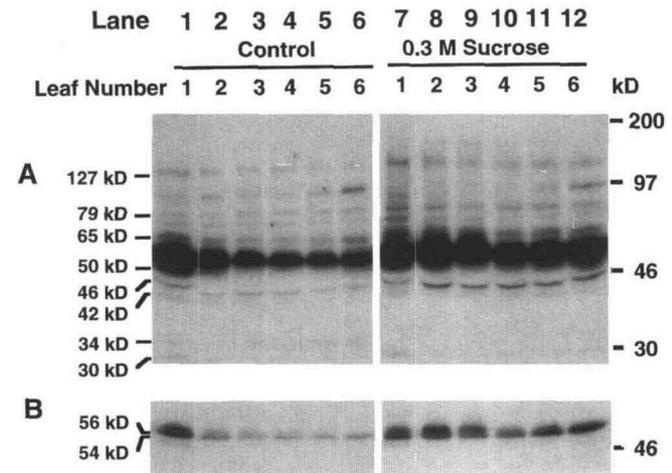
Proteins in the same extracts as those used for the assay of GUS activity were separated by SDS-PAGE, blotted onto a PVDF membrane, and assayed for their autophosphorylating activity. When equal amounts of proteins from control tissues were assayed, proteins from the youngest leaf (Fig. 2A, leaf No. 1) generated the greatest number of bands of proteins with autophosphorylating activity, as well as the bands of the strongest intensities (Fig. 3A, lane 1). A total of 12 ^{32}P-labeled proteins with molecular masses between 127 and 30 kD were identified. Among them, the 56- and 54-kD proteins, which could be separately detected after a shorter exposure time (Fig. 3B, lane 1), were the most heavily labeled with [^{32}P]ATP. The extent of labeling of the 127-, 65-, 56-, 54-, and 50-kD proteins with [^{32}P]ATP

decreased with increasing age of the leaf (Fig. 3, lanes 1–6). By contrast, the labeling intensities of other bands either did not change or increased slightly with increasing age of the leaf (Fig. 3, lanes 1–6).

Treatments of leaf discs, especially those cut from the mature leaves, with 0.3 M Suc resulted in an increase in the labeling of several proteins with [^{32}P]ATP (Fig. 3, lanes 7–12). The most significant increase in labeling was observed with the 65-, 56-, 54-, and 46-kD proteins. By contrast, autophosphorylating activities of other minor bands did not change or decreased, e.g. the 34-kD protein, upon treatment of tissues with Suc. In contrast to treatment with Suc, treatment of leaf discs from mature leaves with water for 24 h did not cause significant changes in the autophosphorylating activities of the 65-, 56-, 54-, and 46-kD proteins (data not shown; Fig. 4B). In the case of the youngest leaf, no significant increase of autophosphorylating activity of the 56- and 54-kD proteins was observed upon treatment with Suc (Fig. 3B, lanes 1 and 7). Autophosphorylation of the 56-kD protein was not detected in extracts from the 9th and 11th leaves (leaf Nos. 5 and 6), with or without treatment with Suc (Fig. 3, lanes 5, 6, 11, and 12).

Sugar-Induced Increases in the Level of the 54-kD Protein with Autophosphorylation Activity

We examined the effects of treatment of leaf discs with various sugars on the levels of the autophosphorylatable proteins. Since the greatest increase in specific activity of GUS upon treatment of leaf discs with 0.3 M Suc was observed in the seventh leaf from the top of the plant (Fig.

Figure 3. Autophosphorylation of proteins in leaves of tobacco. Fifty micrograms of protein in the same extracts as those used for the determination of the specific activity of GUS in the experiment described in the legend to Figure 2 were separated by SDS-PAGE. After proteins were blotted onto a PVDF membrane, autophosphorylation of proteins was performed as described in "Materials and Methods." Autoradiography was performed with one intensifying screen for 13 h (A) or 1.5 h (B). Molecular mass markers used were myosin (200 kD), phosphorylase b (97 kD), ovalbumin (46 kD), and carbonic anhydrase (30 kD). Similar results were obtained in two separate experiments.

A

B
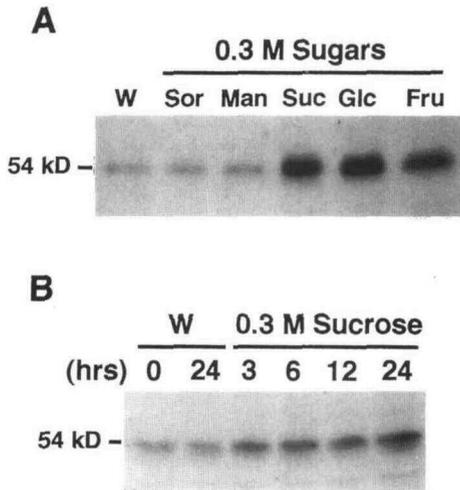
Figure 4. Sugar-induced increase in the level of the 54-kD protein with autophosphorylation activity. Leaf discs from the seventh leaf of a transgenic tobacco plant with the β-amy:GUS fusion gene were treated with water (W) or a 0.3 M solution of Suc, Glc, Fru, mannitol (Man), or sorbitol (Sor) for 24 h in darkness (A), or they were treated with 0 (W) or 0.3 M Suc for 3, 6, 12, and 24 h in darkness (B). Fifty micrograms of protein in each extract were separated by SDS-PAGE, blotted onto a PVDF membrane, and assayed for autophosphorylation. Autoradiography was carried out with one intensifying screen for 6 h (A) or 1.5 h (B). Similar results were obtained in two separate experiments.

2, leaf No. 4), we used leaf discs prepared from the seventh leaf for treatments with sugars. In these experiments, PVDF membranes after the autophosphorylation reaction were exposed for a relatively short time to examine specifically the autophosphorylation of the 54-kD protein. Under these conditions, autophosphorylation of the 56- and 50-kD proteins was too weak to be detected. As shown in Figure 4A, treatment of leaf discs with a 0.3 M solution of Glc or Fru also caused an increase in the level of autophosphorylation of the 54-kD protein. However, a 0.3 M solution of neither mannitol nor sorbitol induced such an increase.

Figure 4B shows the time course of the increase in the level of the autophosphorylatable 54-kD protein after treatment of leaf discs with 0.3 M Suc. The increase in autophosphorylation activity of the 54-kD protein was apparent as early as 3 h after the start of treatment of leaf discs with Suc. The increase in the specific activity of GUS was significant only 6 h after the start of treatment of leaf discs with Suc and was undetectable after 3 h (data not shown).

As shown in Figure 5, the increase in the level of the autophosphorylatable 54-kD protein upon treatment of tissues with Suc was repressed in the presence of 100 μM cycloheximide. These results suggest that the increase in the level of autophosphorylation of the 54-kD protein was due to an increased amount of the 54-kD protein by de novo synthesis. However, other effects of cycloheximide cannot be excluded, such as the increase in the level of the autophosphorylatable 54-kD protein due to the increase of available sites for autophosphorylation by inhibition of the synthesis of protein phosphatases.

Effects of Various Inhibitors and Divalent Cations on Autophosphorylation of Proteins in Vitro

Proteins in extracts from leaf discs that had been treated with 0.3 M Suc for 24 h were separated by SDS-PAGE, blotted onto PVDF membranes, and assayed for autophosphorylation in the presence of various inhibitors. As shown in Figure 6, 2 μM staurosporine and 2 μM K-252a each severely inhibited the autophosphorylation of the 56-, 54-, and 50-kD proteins. Autophosphorylation of these proteins was not inhibited by 2 μM UCN01 (Fig. 6) and 100 μM genistein (data not shown). By contrast, autophosphorylation of the 46- and 42-kD proteins was not inhibited by either staurosporine or K-252a. These results suggest that effects of staurosporine and K-252a are specific and that the 56-, 54-, and 50-kD proteins are Ser/Thr protein kinases that are sensitive to both staurosporine and K-252a.

The standard reaction mixture for autophosphorylation of proteins contains 10 mM $MgCl_2$ and 2 mM $MnCl_2$ (Ferrell and Martin, 1991). A higher level of autophosphorylation of the 56-, 54-, and 50-kD protein kinases was obtained when the reaction mixture contained 2 mM $MnCl_2$ rather than 10 mM $MgCl_2$ (Fig. 7, lanes 2 and 3), a result that suggests that Mn-ATP is more effective than Mg-ATP in autophosphorylation. Previously, higher levels of autophosphorylation of protein kinases from Arabidopsis (Hirayama and Oka, 1992) and tobacco (Shibata et al., 1995) were obtained in the presence of Mn^{2+} than Mg^{2+}. Since EGTA has a higher affinity for Mn^{2+} than for Ca^{2+}, the effects of EGTA on the autophosphorylation of proteins were examined in the presence of only Mg^{2+}. As shown in lanes 4 and 5 in Figure 7, autophosphorylation of protein kinases was completely inhibited by 0.5 mM EGTA, and inhibition by EGTA of autophosphorylation of the 56- and 54-kD protein kinases was overcome by further addition of 0.55 mM $CaCl_2$, the results suggesting that these protein kinases are CDPKs (Roberts and Harmon, 1992). The autophosphorylation of the 56- and 54-kD protein kinases in the absence of EGTA (Fig. 7, lanes 1–3) could have been due to Ca^{2+} that was bound to the enzymes or to the presence of Ca^{2+} as contaminants in solutions of $MgCl_2$ and $MnCl_2$.
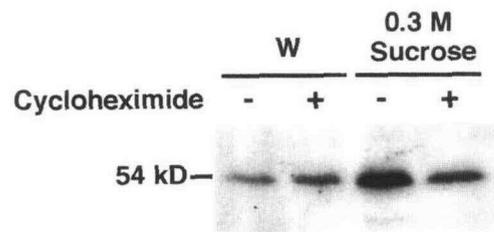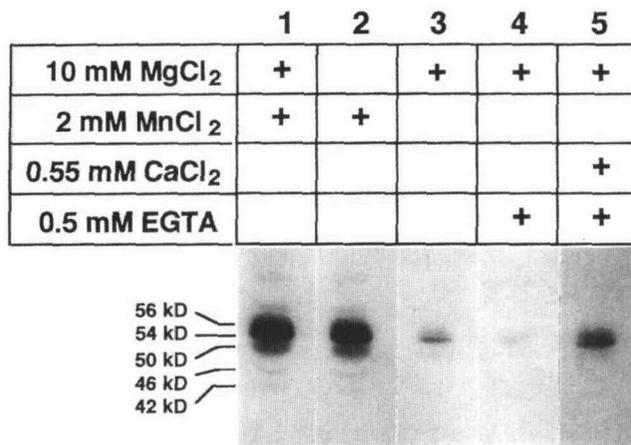

Figure 5. Effects of cycloheximide on the Suc-induced increase in autophosphorylation activity of the 54-kD protein. Leaf discs from the seventh leaf of a transgenic tobacco plant with the β-Amy:GUS fusion gene were treated with water (W) or 0.3 M Suc in the absence (−) or the presence (+) of 100 μM cycloheximide for 6 h in darkness. Fifty micrograms of protein in crude extract were separated by SDS-PAGE, blotted onto a PVDF membranes, and assayed for autophosphorylation. Autoradiography was carried out with one intensifying screen for 5 h. Similar results were obtained in two separate experiments.
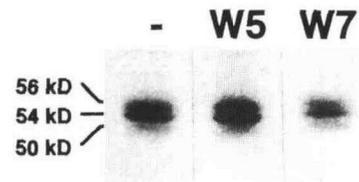
-   +S   +K   +U

56 kD
54 kD
50 kD
46 kD
42 kD
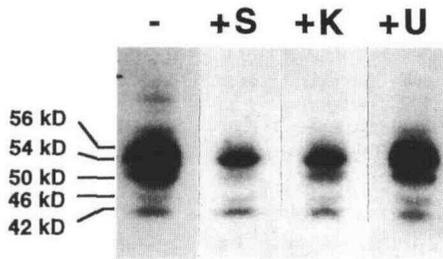
Figure 6. Effects of inhibitors of protein kinase on the autophosphorylation of proteins. Leaf discs from the youngest leaf of a transgenic tobacco plant with the β-Amy:GUS fusion gene were treated with 0.3 M Suc for 24 h in darkness. Fifty micrograms of protein in the extract were separated by SDS-PAGE. After proteins were blotted onto a PVDF membrane, the blot was cut into strips. Each strip was incubated in the standard phosphorylation buffer in the absence (−) or presence of 2 μM staurosporine (+S), 2 μM K-252a (+K), or 2 μM UCN01 (+U) for 30 min. After the strips were washed, they were subjected to autoradiography with one intensifying screen for 6 h. Similar results were obtained in two separate experiments.

The Ca^{2+}-dependent autophosphorylation of the 50-kD protein kinase was also detected by longer exposure time for autoradiography (data not shown).

CDPKs contain calmodulin-like domains with four EF-hand structures (Roberts and Harmon, 1992), and they are sensitive to W-7, an inhibitor of calmodulin, albeit at concentrations higher than those required for inhibition of calmodulin (Harmon et al., 1987). The effects of W-7 on the autophosphorylation of protein kinases were examined in the presence of 0.05 mM $CaCl_2$. As shown in Figure 8, autophosphorylation of the 56- and 54-kD protein kinases

	1	2	3	4	5
10 mM MgCl₂	+		+	+	+
2 mM MnCl₂	+	+			
0.55 mM CaCl₂					+
0.5 mM EGTA				+	+

56 kD
54 kD
50 kD
46 kD
42 kD

Figure 7. Effects of divalent cations on autophosphorylation of proteins. Leaf discs from the youngest leaf of a transgenic tobacco plant with the β-Amy:GUS fusion gene were treated with 0.3 M Suc for 24 h in darkness. Fifty micrograms of protein in the extract were separated by SDS-PAGE. After proteins were blotted onto a PVDF membrane, the blot was cut into strips. Each strip was incubated with [γ-³²P]ATP in a buffer that contained 10 mM MgCl₂ and 2 mM MnCl₂ (lane 1); 2 mM MnCl₂ (lane 2); 10 mM MgCl₂ (lane 3); 10 mM MgCl₂ and 0.5 mM EGTA (lane 4); or 10 mM MgCl₂, 0.5 mM EGTA, and 0.55 mM CaCl₂ (lane 5), for 30 min. After the strips were washed, they were subjected to autoradiography with one intensifying screen for 2.5 h. Similar results were obtained in two separate experiments.

-   W5   W7

56 kD
54 kD
50 kD

Figure 8. Effects of calmodulin inhibitors on the autophosphorylation of proteins. Leaf discs from the youngest leaf of a transgenic tobacco plants with the β-Amy:GUS fusion gene were treated with 0.3 M Suc for 24 h in darkness. Fifty micrograms of protein in the extract were separated by SDS-PAGE. After proteins were blotted onto a PVDF membrane, the blot was cut into strips. Each strip was incubated in the standard phosphorylation buffer that contained 0.05 mM CaCl₂ in the absence (−) or the presence of 200 μM W-5 (W5) or 200 μM W-7 (W7) for 15 min. After the strips were washed, they were subjected to autoradiography with one intensifying screen for 2.5 h. Similar results were obtained in three separate experiments.

was partially inhibited by 200 μM W-7. By contrast, 200 μM W-5, a dechlorated form and inactive analog of W-7, did not have such inhibitory effects.

Cell Fractionation Studies

Homogenates of leaves of tobacco that had been treated with 0.175 M Suc for 24 h were fractionated by differential centrifugation. The 56-, 54-, and 50-kD protein kinases were detected only in small amounts in the soluble fraction and were concentrated in the microsomal fraction (150,000g pellet; data not shown). A plasma membrane-enriched fraction was prepared from the microsomal fraction by aqueous two-phase separation (Mito et al., 1988). The specific activity of vanadate-sensitive ATPase, a marker enzyme of the plasma membrane, in the plasma membrane-enriched fraction was 1.5-fold greater than that in the microsomal fraction (Table I). By contrast, specific activities of azide-sensitive ATPase, nitrate-sensitive ATPase, inosine 5′-diphosphatase, and NADPH-Cyt c reductase, which are marker enzymes of the mitochondrial inner membrane, the tonoplast, the Golgi apparatus, and the ER, respectively, in the plasma membrane-enriched fraction were significantly lower than those in the microsomal fraction (Table I). The same amounts of protein from the microsomal fraction and the plasma membrane-enriched fraction were separated by SDS-PAGE, and an autophosphorylation assay was carried out after the proteins were blotted onto a PVDF membrane. The levels of autophosphorylation of the 56-, 54-, and 50-kD protein kinases were higher in the plasma membrane-enriched fraction than in the microsomal fraction (Fig. 9A).

The plasma membrane vesicles were treated with 2 M NaCl plus 5 mM EGTA or with 0.2 M Na₂CO₃ at 4°C for 30 min, and then they were fractionated into insoluble and soluble fractions by centrifugation. As shown in Figure 9B, the 56-, 54-, and 50-kD protein kinases were solubilized by these treatments, with the treatment with 0.2 M Na₂CO₃ being particularly effective. These results suggest that the 56-, 54-, and 50-kD protein kinases are associated with the periphery of plasma membrane vesicles.

Table I. *Concentration of vanadate-sensitive ATPase in the plasma membrane-enriched fraction*

The specific activities of marker enzymes in the plasma membrane-enriched fraction (PM) were compared to those in the microsomal fraction (M). Relative values of specific activities are shown. Specific activity of enzymes in the microsomal fraction were: vanadate-sensitive ATPase, 385 nmol min^{-1} mg^{-1} protein; azide-sensitive ATPase, 140 nmol min^{-1} mg^{-1} protein; nitrate-sensitive ATPase, 167 nmol min^{-1} mg^{-1} protein; ionsine 5′-diphosphatase, 3.7 μmol min^{-1} mg^{-1} protein; and NADPH-Cyt c reductase, 23 nmol min^{-1} mg^{-1} protein.

| Enzyme	Relative Specific Activity	
	M	PM
Vanadate-sensitive ATPase	1.0	1.5
Azide-sensitive ATPase	1.0	0.014
Nitrate-sensitive ATPase	1.0	0.38
IDPase	1.0	0.42
NADPH-Cyt c reductase	1.0	0.17

DISCUSSION

Both the sugar-inducible expression of the β-Amy:GUS fusion gene in leaf discs of transgenic tobacco and the sugar-inducible accumulation of mRNAs for sporamin and β-amylase in leaf-petiole cuttings of sweet potato were inhibited by staurosporine and by K252a (Fig. 1). These results suggest that the activity of a Ser/Thr protein kinase(s) is involved in the mechanism of regulation. Since inhibitors of types 1 and 2A protein phosphatases have also been shown to have strong inhibitory effects (Takeda et al., 1994), sugar-inducible expression of genes, or at least that of genes for sporamin and β-amylase, seems to be under complex regulatory mechanisms that include both the phosphorylation and the dephosphorylation of proteins.

Similar to Glc-regulated gene expression in yeast (Rose et al., 1991) and mammals (Epstein et al., 1992), sugar-regulated gene expression in plant cells may involve the sensing of incoming sugars by hexokinase (Jang and Sheen, 1994) and/or of energy metabolic status of the cell. The level of phosphate (Sadka et al., 1994) or of some other metabolite might act as the actual signal, and the signaling molecule could be a regulator of the activity of a protein kinase and/or protein phosphatase. Many protein kinases and protein phosphatases are regulated by effector molecules. For example, Suc-P synthase kinase is inhibited by Glc-6-P and Suc-P synthase kinase-protein phosphatase is inhibited by phosphate in spinach leaves (Huber and Huber, 1992). Alternatively, the activity of the protein kinase and/or protein phosphatase involved in the mechanism of sugar-modulated gene expression might be regulated by $[Ca^{2+}]_{cyt}$. The sugar-inducible expression of genes for sporamin and β-amylase is inhibited by EGTA, inhibitors of calmodulin and inhibitors of Ca^{2+} channels (Ohto et al., 1995). Furthermore, results of experiments with transgenic tobacco plants that expressed the Ca^{2+}-specific photoprotein aequorin (Knight et al., 1991) indicated indirectly that an increase in $[Ca^{2+}]_{cyt}$ occurs in leaf cells during incubation of the tissue with a 0.2 M solution of Suc, Glc, or Fru

but not with a 0.2 M solution of sorbitol or mannitol (Ohto et al., 1995).

In this study, we examined autophosphorylatable proteins in leaves of tobacco. The autophosphorylating activities of some of the proteins in leaves varied significantly, depending on the position of leaves in plants (Fig. 3). For example, the autophosphorylation activity of the 54-kD protein and of several other proteins was highest in the top leaf of the plant, and the level of autophosphorylation of these proteins decreased with increasing age of the leaf. However, the autophosphorylation activity of the 54-kD protein and of several other proteins in mature leaves increased significantly when discs prepared from these leaves were incubated with 0.3 M Suc (Fig. 3). These results suggest that the responses of cells in the top leaf to Suc that have been transported from the mature leaves might have been reflected in the highest levels of autophosphorylation of the 56-, 54-, and 50-kD proteins. An increase in the autophosphorylation activity of the 54-kD protein was also induced by treatment of mature leaves with a 0.3 M solution of Glc or Fru, but not with a 0.3 M solution of mannitol or sorbitol (Fig. 4A), and it was inhibited by cycloheximide
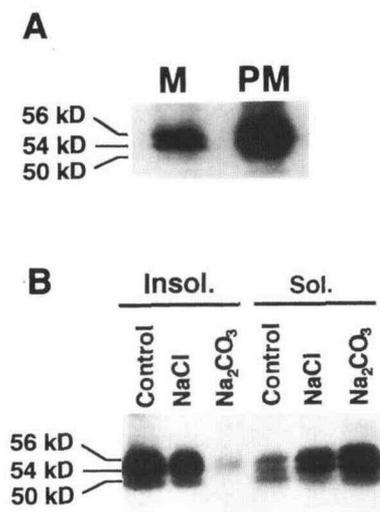
Figure 9. Association of autophosphorylatable protein kinases with the plasma membrane. A, The microsomal fraction (M) was prepared from tobacco leaves that had been treated with 0.175 M Suc, and the plasma membrane-enriched fraction (PM) was isolated from the microsomal fraction by two-phase separation. Fifty micrograms of protein in the microsomal fraction and the plasma membrane-enriched fraction were separated by SDS-PAGE and blotted onto a PVDF membrane. The autophosphorylation assay was carried out in the standard phosphorylation buffer that contained 0.05 mM CaCl$_2$. Autoradiography was carried out without an intensifying screen for 3 h. B, The plasma membrane vesicles were treated with 2 M NaCl plus 0.5 mM EGTA or with 0.2 M Na$_2$CO$_3$ at 4°C for 30 min, and the reaction mixture was centrifuged. Fifty micrograms of protein in pellets (insoluble; Insol.) and supernatants (soluble; Sol.) were separated by SDS-PAGE and blotted onto a PVDF membrane. Autophosphorylation was carried out in the standard phosphorylation buffer that contained 0.05 mM CaCl$_2$. Autoradiography was carried out without an intensifying screen for 70 min. Similar results were obtained in two separate experiments.

(Fig. 5). These results suggest that the level of the 54-kD protein, and possibly of several other autophosphorylatable proteins, is subject to regulation by the carbohydrate metabolic signal rather than being strictly regulated by the developmental stages of the leaf.

The presence of CDPKs has been reported in many plant species and tissues (for review, see Roberts and Harmon, 1992). Each of these CDPKs has an N-terminal kinase domain, a junction domain, and a C-terminal calmodulin-like domain. The kinase activities of highly purified CDPK from pea (Li et al., 1991) and *Dunaliella* (Yuasa and Muto, 1992) are sensitive to inhibition by staurosporine and by K252a. The junction domain is homologous to the autoinhibitor domain of mammalian Ca^{2+}/calmodulin-dependent protein kinase II, and the junction domain of CDPKs functions as an autoinhibitor (Harmon et al., 1994; Harper et al., 1994). Binding of Ca^{2+} to the calmodulin-like domain probably releases the catalytic domain from inhibition by the junction domain. The kinase activities of CDPKs purified from soybean cells (Harmon et al., 1987) and *Dunaliella* (Yuasa and Muto, 1992) are inhibited by inhibitors of calmodulin, such as W-7 or trifluoperazine. Autophosphorylation of the 56-, 54-, and 50-kD proteins was inhibited by staurosporin and K-252a (Fig. 6) and it required Ca^{2+} (Fig. 7). Furthermore, it was partially inhibited by 200 μM W-7 but not by W-5 (Fig. 8). These results suggest that the 56-, 54-, and 50-kD proteins are CDPKs. The weak inhibitory effect of W-7 could be due to the immobilization of proteins on membranes. In Arabidopsis and soybean, CDPKs are encoded by a multigene family (Roberts and Harmon, 1992). The 56-, 54-, and 50-kD protein kinases could be members of a multigene family for CDPKs in tobacco. Indeed, our recent analysis of cDNAs indicates that multiple forms of mRNA for CDPK are present in leaves of tobacco (M. Ohto, M. Nakakita, M. Sasaki, and K. Nakamura, unpublished results).

CDPKs have been found in various subcellular locations such as the soluble fraction (Putnum-Evans et al., 1990; DasGupta, 1994), the plasma membrane (Schaller et al., 1992; Verhey et al., 1993), the symbiosome membranes of soybean root nodules (Weaver and Roberts, 1992), and the nuclei (Li et al., 1991). The 56-, 54-, and 50-kD protein kinases in the leaves of tobacco were localized in the membrane fraction enriched for the plasma membrane, and they were released from membrane vesicles by washing with NaCl plus EGTA or with Na_2CO_3 (Fig. 9). It is suggested that these CDPKs associated with the surface of the plasma membrane.

The sugar-inducible CDPKs described herein might play important roles in the regulation of various cellular activities in response to increases in $[Ca^{2+}]_{cyt}$ that are induced by elevated availability of metabolizable sugars (Ohto et al., 1995). They might regulate the activities of various proteins or enzymes by phosphorylation and, thereby, alter cellular activities to meet the demands of such conditions. Since the basal levels of both GUS activity and the autophosphorylation activities of CDPKs were higher in younger leaves than in mature leaves (Figs. 2 and 3) and since the sugar-induced increase in the autophosphorylat-

ing activity of the 54-kD CDPK (Fig. 4B) occurred earlier than the sugar-induced increase in the activity of GUS encoded by the β-Amy:GUS fusion gene (Takeda et al., 1994), the sugar-inducible CDPK might somehow be positively linked to the regulation of expression of the β-Amy: GUS fusion gene. In general, protein kinases can participate in the regulation of gene expression by the phosphorylation of transcription factors that regulate the expression of target genes. However, since the major sugar-inducible CDPKs described in this paper are associated with the plasma membrane, it seems unlikely that transcription factors are the natural substrates of these CDPKs.

The substrates for the plasma membrane-associated CDPKs described herein could also be proteins associated with the plasma membrane. A nodule-specific membrane protein of soybean that is homologous to metabolite transporters from other organisms is phosphorylated in isolated symbiosome membranes in situ by CDPK, and the malate-transport activity in symbiosome membrane vesicles parallels the state of phosphorylation of this membrane protein (Weaver and Roberts, 1992). The CDPK partially purified from the oat plasma membrane is capable of phosphorylating an H^+-ATPase in vitro (Schaller et al., 1992).

The activities of sugar transporters in the plasma membrane of leaf cells of tobacco could be regulated by plasma membrane-associated CDPK. Several H^+/sugar transporters for mono- and disaccharides that catalize the energy-dependent uptake of the respective sugars are present in higher plant cells (Sauer and Tanner, 1993). Activation of the H^+/sugar symporter either directly by phosphorylation by a sugar-inducible CDPK or indirectly via the activation of an H^+-ATPase by CDPK-catalyzed phosphorylation could enhance the rate of entry of sugars into the cell. In such a case, the sugar-induced increase in the level of CDPKs might represent potentiation or auto-amplification of the size of the sink in cells by increasing the import of sugars in response to signals that are related to carbohydrate metabolism, and the sugar-inducible CDPKs might indirectly be involved in the up-regulation of expression of the β-Amy:GUS fusion gene. Future studies of the effects of inhibitors of CDPKs on the uptake of sugars by cells and on the substrates of the sugar-inducible CDPKs are now required to examine these possibilities.

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