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Okadaic Acid Mimics Nitrogen-Stimulated Transcription of the NADH-Glutamate Synthase Gene in Rice Cell Cultures[1]

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Okadaic acid (OKA), a potent and specific inhibitor of protein serine/threonine phosphatases 1 and 2A, induced the accumulation of NADH-glutamate synthase (GOGAT) mRNA within 4 h in rice (*Oryza sativa* L.) cell cultures. In contrast to the transient accumulation of NADH-GOGAT mRNA by NH_4^+, OKA caused a continuous accumulation for at least 24 h. The induction of NADH-GOGAT mRNA by OKA was not inhibited in the presence of methionine sulfoximine, which inhibited the NH_4^+-induced accumulation of mRNA. These results suggest that the OKA-sensitive protein phosphatase is involved in the regulation of NADH-GOGAT gene expression and probably plays a role in the signal transduction pathway downstream from NH_4^+, although a signal transduction pathway other than that of nitrogen sensing could be responsible. Nuclear run-on assays demonstrated that the accumulation of NADH-GOGAT mRNA induced by the supply of either NH_4^+ or OKA was mainly regulated at the transcription level. OKA effects were synergistic to the NH_4^+-induced expression of the NADH-GOGAT gene. In the presence of K-252a, a protein kinase inhibitor, the accumulation of NADH-GOGAT mRNA induced by either NH_4^+ or OKA was reduced. The possible roles of protein phosphatases in the regulation of NADH-GOGAT gene expression are discussed.

In most plants, Gln synthetase (GS; EC 6.3.1.2) and Glu synthase (GOGAT) are key enzymes in the assimilation of NH_4^+ derived from both external nitrogen sources and internal nitrogen metabolic processes such as photorespiration, phenylpropanoid biosynthesis, amino acid catabolism, and fixation of dinitrogen in legumes. GS catalyzes the synthesis of Gln from NH_4^+ and Glu in an ATP-dependent manner. GOGAT catalyzes the reductive transfer of the amide group of Gln to 2-oxogluarate to form two Glu molecules. This GS/GOGAT cycle, as defined by Lea and Miflin (1974), is now generally accepted to be the major route of NH_4^+ assimilation in plants (Lea et al., 1990; Sechley et al., 1992).

In higher plants, GOGAT exists as two molecular species that use either reduced ferredoxin (Fd) or NADH as a reductant (Lea et al., 1990; Sechley et al., 1992). Fd-GOGAT (EC 1.4.7.1) is found in chloroplasts and is essential in the reassimilation of NH_4^+ generated during photorespiration (Lea et al., 1990; Sechley et al., 1992). The molecular structure and regulatory mechanisms of Fd-GOGAT have been well studied in a number of plant species (Sakakibara et al., 1991; Zehnacker et al., 1992; Avila et al., 1993; Nalbantoglu et al., 1994; Suzuki and Rothstein, 1997; Coschigano et al., 1998).

NADH-GOGAT (EC 1.4.1.14) is located in the plastids of non-photosynthetic tissues such as roots and nodules (Hayakawa et al., 1999; Trepp et al., 1999a). NADH-GOGAT cDNA clones have been obtained from alfalfa (Gregerson et al., 1993), Arabidopsis (Lam et al., 1996), and rice (*Oryza sativa* L.; Goto et al., 1998). Genomic clones for NADH-GOGAT in alfalfa (Vance et al., 1995) and rice (Goto et al., 1998) have been isolated and characterized. In legume root nodules, NADH-GOGAT mediates the process of symbiotic nitrogen fixation (Temple et al., 1998). In alfalfa root nodules, the expression of the NADH-GOGAT gene could be regulated temporally and spatially during the development of effective nodules (Gregerson et al., 1993; Vance et al., 1995; Trepp et al., 1999a, 1999b).

In non-legumes, NADH-GOGAT is important, along with cytosolic GS, in the primary assimilation of NH_4^+ and in the reassimilation of NH_4^+ released during amino acid catabolism and seed germination (Lea et al., 1990; Lam et al., 1996). In a series of studies with rice, NADH-GOGAT was thought to be responsible in young organs for the synthesis of Glu from the Gln that is transported from senescing organs and roots (Yamaya et al., 1992; Hayakawa et al., 1993, 1994). In roots, the mRNA and protein for NADH-GOGAT accumulated markedly within a few hours of supplying low concentrations of NH_4^+ (Yamaya et al., 1995; Hirose et al., 1997). The identical response in the expression of the NADH-GOGAT gene was observed in rice cell cultures (Hayakawa et al., 1990; Watanabe et al., 1996). The NH_4^+-induced accumulation of NADH-GOGAT protein in rice roots occurred in two cell layers of the root surface, the epidermis and the exodermis (Ishiyama et al., 1998). Furthermore, Gln or its downstream metabolites, but not NH_4^+ itself, could be a signal substance for the accumulation of NADH-GOGAT mRNA in the roots (Hirose et al., 1997). Thus, the expression of the NADH-GOGAT gene in rice plants is regulated in an age-, cell type-, and nitrogen-responsive manner.

Protein phosphorylation plays a key role in diverse biological processes in eukaryotes (Hunter, 1995). The availability of specific inhibitors of protein kinases and phos-

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phatases (MacKintosh and MacKintosh, 1994) have allowed us to elucidate the roles of their target enzymes in signal transduction pathways. To investigate whether the protein phosphorylation mechanism is involved in intercellular pathways mediating NADH-GOGAT gene regulation, we examined the effects of okadaic acid (OKA), an inhibitor of PP1- and PP2A-related enzymes (Bialojan and Takai, 1988), on the expression of the NADH-GOGAT gene in relation to the inducible effects of NH_4^+. We found that NADH-GOGAT mRNA accumulation was increased by OKA due to an increased rate of transcription of the gene in suspension-cultured rice cells.

MATERIALS AND METHODS

Cell Culture and Inhibitor Treatment

The original callus cultures were derived from rice (*Oryza sativa* L. cv Sasanishiki) embryos in 1996. The stock suspension cultures were maintained by transferring approximately 1 g fresh weight of cells to 125 mL of fresh R-2 medium containing 3% (w/v) Suc and 4.5 μM 2,4-D (Ohira et al., 1973) in a 500-mL flask at 7-d intervals. The suspension cultures were shaken on a rotary shaker at 120 rpm at 25°C. When the cells were treated with various reagents, 7-d-old cells were further cultured with nitrogen-free R-2 medium for 2.5 d and used as the inoculum (Watanabe et al., 1996). To reduce variations among individual cultures, the nitrogen-starved cells were first pooled in a batch with mixing and inoculated into 20 mL of individual nitrogen-free R-2 medium in a 100-mL flask. Afterward, the cells were treated with various reagents for the time required (as described below and in the figure legends). MES-NaOH buffer (50 mM, pH 5.8) was supplied to media to reduce the pH change during the subsequent treatments with various reagents. Suspension-cultured cells were harvested by vacuum filtration through Miracloth (Calbiochem-Novabiochem, San Diego) and weighed. The collected cells were quick-frozen in liquid nitrogen and stored at −80°C.

Chemicals

OKA and calyculin A were from Wako Pure Chemical Industries (Osaka), 1-norokadaone, staurosporine, and K-252a from Nacalai Tesque (Kyoto), and all were dissolved in DMSO at 1 mM as a stock solution. MSX was purchased from Nacalai Tesque and dissolved in water at 40 mM as a stock solution. When inhibitors dissolved in DMSO were used, all treatments were carried out in the presence of the same volume of DMSO.

Isolation of RNA and RNA Gel-Blot Analysis

Total RNA was extracted from suspension-cultured rice cells as described previously (Hirose et al., 1997). Total RNA (10 μg) was fractionated on a 1.0% (w/v) agarose-formaldehyde gel, separated by electrophoresis, and then transferred to a nylon membrane (Nytran, Schleicher & Schuell, Dassel, Germany) by capillary transfer using 10× SSC (1× SSC = 0.15 M NaCl and 15 mM sodium citrate, pH 7.0). Ethidium bromide was included in the sample loading buffer to facilitate the confirmation of equal sample loading and transfer. When using digoxigenin-labeled DNA probes, subsequent procedures throughout the chemiluminescent detection were performed as described previously (Yamaya et al., 1995). When radioactive DNA probes were used, the hybridization probes were random-prime labeled in the presence of [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Uppsala, specific activity 110 TBq/mmol) using High Prime (Boehringer Mannheim, Basel). Nylon membranes were incubated with ^{32}P-labeled probes at 42°C for 16 h in hybridization buffer containing 50% (v/v) formamide, 40 mM 1,4-piperazinediethanesulfonic acid (PIPES)-NaOH (pH 6.5), 0.5 M NaCl, 1 mM EDTA, 0.4% (w/v) SDS, 100 μg/mL poly(A), and 100 μg/mL yeast tRNA. The hybridized filters were washed twice in 2× SSC/0.1% (w/v) SDS at 42°C for 15 min, then once in 0.1× SSC/0.1% (w/v) SDS at 50°C for 15 min. Filters were air-dried and subjected to autoradiography using x-ray film (X-Omat AR, Eastman-Kodak, Rochester, NJ) with an intensifying screen at −80°C. The hybridization signals were scanned with a bioimaging analyzer (FLA2000, Fujix, Tokyo).

Isolation of Nuclei

Nuclei were isolated using a modification (Suzuki et al., 1994) of the method of Luthe and Quatrano (1980). All subsequent manipulations were carried out at 4°C. Frozen suspension-cultured rice cells (5–10 g fresh weight) were pulverized in liquid nitrogen. The powdered cells were suspended in 10 volumes of NIB (1 M hexylene glycol, 10 mM PIPES-KOH [pH 7.0], 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.5% [v/v] Triton X-100) by stirring in a beaker until thawed. The resultant slurry was filtered through two layers of gauze and two layers of Miracloth, followed by centrifugation at 1,000*g* for 10 min. Crude nuclear pellets were washed twice with NIB and once with NIB without Triton X-100, and suspended in 10 mL of NIB without Triton X-100. Nuclei were further purified by centrifugation at 3,000*g* in a swinging bucket rotor (R10S, Hitachi, Tokyo) for 30 min in a discontinuous gradient of Percoll: 5 mL of a 80% (w/v) Suc cushion, 5 mL of 80% Percoll, 25 mL of 30% Percoll, and 10 mL of suspension of the crude nuclei. The Percoll solutions contained 0.8 M Suc, 5 mM PIPES-KOH (pH 7.0), and 5 mM $MgCl_2$. The nuclei fraction that banded at the interface between the 30% and 80% Percoll layers was collected with a Pasteur pipette, washed twice with a nuclear resuspension buffer (1 M hexylene glycol, 10 mM PIPES-KOH [pH 7.0], 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 20% [v/v] glycerol), and stored at −80°C until use. The DNA in the aliquot of nuclei was quantified as described by Wanner and Gruissem (1991).

In Vitro Transcription

The reactions for transcriptional elongation were performed essentially as described by Gallagher and Ellis (1982) using 100 μg of DNA per sample. Nuclei were incubated in 200 μL of a reaction mixture containing 20 mM

Tris-HCl (pH 7.9), 7.5 mM $MgCl_2$, 75 mM KCl, 0.5 mM concentrations of ATP, GTP, and CTP, 5 mM DTT, 100 units of RNase inhibitor (RNasin, Toyobo, Osaka), 3.7 MBq of [α-^{32}P]UTP (Amersham Pharmacia Biotech, specific activity 110 TBq/mmol), and 10% (v/v) glycerol. The reactions were carried out for 15 min at 30°C. The reaction mixture was then treated with 50 units of RNase-free DNase (Boehringer Mannheim) for 5 min at 30°C in the presence of 25 μg of carrier *Escherichia coli* transfer RNA (Boehringer Mannheim). Afterward, the solution was mixed with proteinase K solution (0.5 mg/mL proteinase K, 10 mM Tris-HCl [pH7.5], 5 mM EDTA, and 1% [w/v] SDS) and incubated for 30 min at 30°C.

The newly synthesized and labeled RNA was extracted with phenol-chloroform and precipitated with 0.1 volume of 5 M NaCl and 2 volumes of ethanol. The RNA pellet was then dissolved in DNase buffer (20 mM HEPES-KOH [pH 7.5], 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$) and incubated with 20 units of RNase-free DNase I (Boehringer Mannheim) in the presence of 2 mM DTT and 20 units of RNasin for 30 min at 30°C. The reaction was again treated with proteinase K solution (0.1 mg/mL proteinase K, 10 mM Tris-HCl [pH7.5], 5 mM EDTA, and 1% [w/v] SDS) for 30 min at 30°C and extracted with phenol-chloroform. The synthesized RNA in aqueous phase solution was then precipitated with cold 10% (w/v) trichloroacetic acid in 10 mM $Na_4P_2O_7$ at 4°C. The precipitate was collected by centrifugation, dissolved in 0.1 M sodium acetate (pH 5.2), and precipitated with ethanol. The final RNA pellet was dissolved in the hybridization buffer. Incorporation of [α-^{32}P]UTP into RNA was assayed as described by Sambrook et al. (1989).

DNA Dot-Blot Hybridization

Equal amounts (1 pmol) of recombinant plasmids were cut at a single restriction site and dot-blotted onto Hybond N^+ membrane (Amersham Pharmacia Biotech) by the direct dot-blot procedure (Hightower and Meagher, 1985). pBluescript (pBS) was used as a negative control. Filters were prehybridized, hybridized at 42°C, and washed as described above for RNA gel-blot hybridization with [α-^{32}P]dCTP-labeled probes. Hybridization was performed in 1.0-mL volumes, with each hybridization containing an equal number of counts (2×10^6 cpm). The autoradiogram was obtained by exposing the blots for 48 h using x-ray film (Kodak X-Omat AR) in the presence of an intensifying screen at −80°C. The hybridization signals were scanned with the bioimaging analyzer (Fujix).

DNA Probes

YK446, a cDNA clone homologous to rice 18S rRNA, was kindly provided by Dr. Hirofumi Uchimiya (University of Tokyo). cDNAs for the tubulin α-1 chain and glyceraldehyde 3-P dehydrogenase were provided by the Rice Genome Research Program, National Institute of Agrobiological Resources (Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan; accession nos. D16089 and D16096, respectively). The 856-bp partial cDNA fragment (Yamaya et al., 1995) and the full-length cDNA (λOSR51) (Goto et al., 1998) for NADH-GOGAT were used for the RNA gel-blot analysis and run-on transcription assay, respectively.

RESULTS

OKA was tested to determine the protein phosphorylation pathways are involved in the regulation of NADH-GOGAT gene expression in rice cell cultures. RNA gel-blot analysis showed that OKA at 1 μM induced the accumulation of NADH-GOGAT mRNA (Fig. 1A). The increase in NADH-GOGAT mRNA reached a maximum 9 to 12 h after the addition of OKA, and this was maintained for at least 24 h after the treatment (Fig. 1A). OKA caused a 7.0 ±

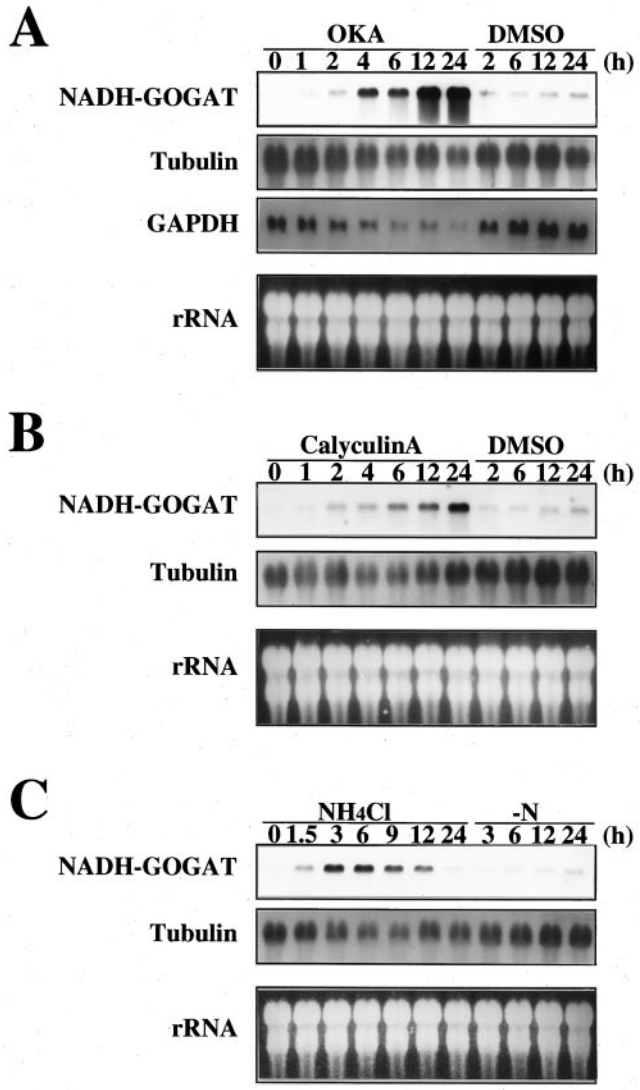

Figure 1. Time-course studies of NADH-GOGAT mRNA accumulation induced by 1 μM OKA (A), 1 μM calyculin A (B), or 20 mM NH_4Cl (C). RNA gel-blot analyses were performed with digoxigenin-labeled cDNA probes for NADH-GOGAT, tubulin, or glyceraldehyde 3-P dehydrogenase (GAPDH). The ethidium-bromide-stained ribosomal bands are shown as a loading control.

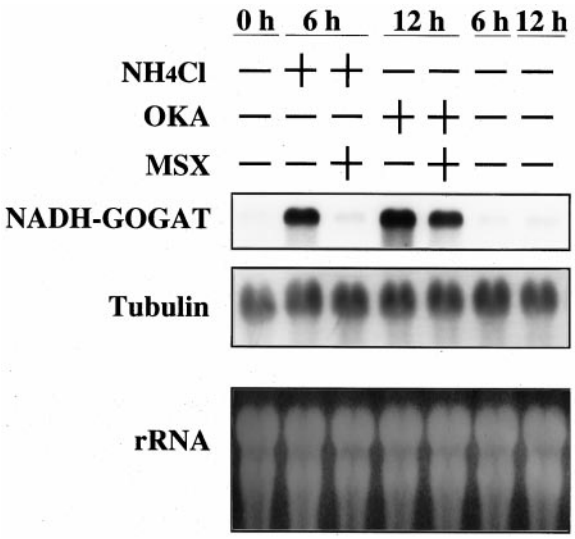

Figure 2. Effects of MSX on the induction of NADH-GOGAT mRNA accumulation by NH_4^+ and OKA. Suspension-cultured rice cells were pretreated with 10 μM MSX for 30 min, and then treated with 20 mM NH_4Cl or 1 μM OKA for 6 or 12 h, respectively. Total RNA was isolated and subjected to RNA gel-blot analysis using digoxigenin-labeled cDNA probes for NADH-GOGAT or tubulin. The ethidium-bromide-stained ribosomal bands are shown as a loading control.

0.5-fold (n = 3) increase in NADH-GOGAT mRNA at 24 h compared with the content at zero time. In contrast, OKA decreased the contents of mRNA for tubulin α-1 chain and glyceraldehyde 3-P dehydrogenase (Fig. 1). Similar effects of OKA in decreasing the contents of mRNA for the tubulin β chain was observed in soybean (Gianfagna and Lawton, 1995).

Our results suggest that OKA did not stimulate the general transcriptional activity, nor did it influence the stability of mRNA under the conditions of this study. Calyculin A, which is structurally unrelated to OKA and has a spectrum of protein phosphatase inhibitory activity different from OKA (Ishihara et al., 1989), also induced NADH-GOGAT mRNA accumulation, but to a lesser extent (Fig. 1B). However, the NH_4^+-dependent induction was transient and more rapid than the OKA-dependent induction (Fig. 1C). Thus, OKA mimics the NH_4^+-induced accumulation of NADH-GOGAT mRNA in rice cell cultures (Watanabe et al., 1996). The inducible accumulation of NADH-GOGAT mRNA by OKA could have been caused by one of the following.

First, OKA could stimulate the expression of NADH-GOGAT gene by inducing endogenous NH_4^+ production. In this case, the induction of NADH-GOGAT mRNA by OKA could be explained on the basis of NH_4^+ action. Second, OKA could stimulate the signal transduction pathway for NADH-GOGAT gene expression downstream from NH_4^+. Third, OKA could stimulate a signal transduction pathway for NADH-GOGAT gene expression other than the nitrogen sensing system. To determine which of these possibilities is the correct one, OKA was added together with MSX, a specific inhibitor of GS that completely inhibits the induction of NADH-GOGAT mRNA accumulation by the supply of NH_4^+ in rice roots (Hirose et al., 1997). As shown in Figure 2, the inhibitor blocked NH_4^+-dependent NADH-GOGAT mRNA accumulation but had no effect on the OKA-dependent accumulation of NADH-GOGAT mRNA. We therefore concluded that the OKA-sensitive protein phosphatase is involved in the regulation

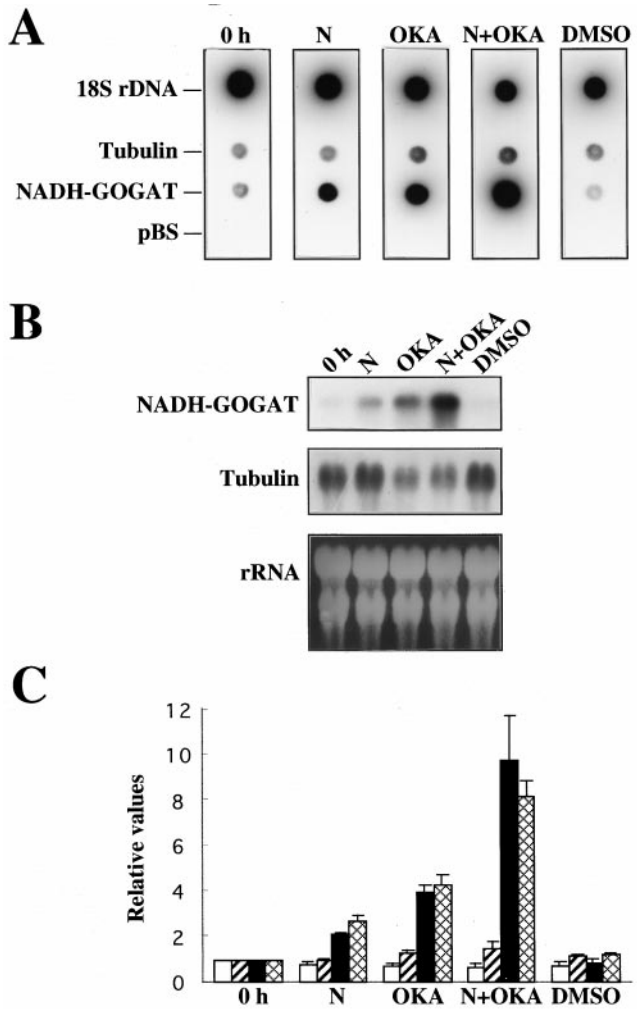

Figure 3. Comparison of the rate of nuclear run-on transcription with mRNA accumulation. A, Suspension-cultured rice cells were treated with 20 mM NH_4Cl (N), 1 μM OKA, their combination (N + OKA), or 0.1% (v/v) DMSO (control) for 6 h. Nuclei were prepared and subjected to transcriptional run-on assays as described in "Materials and Methods." Nylon membrane containing the indicated plasmids were hybridized with the in vitro-labeled RNA and autoradiographed. B, RNA gel-blot analysis was performed using total RNA isolated from the same cells as in A. Hybridization was performed with [α-^{32}P]dCTP-labeled NADH-GOGAT cDNA probes or digoxigenin-labeled tubulin cDNA probes. The ethidium-bromide-stained ribosomal bands are shown as a loading control. C, Signals from run-on transcription and RNA gel-blot analyses were quantified with a bioimaging analyzer and plotted as the increase (-fold) in signal relative to that of zero time. White bars, rDNA gene transcription; hatched bars, tubulin gene transcription; black bars, NADH-GOGAT gene transcription; cross-hatched bars, NADH-GOGAT mRNA accumulation. Error bars represent ±SD for three independent experiments.

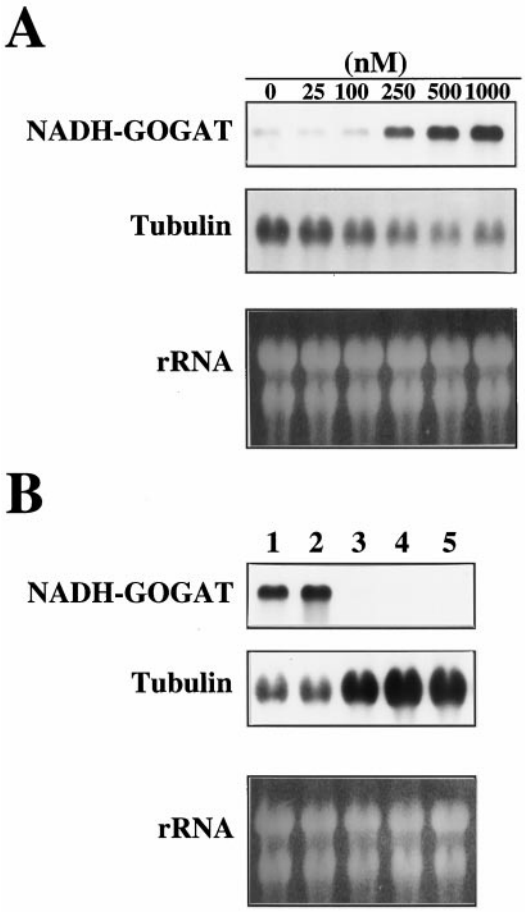

Figure 4. A, Dose dependence of NADH-GOGAT mRNA accumulation induced by OKA. Suspension-cultured rice cells were treated with the indicated concentrations of OKA for 12 h. B, Suspension-cultured rice cells were treated with 0.5 μM OKA (lane 1), 1 μM OKA (lane 2), 0.5 μM 1-norokadaone (lane 3), 1 μM 1-norokadaone (lane 4), or 0.1% (v/v) DMSO (lane 5) for 12 h. RNA gel-blot analyses were performed using the digoxigenin-labeled cDNA probes for NADH-GOGAT or tubulin. The ethidium bromide-stained ribosomal bands are shown as a loading control.

of NADH-GOGAT gene expression and plays a role in the signal transduction pathway downstream from NH_4^+ or in an alternative signal transduction pathway.

To examine whether the induction of NADH-GOGAT mRNA by either NH_4^+ or OKA was caused by an increased rate of gene transcription, nuclear run-on assays were performed with nuclei prepared from control, NH_4^+-treated, and OKA-treated cells for 6 h (Fig. 3A). The radioactivities hybridized with the respective probes were quantified and the values relative to that of zero time were calculated (Fig. 3C). The relative increase in the transcription rate of the NADH-GOGAT gene stimulated by NH_4^+ and OKA was 2.0 ± 0.1-fold (n = 4) and 3.9 ± 0.4-fold (n = 3), respectively (Fig. 3C). However, the relative increase in the control sample was 0.85 ± 0.18-fold (n = 3) (Fig. 3C). To make a comparison with the transcription rate, we measured the contents of NADH-GOGAT mRNA in the same batch of suspension-cultured rice cells used for the isolation of nuclei. The contents of NADH-GOGAT mRNA in the presence of NH_4^+ or OKA at 6 h were 2.7 ± 0.2-fold (n = 4) and 4.3 ± 0.4-fold (n = 3) higher than those in the initial level, respectively (Fig. 3, B and C). Thus, the accumulation of NADH-GOGAT mRNA induced by either NH_4^+ or OKA was correlated with the increase in transcriptional rates. These results suggest that the accumulation of NADH-GOGAT mRNA induced by either NH_4^+ or OKA is mainly caused by the transcriptional activation of NADH-GOGAT gene.

Based on our previous studies showing that the supply of NH_4^+ induced NADH-GOGAT mRNA accumulation in both roots and cell cultures (Yamaya et al., 1995; Watanabe et al., 1996; Hirose et al., 1997; Fig. 1C), a possible interaction between NH_4^+ and OKA was investigated. The cells were treated simultaneously with 20 mM NH_4Cl and 1 μM OKA for 6 h. OKA potentiated the NH_4^+-dependent increase of the NADH-GOGAT mRNA accumulation and gene transcription rate by 8.1 ± 0.7-fold (n = 3) and 9.7 ± 2.0-fold (n = 3), respectively (Fig. 3C). Thus, OKA and NH_4^+ showed a slightly synergistic but nonadditive effect, suggesting that NH_4^+- and OKA-dependent transcriptional activation of the NADH-GOGAT gene share some common components in their signal transduction pathways.

The increase in the accumulation of NADH-GOGAT mRNA was correlated with the increase in OKA concentration (Fig. 4A). When 1-norokadaone, an OKA analog with low inhibiting activity on protein phosphatase (Takai et al., 1992), was used as a negative control, no induction of NADH-GOGAT mRNA was observed (Fig. 4B, lanes 3 and 4). Therefore, OKA apparently acts as a protein phosphatase inhibitor.

When suspension-cultured rice cells were pretreated with 1 μM staurosporine or K-252a, protein Ser/Thr kinase

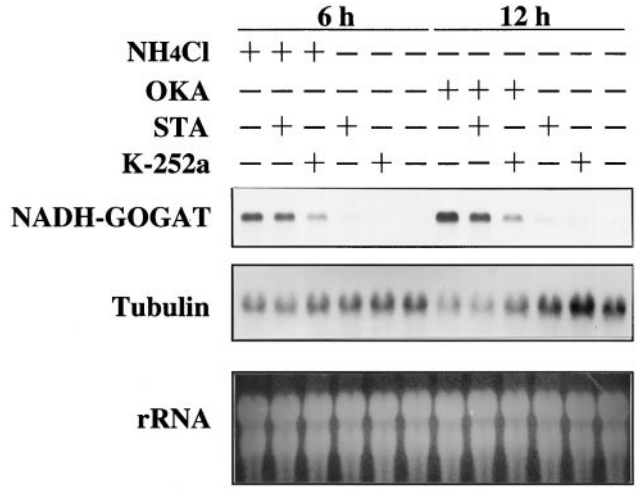

Figure 5. Effects of protein kinase inhibitors on the induction of NADH-GOGAT mRNA by NH_4^+ and OKA. Suspension-cultured rice cells were pretreated with 1 μM staurosporine (STA) or 1 μM K-252a for 1 h, and then treated with 20 mM NH_4Cl or 1 μM OKA for 6 or 12 h, respectively. Total RNA was isolated and subjected to RNA gel-blot analysis using the digoxigenin-labeled cDNA probes for NADH-GOGAT or tubulin. The ethidium-bromide-stained ribosomal bands are shown as a loading control.

inhibitors (Tamaoki et al., 1986; Nakanishi et al., 1988), followed by OKA or NH_4^+, K-252a reduced the increase of NADH-GOGAT mRNA (Fig. 5). However, staurosporine did not cause the reduction. These results suggest that either: (a) K-252a-sensitive protein kinase may be involved in signal transduction pathways for both OKA- and NH_4^+-dependent accumulation of NADH-GOGAT mRNA, or (b) K-252a is more potent or more cell permeable than staurosporine, as indicated by MacKintosh and MacKintosh (1994), in rice cell cultures. 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride (H-7, 100 μM), a protein kinase C inhibitor (Hidaka et al., 1984), did not inhibit the increase in NADH-GOGAT mRNA, suggesting that protein kinase C is not involved in the OKA- and NH_4^+-dependent increase in NADH-GOGAT mRNA (N. Hirose and T. Yamaya, unpublished data).

DISCUSSION

In plant systems, the use of PP1/PP2A inhibitors has led to the identification of a role for reversible protein phosphorylation in various processes, including the response to hormones, pathogens, and environmental stimuli (Smith and Walker, 1996; Luan, 1998). OKA is capable of inducing specific genes at the transcriptional level in higher plants (Dominov et al., 1992; Raz and Fluhr, 1993; Gianfagna and Lawton, 1995; Lue and Lee, 1995; Rojo et al., 1998). In this study, we showed that OKA induced the accumulation of NADH-GOGAT mRNA (Fig. 1A), and that this induction was mainly caused by the increased rate of the transcription (Fig. 3). The concentrations and duration of the treatment of OKA used here were identical to those used in other in vivo experiments with plant tissues and cells (Raz and Fluhr, 1993; Sheen, 1993).

Among the mRNAs examined in this study, the stimulatory effect of OKA was specific to mRNA for NADH-GOGAT. We suggest that PP1-related and/or PP2A-related enzyme activity is involved in the signal transduction pathway that regulates the transcriptional activity of the NADH-GOGAT gene. Because OKA inhibits PP2A-related enzyme with greater potency than PP1-related enzyme, and because calyculin A inhibits PP1-related enzyme with a 10- to 100-fold greater potency than OKA (Cohen et al., 1990), the difference in action of these inhibitors on NADH-GOGAT gene expression is probably a result of the inhibition of PP2A-related enzyme by OKA (Fig. 1, A and B). It should be noted that the actual cellular concentrations of OKA and calyculin A were not determined in the present study. Furthermore, we do not know how many PP1 or PP2A molecules are involved. Thus, there is insufficient evidence at present to conclusively state that inhibition of PP2A-related enzyme is responsible for the stimulation of NADH-GOGAT gene regulation in rice cell cultures.

Induction of NADH-GOGAT mRNA by NH_4^+ was mainly caused by transcriptional activation (Fig. 3). The transcriptional rate of NADH-GOGAT gene increased about 2-fold at 6 h after the addition of NH_4^+ and then declined to the initial rate by 24 h. We therefore concluded that the transient accumulation of NADH-GOGAT mRNA by NH_4^+ (Fig. 1C) was caused by the transient induction of transcriptional activity. In contrast, the transcription rate of NADH-GOGAT gene increased about 4-fold 6 h after the addition of OKA and then elevated to 8-fold after 24 h. The sustained increase of NADH-GOGAT mRNA accumulation induced by OKA (Fig. 1A) was caused by the sustained induction of transcriptional activity. This sustained induction could be expected from a constitutive biochemical type of inhibition by OKA. Simultaneous addition of both OKA and NH_4^+ potentiated the transcription of the NADH-GOGAT gene (Fig. 3).

These results suggest that OKA and NH_4^+ may act through the same signal transduction pathway (Fig. 3). In detached maize leaves, the nitrate-dependent accumulation of transcripts of genes for nitrate reductase, nitrite reductase, and plastidial GS were inhibited by the pretreatment with OKA (Sakakibara et al., 1997). OKA inhibits the nitrogen-responsive expression of *C4Ppc1*, a C_4-form PEP-carboxylase gene (Suzuki et al., 1994). Protein phosphatases possibly play a leading role in nitrogen signal transduction in higher plants. In this context, Gln or its metabolites might stimulate the transcription of the NADH-GOGAT gene by directly or indirectly inactivating PP1/2A-related enzymes. We previously suggested that Gln or its metabolite acts as a metabolic signal for the induction of the NADH-GOGAT gene in rice roots (Hirose et al., 1997). Therefore, we cannot exclude the possibility that OKA stimulates the expression of NADH-GOGAT gene by inducing endogenous Gln or its metabolite production in a compartment where the expression of the NADH-GOGAT gene is regulated.

We treated rice cell cultures with azaserine, an inhibitor of Gln amidotransferases, to examine whether Gln or its metabolite is a direct inducer for the Gln-dependent expression of the NADH-GOGAT gene. Pretreatment with 250 μM azaserine for 30 min inhibited both the Gln- and the OKA-dependent accumulation of NADH-GOGAT mRNA (N. Hirose and T. Yamaya, unpublished data). These results suggest the following possibilities. First, metabolites from Gln are a direct inducer of the NADH-GOGAT gene and OKA stimulates their production. Second, the inhibition of both Gln- and OKA-dependent accumulation of NADH-GOGAT mRNA by azaserine is caused by the stringent effects of the reagent on cellular metabolism, such as the inhibition of transcription caused by the lack of nucleotides. At this time, we have no direct and rigorous evidence to distinguish those possibilities. However, we assume at present that Gln is the most promising signal for the expression of the NADH-GOGAT gene, because the supply of Gln metabolites such as 5 mM carbamoylphosphate, 5 mM glucosamine-6-phosphate, 10 mM anthranilic acid, and 10 mM *p*-aminobenzoic acid to rice roots for 3 h failed to increase the accumulation of NADH-GOGAT transcripts (N. Hirose and T. Yamaya, unpublished data).

Although many types of protein phosphatases have been characterized at the molecular level, much less is known about their function in vivo (Luan, 1998). Our studies on the regulation of the NADH-GOGAT gene will serve to uncover the role of protein phosphatase in the signal transduction pathway in plants.

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LITERATURE CITED

Avila C, Márquetz AJ, Pajuelo P, Cannell ME, Wallsgrove RM, Forde BG (1993) Cloning and sequence analysis of a cDNA for barley ferredoxin-dependent glutamate synthase and molecular analysis of photorespiratory mutants deficient in the enzyme. Planta **189:** 475–483

Bialojan C, Takai A (1988) Inhibitory effects of a marine-sponge toxin, okadaic acid, on protein phosphatase: specificity and kinetics. Biochem J **256:** 283–290

Cohen P, Holmes CFB, Tsukitani Y (1990) Okadaic acid: a new probe for the study of cellular regulation. Trends Biochem Sci **15:** 98–102

Coschigano KT, Melo-Oliveira R, Lim J, Coruzzi GM (1998) Arabidopsis *gls* mutants and distinct Fd-GOGAT genes: implications for photorespiration and primary nitrogen assimilation. Plant Cell **10:** 741–752

Dominov JA, Stenzler L, Lee S, Schwarz JJ, Leisner S, Howell SH (1992) Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. Plant Cell **4:** 451–461

Gallagher TF, Ellis RJ (1982) Light-stimulated transcription of genes for two chloroplast polypeptides in isolated pea leaf nuclei. EMBO J **1:** 1493–1498

Gianfagna TJ, Lawton MA (1995) Specific activation of soybean defense genes by the phosphoprotein phosphatase inhibitor okadaic acid. Plant Sci **109:** 165–170

Goto S, Akagawa T, Kojima S, Hayakawa T, Yamaya T (1998) Organization and structure of NADH-dependent glutamate synthase gene from rice plants. Biochim Biophys Acta **1387:** 298–308

Gregerson RG, Miller SS, Twary SN, Gantt JS, Vance CP (1993) Molecular characterization of NADH-dependent glutamate synthase from alfalfa nodules. Plant Cell **5:** 215–226

Hayakawa T, Hopkins L, Peat LJ, Yamaya T, Tobin AK (1999) Quantitative intercellular localization of NADH-dependent glutamate synthase protein in different types of root cells in rice plants. Plant Physiol **119:** 409–416

Hayakawa T, Kamachi K, Oikawa M, Ojima K, Yamaya T (1990) Response of glutamine synthetase and glutamate synthase isoforms to nitrogen sources in rice cell cultures. Plant Cell Physiol **31:** 1071–1077

Hayakawa T, Nakamura T, Hattori F, Mae T, Ojima K, Yamaya T (1994) Cellular localization of NADH-dependent glutamate synthase protein in vascular bundles of unexpanded leaf blades and young grains of rice plants. Planta **193:** 455–460

Hayakawa T, Yamaya T, Mae T, Ojima K (1992) Purification, characterization, and immunological properties of NADH-dependent glutamate synthase from rice cell cultures. Plant Physiol **98:** 1317–1322

Hayakawa T, Yamaya T, Mae T, Ojima K (1993) Changes in the content of two glutamate synthase proteins in spikelets of rice (*Oryza sativa*) plants during ripening. Plant Physiol **101:** 1257–1262

Hidaka H, Inagaki M, Kawamoto S, Sasaki Y (1984) Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry **23:** 5036–5041

Hightower RC, Meagher RB (1985) Divergence and differential expression of soybean actin genes. EMBO J **4:** 1–8

Hirose N, Hayakawa T, Yamaya T (1997) Inducible accumulation of mRNA for NADH-dependent glutamate synthase in rice roots in response to ammonium ions. Plant Cell Physiol **38:** 1295–1297

Hunter T (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell **80:** 225–236

Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Fusetani N, Watabe S, Hashimoto K, Uemura D, Hartshorne DJ (1989) Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. Biochem Biophys Res Commun **159:** 871–877

Ishiyama K, Hayakawa T, Yamaya T (1998) Expression of NADH-dependent glutamate synthase protein in epidermis and exodermis of rice roots in response to the supply of nitrogen. Planta **204:** 288–294

Lam HM, Coschigano KT, Oliveira IC, Melo-Oliveira R, Coruzzi GM (1996) The molecular-genetics of nitrogen assimilation into amino acid in higher plants. Annu Rev Plant Physiol Plant Mol Biol **47:** 569–593

Lea PJ, Miflin BJ (1974) Alternative route for nitrogen assimilation in higher plants. Nature **251:** 614–616

Lea PJ, Robinson SA, Stewart GR (1990) The enzymology and metabolism of glutamine, glutamate and asparagine. *In* BJ Miflin, PJ Lea, eds, The Biochemistry of Plants, Vol 16: Intermediary Nitrogen Metabolism. Academic Press, San Diego, pp 121–157

Luan S (1998) Protein phosphatase and signaling cascades in higher plants. Trends Plant Sci **3:** 271–275

Lue MY, Lee H (1995) Protein phosphatase inhibitors enhance the expression of an α-amylase gene, *αAmy3,* in cultured rice cells. Biochem Biophys Res Commun **205:** 807–816

Luthe DS, Quatrano RS (1980) Transcription of isolated nuclei. I. Isolation of nuclei and elimination of endogenous ribonuclease activity. Plant Physiol **65:** 305–308

MacKintosh C, MacKintosh RW (1994) Inhibitors of protein kinases and phosphatases. Trends Biochem Sci **19:** 444–448

Nakanishi S, Yamada K, Kase H, Nakamura S, Nonomura Y (1988) K-252a, a novel microbial product, inhibits smooth muscle myosin light chain kinase. J Biol Chem **263:** 6215–6219

Nalbantoglu B, Hirasawa M, Moomaw C, Nguyen H, Knaff DB, Allen R (1994) Cloning and sequencing of the gene encoding spinach ferredoxin-dependent glutamate synthase. Biochim Biophys Acta **1183:** 557–561

Ohira K, Ojima K, Fujiwara A (1973) Studies on the nutrition of rice cell culture. I. A simple, defined medium for rapid growth in suspension culture. Plant Cell Physiol **14:** 1113–1121

Raz V, Fluhr R (1993) Ethylene signal is transduced via protein phosphorylation events in plants. Plant Cell **5:** 523–530

Rojo E, Titarenko E, León J, Berger S, Vancanneyt G, Sánchez-Serrano JJ (1998) Reversible protein phosphorylation regulates jasmonic acid-dependent and -independent wound signal transduction pathway in *Arabidopsis thaliana*. Plant J **13:** 153–165

Sakakibara H, Kobayashi K, Deji A, Sugiyama T (1997) Partial characterization of the signaling pathway for the nitrate-dependent expression of genes for nitrogen-assimilatory enzymes using detached maize leaves. Plant Cell Physiol **38:** 837–843

Sakakibara H, Watanabe M, Hase T, Sugiyama T (1991) Molecular cloning and characterization of complementary DNA encoding for ferredoxin-dependent glutamate synthase in maize leaf. J Biol Chem **266:** 2028–2035

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sechley KA, Yamaya T, Oaks A (1992) Compartmentation of nitrogen assimilation in higher plants. Int Rev Cytol **134:** 85–163

Sheen J (1993) Protein phosphatase activity is required for light-inducible gene expression in maize. EMBO J **12:** 3497–3505

Smith RD, Walker JC (1996) Plant protein phosphatases. Annu Rev Plant Physiol Plant Mol Biol **47:** 101–125

Suzuki A, Rothstein S (1997) Structure and regulation of ferredoxin-dependent glutamate synthase from *Arabidopsis thaliana*: cloning of cDNA, expression in different tissues of wild-type and *glt*S mutant strains, and light induction. Eur J Biochem **243:** 708–718

Suzuki I, Crétin C, Omata T, Sugiyama T (1994) Transcriptional and posttranscriptional regulation of nitrogen-responding expression of phospho*enol*pyruvate carboxylase gene in maize. Plant Physiol **105:** 1223–1229

Takai A, Murata M, Torigoe K, Isobe M, Mieskes G, Yasumoto T (1992) The inhibitory effect of okadaic acid derivatives on protein phosphatases: a study on structure-affinity relationship. Biochem J **284:** 539–544

Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M, Tomita F (1986) Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase. Biochem Biophys Res Commun **135:** 397–402

Temple SJ, Vance CP, Gantt JS (1998) Glutamate synthase and nitrogen assimilation. Trends Plant Sci **3:** 51–56

Trepp GB, Plank DW, Gantt JS, Vance CP (1999a) NADH-glutamate synthase in alfalfa root nodules: immunocytochemical localization. Plant Physiol **119:** 829–837

Trepp GB, van de Mortel M, Yoshioka H, Miller SS, Samac DA, Gantt JS, Vance CP (1999b) NADH-glutamate synthase in alfalfa root nodules: genetic regulation and cellular expression. Plant Physiol **119:** 817–828

Vance CP, Miller SS, Gregerson RG, Samac DA, Robinson DL, Gantt JS (1995) Alfalfa NADH-dependent glutamate synthase: structure of the gene and importance in symbiotic N_2 fixation. Plant J **8:** 345–358

Wanner LA, Gruissem W (1991) Expression dynamics of the tomato *rbcS* gene family during development. Plant Cell **3:** 1289–1303

Watanabe S, Sakai T, Goto S, Yaginuma T, Hayakawa T, Yamaya T (1996) Expression of NADH-dependent glutamate synthase in response to the supply of nitrogen in rice cells in suspension culture. Plant Cell Physiol **37:** 1034–1037

Yamaya T, Hayakawa T, Tanasawa K, Kamachi K, Mae T, Ojima K (1992) Tissue distribution of glutamate synthase and glutamine synthetase in rice leaves: occurrence of NADH-dependent glutamate synthase protein and activity in the unexpanded non-green leaf blades. Plant Physiol **100:** 1427–1432

Yamaya T, Tanno H, Hirose N, Watanabe S, Hayakawa T (1995) A supply of nitrogen causes increase in the level of NADH-dependent glutamate synthase protein and in the activity of the enzyme in the roots of rice seedlings. Plant Cell Physiol **36:** 1197–1204

Zehnacker C, Becker TW, Suzuki A, Carrayol E, Caboche M, Hirel B (1992) Purification and properties of tobacco ferredoxin-dependent glutamate synthase, and isolation of corresponding cDNA clones. Planta **187:** 266–274